IDENTIFYING FOXA1 INTERACTING PARTNERS: INSIGHT INTO

ANDROGEN REGULATED PROSTATIC GENES

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Dissertation completed under the direction of Dr. Robert Matusik, PhD

This research project is to identify FoxA1 interacting partners. In this study, a potential FoxA1 binding partner, Upstream Stimulatory Factor 2 (USF2), has been examined; its DNA binding activity was determined and the function of USF2 was monitored in USF2 knockdown LNCaP cells. FLAG-6His-FoxA1 stable expressing LNCaP cell lines were established. The FoxA1 protein complex from LNCaP tagged-FoxA1 cells was purified and analyzed by Liquid Chromatography Tandem Mass Spectrometry.

USF2 has been confirmed to interact with FoxA1 using ImmunoPrecipitation and glutathione-S-transferase pull-down assays. Our study also indicates, as FoxA1, USF2 binds to prostatic specific gene promoters such as probasin promoter, Spermine Binding Protein promoter, and the Prostate Specific Antigen core enhancer. PSA mRNA levels were increased in USF2 knocked down LNCaP cells compared with control LNCaP cells when the LNCaP cells were treated with DHT (10^{-8} M).

Using LC-MS/MS analysis, a total of 16 proteins that appear to interact with Foxa1 have been identified and eight of them have been previously reported as AR interacting proteins. Further work is needed to confirm the binding between FoxA1 and these identified proteins and to perform functional studies of the confirmed FoxA1 binding partners.

This study has identified FoxA1 interacting partners which helps unravel the critical transcription factors that control cell type specific gene expression and to further understand the role of FoxA1 during prostate development. This work gives us an insight into the essential mechanism of androgen regulation of the prostate gland and of hormonal regulation of prostate cancer.

To my parents for their love and support …

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ACTH   Adrenocorticotropic Hormone

AIS   Androgen Insensitivity Syndrome

AR   Androgen Receptor

ARBS   Androgen Receptor Binding Site

ARE   Androgen Response Element

ARR   Androgen Response Region

bHLH-zip   basic-Helix-Loop-Helix-leucine-zipper

BPH   Benign Prostatic Hyperplasia

CAT   Chloramphenicol Acetyltransferase

ChIP   Chromotin ImmunoPrecipitation

Co-IP   Co-Immunoprecipitation

CRPC   Castration Resistant Prostate Cancer

DHEA   Dihydroepiandrosterone

DHT   Dihydrotestosterone

DNADPK   DNA-dependent protein kinase

DBHS   Drosophila Behavior Human Splicing

ELISA   Enzyme-linked Immunosorbent Assays

EMSA   Electrophoretic Mobility Shift Assays

ER   Estrogen Receptor

FH   Fork head domain

GST   Glutathione-S-transferase

GNL3L	Guanine nucleotide binding protein-like 3-like
hKLK2	human glandular Kallikrein 2
HNF-3	Hepatocyte Nuclear Factor-3
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LC-MS/MS	Liquid Chromatography/Tandem Mass Spectrometry
LH	Lutenizing Hormone
LHRH	Lutenizing Hormone Releasing Hormone
Luc	Luciferase
mE-RABP	mouse Epididymal Retinoic Acid Binding Protein
MMTV	mouse mammary tumor virus
MS	Mass Spectrometry
MSRC	Mass Spectrometry Research Center
NE	Neuroendocrine
NFI	Nuclear Factor I
NPM	Nucleophosmin
NS	Nucleostemin
PAP	Prostatic Acid Phosphatase
PB	Probasin (PROstatic BASic protein)
PCR	Polymerase Chain Reaction
PDEF	Prostate Derived ets Factor
PMSF	Phenylmethylsulfonyl fluoride
PSA	Prostate Specific Antigen
PSE	Prostate Specific Enhancer

PSF	polypyrimidine tract-binding protein-associated splicing factor
PSMA	Prostate-Specific Membrane Antigen
PSP1	Paraspeckle Protein 1
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
SBP	Spermine Binding Protein
Shh	Sonic hedgehog
SHP	Small Heterodimer Partner
TF	Transcription Factor
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate
UGS	Urogenital Sinus
USF	Upstream Stimulatory Factor

CHAPTER I

INTRODUCTION

Prostate Overview

A. Prostate Structure and Function

The prostate is an accessory gland of the male reproductive system. The gland is slightly larger than a walnut and it surrounds the urethra as it leaves the bladder (Aumueller et al., 1994). The prostate consists of glandular tissue and muscle tissue. The glandular tissue produce about 20% of seminal fluid and other substances including proteins such as proteolytic enzymes, prostatic acid phosphatase, prostate-specific antigen and a high concentration of zinc (Aumuller and Seitz, 1990). Although those secretions are necessary for fertility, many of the functions of these proteins are unknown (Aumuller and Seitz, 1990). The muscle tissue in the prostate help expel the prostatic fluid in ducts.
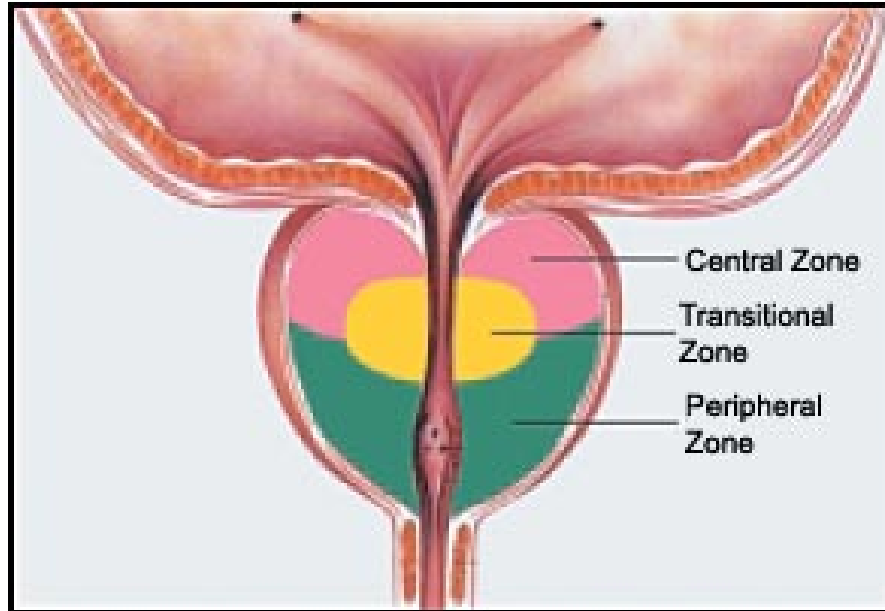
Figure 1. The adult prostate structures. The prostate is below the bladder and wraps around the urethra. The three zones of prostate are central zone, transitional zone, and peripheral zone. Image taken from

http://www.prostate-cancer.org/education/sidefx/Hai_PhotoselectiveVaporization.html

The prostate can be divided into three zones based upon anatomy and histology: peripheral zone, central zone, and transition zone (Figure 1) (Kumar and Majumder, 1995). The peripheral zone is a large area of the prostate gland that is located in the back of the prostate gland and surrounds the distal urethra (Kumar and Majumder, 1995). More than 50% prostate cancer, specifically prostatic adenocarcinoma, develops in this area. The central zone is located in the base right beneath the bladder and surrounds the ejaculatory ducts (Kumar and Majumder, 1995). Prostate cancer in this zone tends to be more aggressive even through the chance of developing prostate cancer in this zone is very low. The transition zone is a small area that is located in the internal part of the

prostate; it surrounds the proximal urethra (Kumar and Majumder, 1995). Prostate cancer can also occur in this zone and over growth of this zone as well as the central zone can lead to the disease of benign prostatic hyperplasia.

B. Prostate Diseases

Prostatitis. Prostatitis is an inflammation of the prostate gland that can cause pain in the groin, painful and difficult urination (De Marzo et al., 2007). Some forms of prostatitis that are related to bacterial infections are well studied and can be treated effectively. Most forms of prostatitis are not well understood and are difficult to diagnose and treat. There are mainly four categories of prostatitis: 1. acute prostatitis, 2. chronic bacterial prostatitis, 3. chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), and 4. asymptomatic inflammatory prostatitis (Hua and Schaeffer, 2004; Rivero et al., 2007; Simardi et al., 2004). Acute prostatitis and chronic bacterial prostatitis are both due to bacterial infection of prostate gland (De Marzo et al., 2007). More than 90% prostatitis patients are diagnosed as CP/CPPS, also known as chronic nonbacterial prostatitis with pelvic pain. Asymptomatic inflammatory prostatitis patients usually have leukocytosis symptoms, but barely have genitourinary pain (Simardi et al., 2004).

BPH (Benign Prostatic Hyperplasia). When males are in their 50's the prostate gland may start to enlarge. Studies show that about 50% of the men over 50 have an enlarged prostate. The benign overgrowth of the prostatic stromal and epithelial cells is so called Benign Prostatic Hyperplasia. When the prostate grows continuously, eventually the gland can no longer grow outwardly due to being restricted by the layer of tissue that surrounds the gland, so the gland will grow inwardly, pinching the urethra. When the

urethra is pinched, the flow of urine is obstructed, and the bladder experiences back pressure, which would cause the bladder wall to become thicker and more irritable. The bladder will start contracting more frequently (frequent urge to urinate), and eventually, the bladder is weakened substantially and unable to empty all its content.

The cause for BPH is not known, but many potential factors are thought to contribute to BPH (Isaacs and Coffey, 1989). Androgen is considered to be a passive factor in causing BPH, since BPH cannot occur in the absence of androgen and applying extra androgen does not aggravate the symptoms of BPH. In particular, dihydrotestosterone (DHT), a substance derived from testosterone in the prostate, is thought to play a main role in BPH (Isaacs and Coffey, 1989). Even as testosterone drops in older men, DHT can continue to be produced and stored substantially in the prostate. DHT can encourage cell growth in the prostate (Isaacs and Coffey, 1989). It has been observed that older men that do not produce DHT do not develop BPH.

Prostate Cancer. The most common prostate cancer is adenocarcinoma. At the beginning cancer grows in the interior of the prostate. Early stage prostate cancer is not lethal and can usually be treated successfully. The cancer cells can spread to surrounding tissues, such as the seminal vesicles and bladder and may metastasize to other parts of the body, particularly the bones and lymph node (Coffey and Pienta, 1987). The metastasized tumors are often lethal. Prostate cancer is the most common cancer in men and is the second leading cause of cancer death and the leading type of new cancer in American males. Although the causes of prostate cancer are not completely understood, several factors may contribute to prostate cancer:

1. Age. Overall, cancer is an aging disease, so age is the strongest risk factor associated with prostate cancer (Greenlee et al., 2001). Most prostate cancers are found after age 50 and the onset of cancer increases rapidly as the age increases.

2. Race. Studies have found that the possibility of getting prostate cancer is different in different races. Lower incidence of prostate cancer is found in Asian and Hispanic/Latino men than in non-Hispanic whites (Gann, 2002). African-American men have the highest risk of being diagnosed with prostate cancer and are more likely to die of prostate cancer. The reasons for the racial differences are not clear.

3. Family history. Like most cancers, inherited genes have been found and seem to be related to prostate cancer risk. These genes or genetic factors can be inherited, so a man having a father or brother or other relatives with prostate cancer may have a high risk of developing this disease (Gann, 2002). For example, MSMB, a gene coding for beta-microseminoprotein, and POU5F1P1, a processed pseudogene homologous to the pluripotency factor OCT4 have been identified recently as candidate susceptibility genes for prostate cancer in genome-wide association studies (Kote-Jarai et al., 2010). The over-expression of these genes in prostatic carcinoma in addition to their genomic location and the putative function of their gene products render them good candidates to harbour functional genetic variants which modulate prostatic cancer susceptibility.

4. Diet. Studies have found that Asian men are less likely of getting prostate cancer than American men, but for American born Asian the risk of getting prostate cancer is the same as American men within several generations (Lee et al., 2007). The facts indicate that diet may be one of the risk factor for prostate cancer. Men who eat more red meat or high-fat dairy products appear to have a higher chance of getting the disease.

5. Inflammation and infection. Studies have found that inflammation is often observed in the prostate tissues that are cancerous. Even though the link between the two is not very clear, some evidence suggests that inflammation of the prostate gland may be considered as a risk factor of prostate cancer. Sexually transmitted infections may also increase the risk of prostate cancer, like gonorrhea or chlamydia, which can cause prostate inflammation.
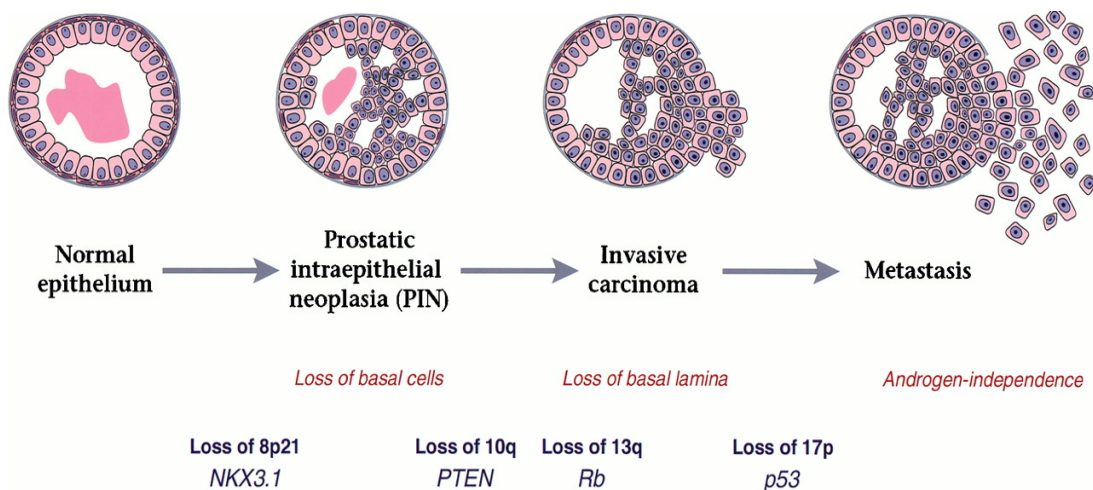
Figure 2. Human prostate cancer progression pathway. Progression stages of prostate cancer are correlated with specific gene changes. (Abate-Shen and Shen, 2000)

It is not clear how prostate cancer initiates. More research has been done to understand how the cancer progresses and to analyze chromosomal alterations during the progression. Stages of the progression are correlated with losses and/or gains of specific chromosome regions (Figure 2). Studies have found that loss of chromosomes 6q, 7q, 8p, 10q, 13q, 16q, 17p, and 18q are frequently identified in prostate cancer patients, which may correlate with the loss of candidate tumor suppressor genes (Latil et al., 1994; Zenklusen et al., 1994; Takahashi et al., 1995; Cooney et al., 1996; Cunningham et al., 1996; Elo et al., 1995; Latil et al., 1997; Saric et al., 1999).

At the early stage of prostate cancer, when carcinoma is limited in the prostate capsule, surgery and radiation therapy are routinely used. Localized disease usually has a high survival rate. Prostate cancer can develop to more aggressive stages characterized by local invasion of the seminal vesicles and even metastasis to the bone or lymph nodes. Androgen ablation is the therapeutic choice for these advanced diseases. Prostate cancer always progresses to androgen independent disease after hormone ablation therapy and become more aggressive and results in lethality.

Prostate Development and Hormone Regulation

A. Prostate Development

The urogenital sinus develops from endodermal cells to form the prostate. Several glandular and non-glandular components tightly fused within a capsule to form the adult prostate gland, which develops from the condensation of mesenchyme, urethra and

Wolffian ducts. At the early weeks of human fetal development, the male and female urogenital systems are identical. During the 9th week of the embryonic development, the wall of the proximal portion of the male urethra represents the prostate glands. At week 10, the epithelial buds in the presumptive prostatic urethra are induced by the androgen responsive urogenital sinus mesenchyme. Initially, solid epithelial cell develop into buds and grows into the surrounding mesoderm, this presents the presumptive peripheral glandular area. Later, the budding gives rise to the internal glandular area and keeps extending and branching under mesodermal control. Lumens starts to form within the epithelial cords and cellular end buds form primitive acini by the 11th week. Mesenchymal cells differentiate into smooth muscle, fibroblasts and blood vessels. During the 12th week, the epithelium continues to grow and proliferate and the stroma of the gland becomes thinner while the ducts and acini expand. At week 13~15, the embryonic testosterone level have reached its peak, the cuboidal epithelial cells start to differentiate by interacting with androgen mediated mesenchyme. At first, the proximal regions of the large ducts progress distally. At the end of the 15th week, the secretory cells are functional; the basal cells have developed and scattered neuroendocrine cells are present. The gland keeps developing and matures as the embryonic testosterone levels elevate. When the testosterone levels fall during the third trimester, the gland enters a quiescent state.

The quiescent state lasts until puberty, when the testosterone levels increase again and epithelium proliferates and gives rise to the complex folding in the mature gland. The prostate size will double in size during puberty and the epithelial cells will express

androgen regulated factors and the gland will reach its full secretory state. By 45 to 50 years, the testosterone levels drop again and the prostate undergoes a period of mild involution.
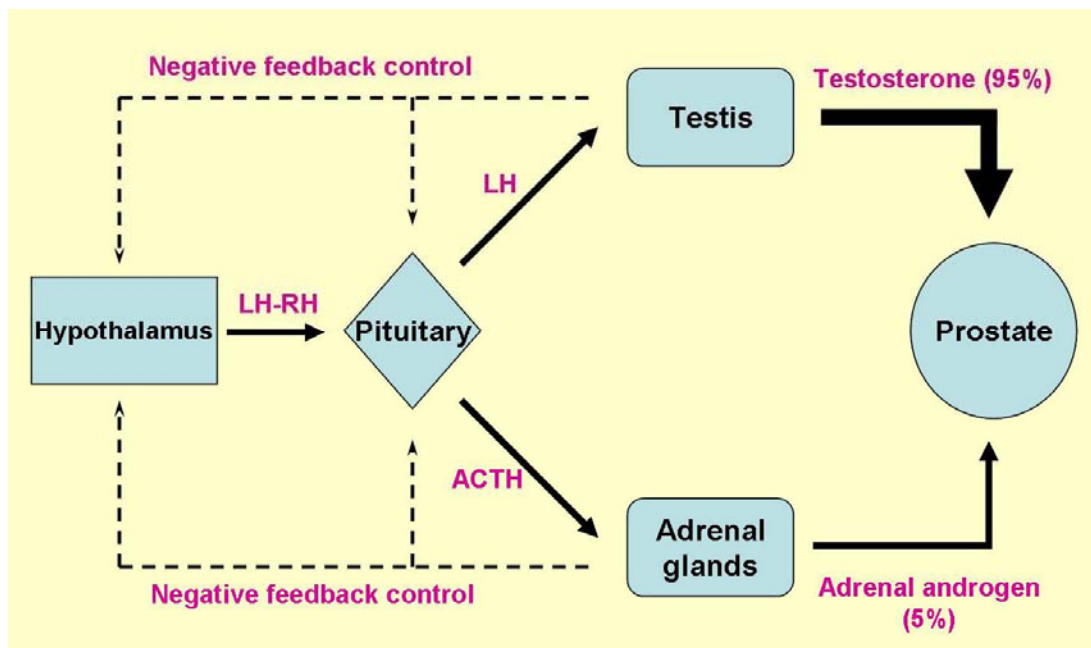
Figure 3. The male endocrine pathway. The hypothalamus releases LHRH, which stimulates the pituitary to generate LH and ACTH. LH stimulates the testis to produce testosterone, while ACTH stimulates adrenal glands to synthesize adrenal androgens. Both testosterone and adrenal androgens can target the prostate. In addition, androgens also function as a negative feedback to the pituitary and the hypothalamus to control hormone production. (Krongrad and Droller, 1993)

B. Prostate Hormone regulation

Androgen plays an important role in prostate development. The maturation of prostate is stimulated by androgens (testosterone) that are primarily secreted by the testis and a small amount that is secreted by adrenal glands (referred to as the adrenal androgens). The endocrine pathway that ultimately results in synthesis of androgen

begins in the hypothalamus. The hypothalamus synthesizes and releases Lutenizing Hormone Releasing Hormone (LHRH), which stimulates the pituitary to secret Lutenizing Hormone (LH) and Adrenocorticotropic Hormone (ACTH). LH stimulates the testis to release testosterone and ACTH stimulates the adrenal glands to produce the adrenal androgens such as testosterone, dihydroepiandrosterone (DHEA), DHEA sulfate, and androstenedione. Both the testicular and adrenal androgens stimulate the prostate, but testosterone is responsible for the primary effects (Figure 3). Testosterone circulates in the blood either bound to albumin or sex-hormone binding globulin or freely by itself. The free testosterone can cross the plasma membrane and reach the prostate by diffusion. The testosterone will be converted to DHT by the enzyme 5 alpha-reductase. DHT is the most active androgen that can bind the androgen receptor (AR). AR is part of the superfamily of nuclear receptors which functions by binding small cytoplasmic steroid or non-steroid ligands. AR contains a DNA binding domain, a hinge region, a hormone binding domain, and a N-terminal variable domain. When AR is inactive, it is associated with heat shock proteins and located in cytoplasm. When DHT binds to the androgen receptor, the heat shock proteins are released. The ligand-binding AR dimerizes and translocates to the nucleus to bind androgen responsive elements in DNA and thus to activate transcription of target genes which involved in the growth and survival of the cell (Figure 4).
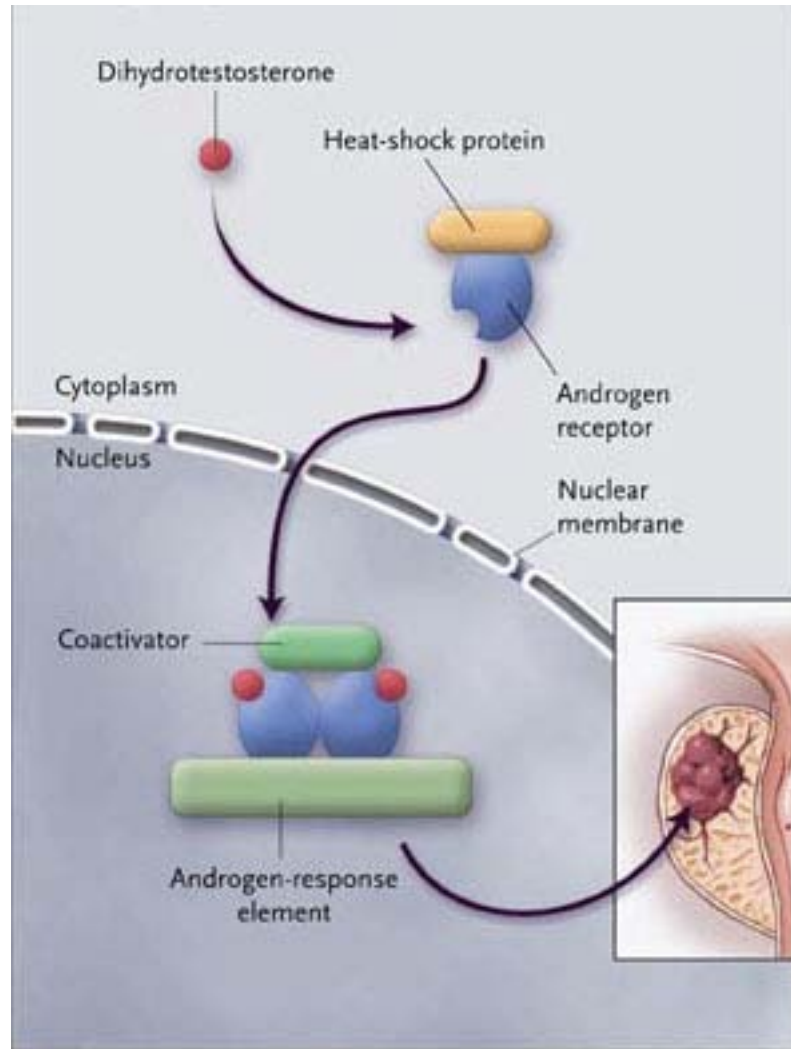
Figure 4. AR action in the prostate. DHT binds AR and causes the release of heat shock protein. Ligand binding induces receptor dimerization followed by translocation to the nucleus. Dimerized AR and AR coactivator together bind to androgen-response element in the promoter region of androgen-regulated genes to active the genes and lead to biological responses. (Debes and Tindall, 2004)

Androgen and AR are critical for prostate development and normal prostate function (Abate-Shen and Shen, 2000; Heinlein and Chang, 2004; Cunha et al., 1987; Roy et al., 1999; Yeh et al., 2002). In AR knockout mice or testicular feminized mice or mice with inactivating mutations of AR, the prostate does not develop (Heinlein and Chang, 2004;

Cunha et al., 1987; Yeh et al., 2002; Quigley et al., 1995; Lyon and Hawkes, 1970). In the mature prostate, the proliferation and differentiation of the prostatic epithelial cells is mainly regulated by androgens (Feldman and Feldman, 2001; Heinlein and Chang, 2004; Cunha et al., 1987). Androgens promote cell survival and inhibit apoptosis (Feldman and Feldman, 2001; Heinlein and Chang, 2004). To maintain the normal size of the prostate gland, the number of cells that undergo proliferation and apoptosis have to be kept in balance.

AR is required for normal prostate function and is expressed in PIN and early carcinoma, advanced carcinoma, and metastatic carcinoma. Androgens regulate the proliferation rate of epithelial cells, which become the adenocarcinoma. So androgens that increase AR activity may result in uncontrolled cell proliferation of the cancer. PSA, an androgen regulated gene, can be monitored in the bloodstream as a marker for prostate cancer.

Initially, the prostate cancer growth is dependent on androgens; when androgens are removed, the tumor undergoes apoptosis and regression. When Clarence V. Hodges and Charles B. Huggins reported this observation in 1941, androgen ablation therapy was used to treat prostate cancer patients and it was very effective for a certain period of time (Huggins and Hodges, 1941; Taplin et al., 1995; Brewster and Simons, 1994). However, most of the tumors eventually become refractory to androgen ablation and develop into androgen-independent tumors. The mechanisms behind this progression is still not very clear, it appears to vary from patient to patient and has been difficult to study.

The mechanisms for the development of Castration Resistant Prostate Cancer (CRPC) can be divided into main two pathways: one involves the androgen receptor and another bypasses the androgen receptor (Figure 5) (Grossmann et al., 2001; Debes and Tindall, 2004). When the androgen receptor remains involved in prostate cancer cells survival, then AR amplification, AR mutations, alteration of AR coactivators or deregulation of growth factors/cytokines may occur (Feldman and Feldman, 2001):
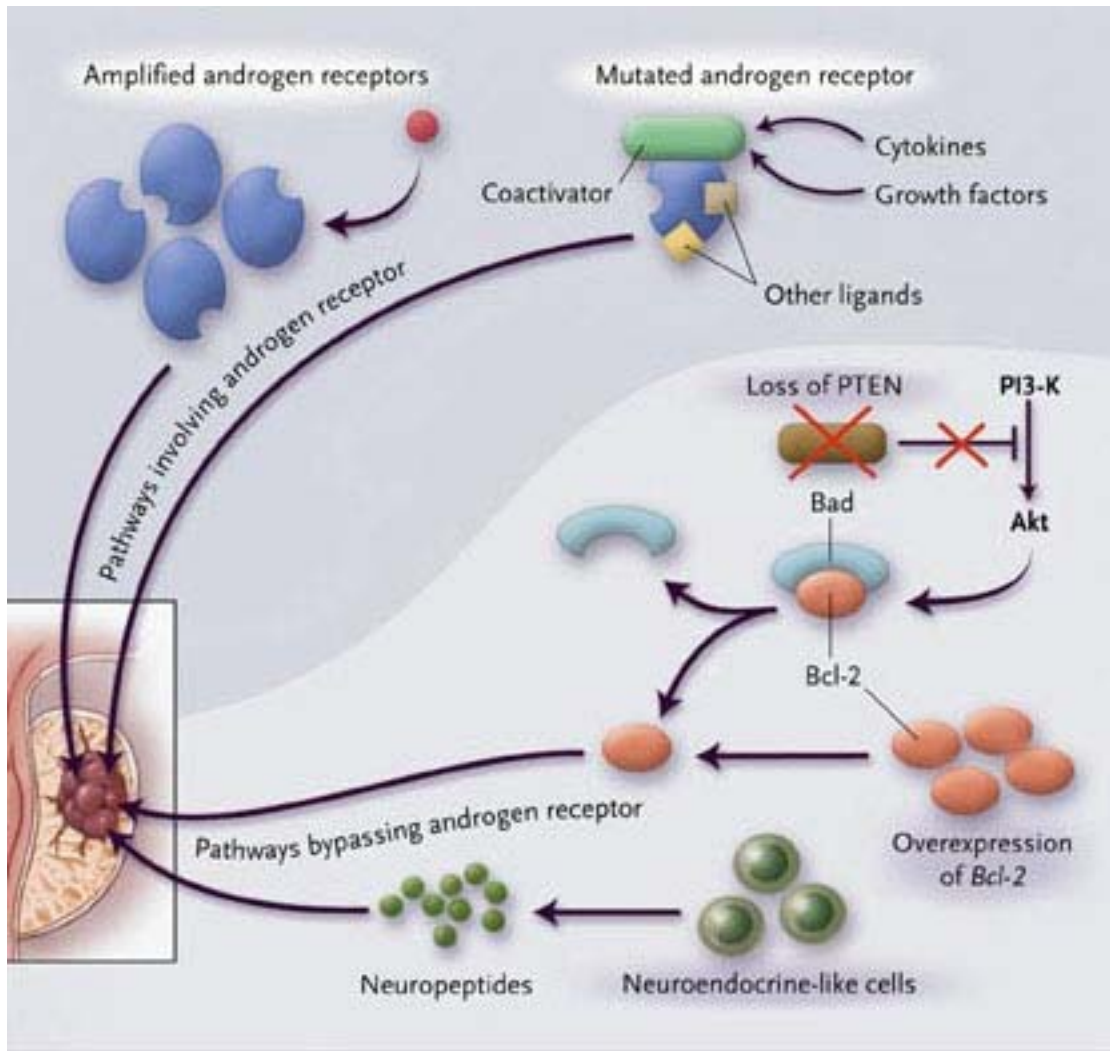
Figure 5. Androgen-Independent Progression of Prostate Cancer. Multiple mechanisms for a cancer cell to escape androgen deprivation therapy and become androgen independent. (Debes and Tindall, 2004)

1. Amplification of AR functions to enhance the AR response to low levels androgen. Androgen receptor gene amplification is observed in about a third of patients with CRPC, but not in hormone dependent prostate cancer patients. The AR gene amplification causes the overexpression of the androgen receptor and the receptors can response to low levels of androgens (See review by Debes and Tindall (Debes and Tindall, 2004)). Interestingly,

studies have found that the CRPC patients with amplification of the androgen receptor gene survive longer than patients without amplification of the gene (Debes and Tindall, 2004).

2. Mutation of AR results in its binding of other ligands or antiandrogens. The androgen receptor gene in androgen-independent tumors may have mutations, which can increase the number of ligands that can activate the receptor (Debes and Tindall, 2004). In normal prostate cells AR is activated by DHT, when it is mutated AR can respond to other steroids or antiandrogens which could activate the downstream gene expression (Debes and Tindall, 2004). This mechanism accounts for failure to respond to therapy in a small portion of CRPC patients.

3. Overexpress of AR coactivators. The AR action pathway in prostate involves different coactivators, growth factors or cytokines which could facilitate the activation of androgen receptor. In some CRPC patients, several growth factors such as insulin-like growth factor I or cytokines such as interleukin-6 or other activators have been found to be overexpressed (Debes and Tindall, 2004).

Another type of pathways that androgen-dependent prostate cancer cells use to survive is to completely bypass the androgen receptor. There are two subtypes:

1. Neuroendocrine cells. In androgen-independent prostate cancer neuroendocrine cells are more widespread compared to androgen-dependent prostate cancer. Neuroendocrine cells have a higher survival rate than epithelial cells during most

chemotherapy, endocrine and radiation treatments due to their low proliferation rate. The proliferation of cancer cells that surrounding the neuroendocrine cells is stimulated by the neuropeptides secreted by the neuroendocrine cells (Debes and Tindall, 2004).

2. Deregulation of apoptotic genes. The tumor suppressor gene PTEN and the antiapoptotic gene Bcl-2 play critical roles in androgen-independent prostate cancer. In normal prostate cells, PTEN inhibits the phosphatidylinositol 3-kinase pathway and allows cells to undergo apoptosis. Loss of PTEN function is always found in androgen-independent prostate cancer patients and the inactivation of PTEN increases Akt activity to block apoptosis (Debes and Tindall, 2004). Studies have shown that androgen ablation can cause the overexpression of Bcl-2, which increases cells survival (Debes and Tindall, 2004).

Tissue-specific gene expression

The development of a complex eukaryote requires differential transcription of over 20,000 genes in precise spatial and temporal patterns (Carey, 1998). How could an organism have such diversity while simultaneously maintaining cell specificity and responding dynamically to its environment? In 1974 Gierer proposed the paradigm of combinational gene control which assumes that the regulatory protein added to each new cell must be self-perpetuating, giving that cell a memory (Gierer, 1974; Alberts et al., 1994). The regulatory proteins added during development need not initially change gene expression but may await the expression of a final regulatory protein which then dispatches the cell to its final transformation. This cascade can account for cell

determination where the cell is assigned a developmental fate, and subsequently cell differentiation where the assigned cell now emerges with its own unique character. As few as twenty-five regulatory proteins can potentially specify 10,000 different cell types (Alberts et al., 1994). Thus, the regulatory proteins need not be unique. Rather, they must appear in the proper combination of multiple factors to result in tissue-specific expression (Gierer, 1974). This view has evolved through our understanding transcriptional synergy (Figure 6) (Carey, 1998; Brivanlou and Darnell, Jr., 2002; Fry and Peterson, 2002). A rough estimation suggests that 2000-3000 transcription factors exist in mammals. They are: 1) general transcription factors that are ubiquitously distributed and constitutively important for most genes; 2) conditional transcription factors that are signal-dependent, such as steroid receptors; 3) cell specific factors that are restrictively distributed and expressed in certain cell types during development (Brivanlou and Darnell, Jr., 2002). Combinatory use of these factors could lead to an exponentially large number of regulatory decisions, ensuring that each gene is expressed in the right place and at the right time under a unique regulation (Carey, 1998).

POSITIVE-ACTING TRANSCRIPTION FACTORS

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                    I                        II
             CONSTITUTIVE              REGULATORY
                 Sp1                   (CONDITIONAL)
                CCAAT
                 NF1
         A                                          B
   DEVELOPMENTAL                      SIGNAL DEPENDENT
   "CELL SPECIFIC"

     GATA          STEROID                                    CELL SURFACE
     HNFs          RECEPTOR          INTERNAL             RECEPTOR-LIGAND
     Pit1          SUPERFAMILY       SIGNALS
     MyoD            AR              SREBP                                    LATENT
     Myf5            GR              P53           RESIDENT             CYTOPLASMIC
     Bicold          ER              STEROID       NUCLEAR                 FACTORS
     Hox             PR              'ORPHANS'     FACTORS                  STATs
     Nkx             TR                              ETS                    SMADs
  Winged Helix       RARs                           CREBs                 NFKB/Rel
   Forkhead          RXRs                           ATMs                  CI/GLI (HH)
                     PPARs                        SRF (MADS box)          NOTCH (NICD)
                                                  FOS-JUN               Wnt/B-catenin
                                                   MEF-2                   TUBBY
                                                                           NFAT
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Figure 6. Positive-acting transcription factors. (Matusik et al., 2008)

In the regulatory regions of some well characterized genes, multiple proteins are required to act on the same "enhanceosome" for optimal and specific gene expression. The underlying mechanisms for cell specific gene expression are currently under active studies. Several transcription factors that are specific for pituitary (Sornson et al., 1996), muscle (Arnold and Winter, 1998), testis (Xu et al., 1994), mammary gland (Wakao et al., 1992), and prostate (Bieberich et al., 1996; Oettgen et al., 2000) have been described. For example, the rat prolactin (rPRL) gene promoter has been well characterized. The expression of rPRL gene, which is restricted to the pituitary glandular cells, is under a stringent tissue-specific control. The rPRL gene expression is cell-specific and

hormone-dependent, which result from the regulation of both cell-specific and ubiquitous transcription factors (Gourdji and Laverriere, 1994). Pituitary-specific transcription factor Pit-1 and LSF-1 together with ubiquitous TFs Oct-1, BTF, F2F, and ER are binding at the distal enhancer and/or proximal promoter of rPRL gene to regulate the gene expression (Bodner et al., 1988; Jackson et al., 1992; Gutierrez-Hartmann et al., 1987; Ingraham et al., 1988; Maurer and Notides, 1987; Waterman et al., 1988; Day and Maurer, 1989). When the minimal DNA promoter elements necessary to control tissue specificity have been defined in species ranging from drosophila to man, the complete cascade will appear to require four to eight regulatory proteins, with the mean being only five regulatory proteins (Arnone and Davidson, 1997).

Cell determination for the reproductive glands occurs in the embryo but the final stages of cell differentiation take place after birth and during sexual maturation. For example, androgen resistance prevents normal embryonic and pubertal development of the genotypic male. Mutations in the androgen receptor result in androgen insensitivity syndrome (AIS, formerly termed Tfm) where patients are genotypic males but the resulting phenotype is female or they may appear with ambiguous external genitalia (Kazemi-Esfarjani et al., 1993; Pinsky et al., 1992; Brown et al., 1990; Wiener et al., 1997). In Tfm mice, the Wolffian ducts degenerate and a female-like urethra, a shortened vagina, and external female genitalia develop in the male. The Tfm mice never develop prostates. Clearly, the androgen receptor plays a vital role in the development of the male reproductive tract (Sugimura et al., 1986). The role differs in mesenchymal and epithelial cells. Since the Tfm mouse reflects the AIS human phenotype, the mouse model permits a

dissection of the critical role that the mesenchymal-epithelial cell interactions play in organogenesis (Philip et al., 1994; Cunha, 1994). Cunha's seminal work showed that Tfm mesenchymal cells, when recombined with wild type epithelial cells from the urogenital sinus (UGS) and implanted into the kidney capsule, would histologically develop into an organ that was vaginal-like whereas wild type UGS mesenchymal cells combined with Tfm epithelial cells would result in a differentiated prostate (Cunha and Lung, 1978). Cunha went on to prove that female vaginal stroma could direct epithelium to form a prostate in response to androgens but that these vaginal stromal cells increasingly lost this ability when the newborn mouse reached 20 days of age. However, vaginal epithelium could always be converted to a ductal prostate structure by UGS mesenchymal cells in the presence of androgens (Cunha et al., 1980). Therefore, the androgen receptor is a critical trigger in the mesenchymal cells (UGS or newborn vaginal) to induce prostatic ductal growth and epithelium differentiation (Hayashi et al., 1993; Donjacour and Cunha, 1993). However, AR must function with other TFs to determine prostate function since many organs contain AR but do not express prostatic genes. This process is completed during sexual maturation (Cunha et al., 1987).

Prostatic cell determination during prostate development involves the establishment of unique regulatory programs that eventually lead to cell differentiation resulting in prostate-specific gene expression. Several genes, such as prostate specific antigen (PSA), PB, prostatic steroid-binding protein gene C3 (1), prostate-specific membrane antigen (PSMA), human glandular kallikrein 2 (hKLK2), prostate-specific transglutaminase gene, and prostatic acid phosphatase (PAP) are almost exclusively expressed in prostate. These

genes provide unique insight to prostate specific gene expression. Schuur et al first described three regions located at the upstream enhancer (-4.8kb to -3.8kb) of PSA gene that showed prostate-specific binding (Schuur ER et al., 1996). The core enhancer region (-4168 to -3801bp) of PSA was further characterized as a highly AR-responsive and prostate-specific element (Cleutjens et al., 1997; Huang et al., 1999). Farmer *et al.* showed that this element interacts with a prostate-specific factor and is essential for maximal activation of PSA in transient transfection assays (Farmer et al., 2001). Similarly, using *in vitro* protein-DNA interaction analyses, Celis *et al.* found that an unidentified prostate-specific factor bound to an A/T rich DNA element in the first intron of PBP C3 (1) subunit gene (Celis et al., 1993). These observations indicated the existence of factors that are involved in prostate-specific regulation although the corresponding factors have not yet been identified. These early results are supported by several recent discoveries. The prostate derived ets factor (PDEF) was recently described as a novel prostate-specific transcription factor that activates PSA gene expression (Oettgen et al., 2000). However, more recent observations pointed out that PDEF is also overexpressed in human breast tumors (Ghadersohi and Sood, 2001). Nkx-3.1, a homeobox gene, serves a negative role (Bhatia-Gaur et al., 1999). Phenotypic analyses of Nkx-3.1 knockout mice demonstrated prostate epithelial hyperproliferation (Bhatia-Gaur et al., 1999). A more recent yeast two hybrid screen surprisingly showed that Nkx-3.1 interacts with PDEF and regulates PSA promoter antagonistically (Chen et al., 2002). These findings provide promising prospects for identifying factors contributing to tissue specificity (See review by Matusik et al. (Matusik et al., 2008)).

Prostate Specific Gene Expression

    A. Probasin and probasin promoter.

    Probasin (PB: PROstatic BASic protein) was identified in the rat dorsolateral prostate lobes as a protein of 177 amino acids with an isoelectric point of 11.5. It is specifically expressed in prostatic epithelial cells (Matuo et al., 1982; Yamamoto et al., 1989). The PB gene contains 7 exons (Kasper and Matusik, 2000) and the alternative use of an initiation codon can produce either a secreted protein with a signal peptide or a nuclear protein without the signal peptide (Spence et al., 1989). The PB protein belongs to the lipocalin superfamily of proteins, it shares the lipocalin conserved motifs such as the glycine-X-tryptophan (G-X-W) sequence, the C-X-X-X-C sequence (Newcomer, 1995; Du and Prestwich, 1995), and two conserved cysteines. It most closely shares the similar amino acid sequence and tertiary structure of the lipocalins that include aphrodisin, rat odorant binding protein, rat alpha2 urinary globulin, mouse major urinary globulin, retinol binding protein and bovine beta-lactoglobulin (Kasper and Matusik, 2000). All of the lipocalin proteins have eight antiparallel beta-sheets with a beta-barrel core binding the hydrophobic ligands. Since aphrodisin, OBP, rat alpha2 urinary globulin and MUP are pheromone carriers, it is reasonable to postulate that PB may be a pheromone carrier as well, although the ligand of PB is still unknown (Kasper and Matusik, 2000).

    Although the protein function of PB is unknown, the promoter of the PB gene has been well studied and the prostate-specificity of this gene promoter can be used as a tool to direct gene expression to the prostate. The Chloramphenicol Acetyltransferase (CAT)

reporter gene driven by a -426PB promoter showed specific and high expression in human prostate cell lines (LNCaP and PC-3), but negligible expression in non-prostate cell lines (HepG2, MCF-7, CHO, BL13) by *in vitro* experiments (Brookes et al., 1998). *In vivo* experiments indicate that the large PB promoter fragment (~12 kb or the artificial promoter) (Yan et al., 1997), the small 5'-flanking PB fragment (-426/+28) (Greenberg et al., 1994), and the ARR$_2$PB promoter (Zhang et al., 2000) all targeted gene expression in the prostate in transgenic mice. In the ARR$_2$PB promoter construct, an Androgen Responsive Region, ARR, (-244 to -96 bp of the PB promoter) serves as enhancer that is repeated in front of -286/+28 bp PB promoter. Experiments also confirmed that the -286/+28 bp fragment of PB promoter is sufficient to target the prostate in transgenic mice (Zhang et al., 2000).

Different genes have been targeted to mouse or human prostate using the PB promoters. SV40 early region has been targeted into mouse prostate to create the TRAMP mouse by using the -426/+28 PB promoter; the large T antigen (a deletion removes the small t antigen) has been targeted into the mouse to create the LADY mouse models (Greenberg et al., 1995; Kasper et al., 1998). Additionally, therapeutic genes were targeted to the prostate tumor cells for gene therapy purposes: Adenoviral delivery of pro-apoptotic Bax gene directed by ARR2PB promoter induces apoptosis in LNCaP prostate cancer cells (Lowe et al., 2001; Andriani et al., 2001); Spencer et al. took advantage of the ARR2PB-direted death switch protein caspase-9 to elicit prostate cancer cell suicide (Xie et al., 2001); and Handerson et al. reported that, a novel adenovirus CV787, with the rat PB promoter driving the adenovirus type 5 E1A gene, destroys

prostate cancer cells 10,000 times more efficiently than non-prostate cells and has powerful therapeutic efficiency for human metastatic prostate cancer which is always intractable for surgical treatment (Yu et al., 1999).

The probasin promoter has become the promoter to target genes to prostate epithelial cells in transgenic mice and to be used in gene therapy constructs to target therapeutic genes to prostate cancer cells.

B. Probasin promoter is regulated by androgen receptor and other transcription factors (TF).

Studies show that PB mRNA level dramatically drops below detection limits in castrated animals, but after androgen treatment of the castrated animals PB mRNA level was found to restore to the pre-castration level (Greenberg et al., 1994; Yan et al., 1997). In transgenic mice, the expression level of the PB promoter directed CAT transgene increased consistently with the increasing serum androgen level during animal maturation, and CAT expression reached a peak when the animal reached sexual maturity at six week of age (Yan et al., 1997). These facts suggest that the expression of the rat PB transgene in transgenic mice was specifically androgen-regulated. Two Androgen Responsive Elements (ARE) were identified within the -426 to +28bp of PB 5' flanking region by DNase I footprinting and Electrophoretic Mobility Shift Assays (EMSA) (Rennie et al., 1993; Kasper et al., 1994; Kasper et al., 1999). The two sites were named as Androgen Receptor Binding Site 1 (ARBS1) and ARBS2. They are located at -236 to -223bp and -140 to -117bp, respectively. A single point mutation in either binding site can

dramatically reduce AR binding on both sites and it would cause more than 95% loss of CAT transgene expression (Rennie et al., 1993). These experiments confirmed the dependence of PB promoter activity on AR. Both *in vivo* and *in vitro* experiments showed that this hormone regulatory effect is specific for AR. Therefore, the region of -244 to -96bp designated as being responsible for the full androgen induced activity of the PB promoter. This fragment was named Androgen Responsive Region (ARR). By making deletion constructs over a 10kb fragment of the upstream region of the PB gene, two additional ARBSs (ARBS3 and ARBS4) between -700 to -400 bp have been identified. These sites cooperate with ARBS1 and ARBS2 to confer a potent androgen induced activity (Zhang J-F et al., 2010). AR is expressed not only in prostate tissues, but in non-prostate tissues such as liver, kidney, testis, bladder, seminal vesicle as well, which do not express probasin and AREs are also found in promoters of genes which are expressed in non-prostate cells; thus it is not possible for AR alone to dictate the tissue selectivity of PB.

In order to identify other DNA binding elements within the -286/+28 bp PB promoter, DNA sequences were mutated every ten base pairs to cover the -286 to +28 promoter region resulting in 31 continuous link-mutation constructs (Zhang J-F et al., 2010). Besides ARBS1, ARBS2, and the TATA box, five additional *cis*-acting elements were identified as key regulatory sites (Zhang J-F et al., 2010) (Gao et al., 2003). Three of the five elements are occupied by ubiquitous TFs: NF-1, c-jun, and Oct-1. When these sites were mutated individually, they reduced the bioactivity of the PB promoter in both prostatic and non-prostatic cell lines (Figure 7). The binding of NF-1, c-jun, and Oct-1 to

the PB promoter has been confirmed by EMSA, antibody supershift, and consensus TF site competition (Zhang J-F et al., 2010).
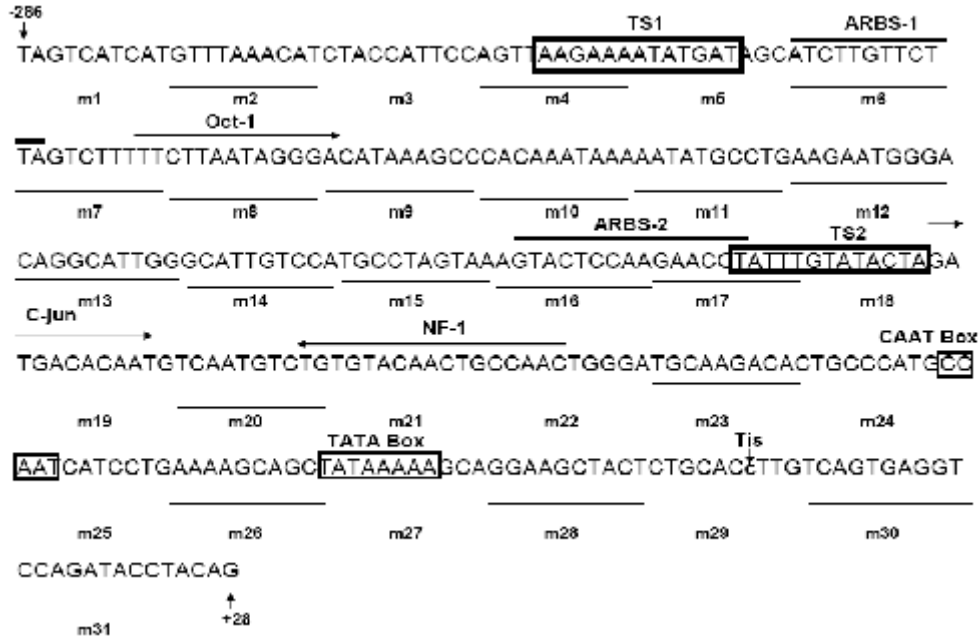
Figure 7. PB promoter. PB proximal promoter region (-286 to +28) linker scanning mutagenesis disclosed five additional key cis-acting elements including two tissue specific elements (TS1 and TS2).

Two elements were referred to as TS1 and TS2 (Tissue Specific). When TS sites are mutated, the bioactivity is reduced only in prostatic cells lines, but not in non-prostatic cells (Gao et al., 2003). TS1 is immediately upstream to ARBS1 and TS2 is immediately downstream to ARBS2 (Gao et al., 2003). DNA alignment of TS1 and TS2 revealed sequence similarity, EMSA and nuclear protein fractionation revealed nearly identical protein binding patterns of TS1 and TS2 (Gao et al., 2003). Southwestern and EMSA disclosed that both TS1 and TS2 harbor a FoxA1 binding sites (Gao et al., 2003). The

FoxA1 binding sites are confirmed to be essential for maximum induction by androgens since point mutations in FoxA1 binding sites block activation of the PB promoter (Gao et al., 2003). Further, southwestern analyses mapped at least two small proteins bound to TS2 at 52 kDa (FoxA1) and 46 kDa (unknown at the time but identified in this thesis as USF2) as well as the large protein at 113 kDa identified as AR plus additional weaker bands (Gao et al., 2003). The FoxA1 and AR bands have been confirmed (Gao et al., 2003). Mutation of the FoxA1 site prevents expression in transgenic mice. Our data strongly suggests that FoxA1 functions as a critical component in a TF complex that controls androgen regulated and prostate-specific expression of PB.

FoxA

Originally the FoxA proteins were called Hepatocyte Nuclear Factor 3 (HNF3). HNF3 proteins were discovered as liver-enriched factors because of their ability to bind the transthyretin gene promoter (Lai et al., 1991; Lai et al., 1990). The three FoxA proteins, FoxA1 (HNF3alpha), FoxA2 (HNF3beta) and FoxA3 (HNF3gamma), are encoded by different genes on different chromosomes (Kaestner et al., 1994), yet they share about 85% sequence identity in the DNA-binding domain (Lai et al., 1991), which exhibits 82% similarity with the Drosophila forkhead protein (Weigel and Jackle, 1990). The FoxA proteins bind to the same consensus binding site but each exhibit different affinity. The forkhead domain (FH) displays a novel protein folding called "winged helix", which contains three alpha-helices flanked by two loops, or "wings" (Clark et al., 1993). Helix 3 makes primary contacts with the DNA major groove and wing 2 makes contacts with the minor groove (Clark et al., 1993). In mouse, FoxA2 is first expressed in

the anterior of the early primitive streak and later in the definitive endoderm, the notochord and the floor plate of the neural tube (Sasaki and Hogan, 1993); FoxA1 is first expressed in the definitive endoderm at the late primitive streak stage(Kaestner et al., 1999); FoxA3 is first expressed in the endoderm after the formation of the gut. Homozygous FoxA2 knockout mice die at embryonic day 10 due to foregut defects and failure to form notochord; homozygous inactivation of FoxA1 results in perinatal lethality (Kaestner et al., 1999); inactivation of FoxA3 is not lethal (Kaestner et al., 1998). Three FoxA proteins are involved in endoderm differentiation, but FoxA2 is important for both formation and maintenance of endoderm. FoxA binding sites were found in numerous liver-expressed gene promoters (Zaret, 1999), and the consensus binding sites 5'-(G/A)(T/C)(C/A)AA(C/T)A-3' were extracted from these reported binding sites (Kaufmann and Knochel, 1996). The FoxA proteins also regulate gene transcription in other gut endoderm-derived lineages, such as lung, pancreas, intestine and thyroid (Kaufmann and Knochel, 1996). To date, nearly 200 FH members have been discovered in a variety of species (Kaestner et al., 2000).

The molecular mechanisms by which FoxA proteins control transcription have only been partially characterized. Three dimensional structure of the FH domain mimics the structure of linker histones H1 and H5, which help to compact chromatin and inhibit transcription (Clark et al., 1993; Zhou et al., 1998). FoxA proteins activate transcription by displacing linker histones from nucleosomes and opening chromatin, making the local DNA region more accessible for other transcription factors (Cirillo et al., 1998). For example, FoxA2 facilitates the binding of glucocorticoid receptor to the

phosphoenolpyruvate carboxykinase gene promoter (Wang et al., 1996; Stafford et al., 2001); while on the vitellogenin B1 gene promoter, FoxA establishes promoter environment favorable to transactivation by estrogen receptor (Robyr et al., 2000). Both amino-terminal and carboxyl-terminal ends of FoxA show transactivation activities (Pani et al., 1992); however, little information exists on co-operative co-regulators that mediate the crosstalk with general transcription machinery. Overproduction of a truncated FoxA protein without both N- and C-terminal domains inhibited the expression of several liver-specific genes (Vallet et al., 1995). Beside its DNA-binding ability, the FH domain also contains a nuclear localization signal (Qian and Costa, 1995). A phosphorylation site for casein kinase was reported for FoxA2 protein, but this phosphorylation event does not affect transcription activity (Qian and Costa, 1995). Direct and indirect evidence point out the cross talk between FoxA transactivation activity and protein kinase A, B, and C pathways. For example, PKA activation improves the recruitment of FoxA to a glucocorticoid responsive element on the tyrosine aminotransferase promoter (Espinas et al., 1995); PKB directly phosphorylates three FH members AFX, FKHR and FKHRL1 to inactivate them (Kops and Burgering, 1999). FoxA1 translocation from nucleus to cytoplasm and inactivation could be caused by TGFbeta treatment; however this effect was abolished by PKC inhibitor (Kumar et al., 2000). Curiously, no report so far could be found about screening for coregulators for FoxA proteins, such as using yeast two hybrid system. Two different groups have identified a novel interaction between estrogen receptor (ER) and a different forkhead member, FKHR, by using ER as a bait in yeast two hybrid screen (Zhao et al., 2001; Schuur et al., 2001). It has been reported that Small Heterodimer Partner (SHP; NR0B2), an atypical orphan nuclear receptor that acts as a

coregulator of various nuclear receptors, physically interacted with FoxA (Kim et al., 2004). Also, FoxA1 is essential for ERalpha binding to the prototypic gene TFF1 as determined by using a combination of genome-wide location, genetic analyses, and functional assays. Furthermore, FoxA1 is required for E2-induced reentry of quiescent breast cancer cells into the cell cycle (Laganiere et al., 2005). Developmental studies showed that FoxA proteins are expressed at a very early stage of mouse embryo development, and in vivo foot printing studies indicated that FoxA is among the first to bind silent genes in early development (Zaret, 1999), Ken Zaret suggested FoxA proteins are "genetic potentiators", which could enhance the activation of genes by facilitating the binding of other TFs during development and differentiation (Zaret, 1999). In conclusion, the expression patterns of FoxA proteins in the early embryo developmental stage and the ability to access as well as to modify compacted chromatin indicate that FoxA proteins are important positive acting TFs that regulate organ differentiation and cell specific gene expression.

Our study shows that FoxA1 is involved in androgen-regulated prostate-specific gene expression. FoxA1 is expressed constitutively in prostate, and prostates rescued from FoxA1-deficient mice displayed basal-like cell hyperplasia (Gao et al., 2003; Gao et al., 2005a). FoxA2 is only expressed in budding prostate during development between E18 to D1 and is dramatically reduced in the adult prostate tissue to barely detectable levels (Mirosevich et al., 2005b). However, FoxA2 is expressed in both normal and cancerous prostate neuroendocrine (NE) cells. Surprisingly, FoxA2 can convert the androgen-dependent PSA promoter to function in an androgen-independent manner

(Mirosevich et al., 2005a). This suggests that FoxA2, or a closely related forkhead protein, may represent a common mechanism by which prostate cancer can progress from androgen-dependent to androgen-independent disease. FoxA2 can regulate androgen-dependent prostatic genes in an androgen-independent fashion, FoxA2 but not FoxA1 is expressed in the epididymis. Furthermore, FoxA2 interacts with the AR to regulate the mouse epididymal retinoic acid binding protein (mE-RABP) gene, an epididymis-specific gene (Yu et al., 2005). Glutathione-S-transferase (GST) pull-down assays determined that both FoxA1 and FoxA2 physically interact with the DNA binding domain of the AR (Gao et al., 2003; Yu et al., 2005). (See review by DeGraff et al. (DeGraff et al., 2009))

USF2

The upstream stimulatory transcription factor (USF) was originally identified as a cellular transcription factor that is critical for maximal expression of the major late promoter of adenovirus both *in vivo* and *in vitro* (Sawadogo and Roeder, 1985; Carthew et al., 1985; Miyamoto et al., 1985). Two different polypeptides of USF were first purified from HeLa cell nuclei with molecular weight of 43 kDa and 44 kDa (Sawadogo et al., 1988). From both human and mouse, USF cDNA clones were isolated and indicated the 43 kDa (USF1) and 44 kDa (USF2) polypeptides were the products of two different genes (Gregor et al., 1990; Sirito et al., 1992; Sirito et al., 1994). Both USF1 and USF2 are expressed in different cell types and they can either form homodimers or heterodimers (Sirito et al., 1994). The DNA consensus sequence of USF is identified by the presence of a central CACGTG motif, which includes the canonical CANNTG that

are recognized by the helix-loop-helix transcription factors (Sawadogo and Roeder, 1985; Murre et al., 1989). Studies have found that USF binding sites exist in a variety of cellular and viral genes (Carthew et al., 1987; Chodosh et al., 1987; Lemaigre et al., 1989; Chang et al., 1989; Scotto et al., 1989; Sato et al., 1990; Greaves and O'Hare, 1991; Potter et al., 1991; Zwartkruis et al., 1991; Bungert et al., 1992; Riccio et al., 1992; Reisman and Rotter, 1993). Interestingly, most USF binding sites are also recognized by other transcription factors, such as Myc (Blackwell et al., 1990; Prendergast and Ziff, 1991; Halazonetis and Kandil, 1991), MAX/Myn (Blackwood and Eisenman, 1991; Prendergast et al., 1991), Mad/Mxi (Ayer et al., 1993; Zervos et al., 1993), and TFE3/TFEB (Beckmann et al., 1990; Carr and Sharp, 1990).

USF2 binds to the conserved E-box (CANNTG) site and belongs to the basic-helix-loop-helix-leucine-zipper (bHLH-zip) family of transcription factors (Gregor et al., 1990; Sirito et al., 1992). USF2 has been confirmed to occupy the same binding site as FoxA1 on the PB promoter (Kivinen et al., 2004). USF2 has a weak interaction with AR together with other proteins that bind to the same site to enhance the transactivation function of AR (Kivinen et al., 2004). Western blot analysis has revealed that USF2 expression is decreased in androgen-independent prostate cancer cell lines, PC-3, DU145, and M12 (Chen et al., 2006). Overexpression of USF2 in PC-3 cells could inhibit anchorage-independent growth and the tumorigenicity of PC-3 cells (Chen et al., 2006). USF2 can inhibit Myc-dependent transformation in rat embryo fibroblast (Luo and Sawadogo, 1996). USF2 knockout mice demonstrated prostate hyperplasia suggesting that USF2 may play an important role in prostate differentiation and prostate

carcinogenesis (Chen et al., 2006).

Hypothesis

In summary, we believe that FoxA1 and its binding partners form a protein complex with AR and other TFs which is capable of directing prostate-specific gene expression and that this or a similar complex plays a critical role in the androgen regulation of tumor growth. Purification and identification of the interacting partners of FoxA1 will facilitate our understanding of how these TFs/AR interactions regulate tumor growth.

The overall hypothesis is that the FoxA1, AR, and other FoxA1 interacting partners co-operate together to direct androgen regulated gene expressions.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

The human prostate carcinoma cell line LNCaP was obtained from American Type Culture Collection. LNCaP cells were cultured in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum.

NeoTag cell line was generated by our lab. The androgen-regulated probasin promoter linked to the neomycin phosphotransferase (Neo) gene was used to generate the ARR_2PBneo transgenic mouse model. All six ARR2PBneo transgenic founder lines expressed the Neo gene in a prostate-specific manner. Line C, which expressed high levels of neo, was crossbred to LPB-Tag 12T-7f transgenic mice (in which the SV40 large T antigen (Tag) was targeted to the prostate by the large probasin (LPB) promoter). Three bigenic males (carrying both Neo and Tag transgenes) were identified. Three separate NeoTag epithelial cell strains were established from three bigenic mice. G418 selection was used to obtain immortalized epithelial cells in culture. Selected cells expressed both the Neo and Tag transgenes, cytokeratins 8 and 18, and were androgen responsive for growth (Wang et al., 2006).

Expression Vectors and Reporter Plasmids

A. Expression Vectors

Mammalian FoxA1 (HNF-3α) expression vector pRB-HNF-3α was kindly provided

by Dr. Kenneth S. Zaret (Fox Chase Cancer Center, Philadelphia, PA). Full-length wild type and FoxA1 subdomain fragments were PCR amplified and directionally cloned into pcDNA3.1D/V5/His-TOPO expression vector (Invitrogen Carlsbad, CA), in frame with the carboxy-terminal V5 epitope and 6⨉His Tag by Dr. Nan Gao (a previous graduate student in Dr. Matusik's lab) (Gao et al., 2003).

pSG5-mUSF2 (containing wild type mouse USF2), pSG5-U2d(7-178) (containing USF2 with 7-178 a.a. deletion), and pSG5-U2dE5 (containing USF2 with exon 5 deletion) vectors were kindly provided by Dr. N Chen (Department of Molecular Genetics, U.T. M.D. Anderson Cancer Center). USF2 (a.a.1-a.a. 235) (USF2 amino acid 1 to 235) was cloned by PCR using pSG5-mUSF2 as a template. All four USF2 sequences were cloned into pGEX-4T-1 to express GST-USF2 fusion proteins: GST-USF2, GST-USF2-ΔE5, GST-USF2-ΔN (Aa 7-178 deletion), and GST-USF2-ΔC (Aa 1- 235). pGEX-4T-1 vector was kindly provided by Dr. Guanglei Zhuang from Dr. Jin Chen's lab (Cancer Biology department, Vanderbilt University Medical Center).

B. Luciferase Reporter Plasmids

ARR$_2$PB-Luc reporter (ARR is the androgen response region of PB promoter) contains an additional androgen response region (-244 to -96 bp of PB promoter) fused upstream of the -286 to +28 bp of PB promoter to enhance the androgen response (Zhang et al., 2000).

A 621 bp fragment of PSA minimal promoter (-610 to +11 *nt*) was amplified by PCR and cloned at the *SmaI* and *XhoI* sites of pGL3-basic luciferase vector (Promega Corp., Madison, WI), thus a 823 bp upstream enhancer fragment (-4758 to -3935 bp) containing

the PSA core enhancer region was obtained by PCR and inserted upstream of the PSA promoter at *SacI* and *SmaI* sites, resulting in PSA-Luc reporter construct.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extract for LNCaP cells was prepared as described previously CITE. All nuclear extracts and purified proteins were stored in buffers containing 1x concentration of Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN). All oligonucleotides for EMSAs were purchased from Integrated DNA Technologies (Coralville, IA). Probes were end labeled with [γ-32P]ATP (Amersham Pharmacia Biotech, Pittsburgh, PA) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA), and purified by 15% PAGE. A typical binding reaction involved a 10-min preincubation with 10 μg of nuclear extract, 1 μg of the nonspecific competitor poly (dI-dC), and buffer D [20 mM HEPES-NaOH (pH 7.9); 100 mM KCl; 0.2 mM EDTA; 1.5 mM MgCl2; 1 mM dithiothreitol; 20% glycerol; and 1 mM phenylmethylsulfonyl fluoride (PMSF)], followed by a 15 min incubation with 200,000 cpm of radiolabeled probe in a total volume of 20 μl. For supershift analysis, antibodies were added after the binding reaction and incubated for an additional 20 min on ice before electrophoresis. All supershift antibodies [USF2 ((N-18)X sc-861X), USF1 ((H-86)X sc-8983X), GATA-3 ((H-48)X sc-9009)] were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The concentration of antibody in each EMSA reaction was 0.2 μg/μl. Complexes were resolved by electrophoresis for 2.5 h at 160 V on a 5% native polyacrylamide gel, which was later dried and processed for autoradiography.

Western Blotting

For Western blotting analysis, cell pellets were collected, and sonicated in cold lysis buffer [0.01 M Tris-HCl (pH 7.4), 0.01 M NaCl, 1 mM EDTA, in 1x concentration of Complete Protease Inhibitor Cocktail], followed by centrifugation at 14,000 rpm.

Samples were loaded onto a 4%-12% Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) gel and separated at 120 volts for 2.5 hours and transfered to polyvinylidine difluoride membrane (Invitrogen, Carlsbad, CA). Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk, and incubated with primary antibodies [1:1000 dilution: anti-FoxA1 (sc-6553), 1:10000 anti-USF2 (Santa Cruz Biotechnology, Santa Cruz, CA)] for 1 hour at room temperature. Membranes were incubated with secondary antibody (anti-rabbit IgG, GE Healthcare Piscataway, NJ), or anti-goat IgG, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. In cases where anti-V5-horseradish peroxidase antibody (Invitrogen, Carlsbad, CA) was used to detect the recombinant V5-tagged FoxA1 proteins, a dilution of 1:5000 was used. The signal was visualized by enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Pittsburgh, PA).

Chromatin Immunoprecipitation (ChIP)

The procedure and PCR primers used in this study is listed in Table 1.

Table 1. Primers Used in ChIP	
Name	Sequence
PB-F	TCCCAGTTGGCAGTTGTA
PB-R	CACCAACATCTATCTGATTGGAG
PBdis-F	TTCTATTGGTGCCCAACGAGGCTA
PBdis-R	AGGTCAATGCTAGCAGCAAACCAC
SBP-F	GCCCCTACTGACCCAGTATAGA
SBP-R	GAACTTTGTTTTCTGCTTATCCCTCAG
SBPdis-F	TGCCTGTTTCAATAGGACGAGGGT
SBPdis-R	GATGGCTCAGAAGATGGGAATTGCTG
PSA-F	ACAGACCTACTCTGGAGGAA
PSA-R	AAGACAGCAACACCTTTTTTTTC
PSAdis-F	GATGGTGTTTCACCGTGTTG
PSAdis-R	AGAGTGCAGTGAGCCGAGAT
PSA-RTPCR-F	CTGCCCACTGCATCAGGAACAAA
PSF-RTPCR-R	AGCTGTGGCTGACCTGAAATACCT
hGAPDHf	TGCACCACCAACTGCTTAGC
hGAPDHr	GGCATGGACTGTGGTCATGAG

ChIP assays were performed according to manufacturer's protocol (Millipore, Billerica, MA). Briefly, LNCaP cells were serum starved for 24 hours in RPMI 1640 with 5% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT), and cells were subsequently treated in the presence or absence of 10^{-8} M DHT for 12 hours and then washed with PBS and cross-linked with 1% formaldehyde at 37°C for 10 minutes. Cells were scraped into conical tube, centrifuged for 4 min at 2000 rpm at 4°C, and resuspended in SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1x proteinase inhibitor cocktail] at a concentration of 500,000 cells/100 μl., after which cells were sonicated 10 times for 10 seconds pulses at an output level 2-3 (Fisher Sonic Dismembrator, model 50; Fisher Scientific, Pittsburgh, PA). Cells were centrifuged, and supernatants were collected and diluted in ChIP dilution buffer [0.01% SDS, 1.1% Triton

X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl], followed by preclearing for 30 min with 3 μg of sonicated salmon sperm DNA with protein A agarose (80 μl of 50% slurry in 10 mM Tris-HCl, 1 mM EDTA). Immunoprecipitation (IP) was performed overnight at 4°C with specific antibodies (anti-AR, anti-FoxA1, anti-USF2, anti-FoxA3 antibodies were used for IP, water was used as negative control). Protein A agarose with salmon sperm DNA was then added and incubated for 1 hour to collect immune complexes. Beads were then sequentially washed in low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl) and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA). The complex was eluted twice with 250 μl of elution buffer (1% SDS, 0.1 M NaHCO$_3$) and eluates were pooled. Formaldehyde cross-linking was then reversed by adding 20 μl 5 M NaCl and incubating for 8 hours at 65°C. Eluates were incubated for an additional 1 hour at 45°C with Proteinase K. DNA was extracted using QIAquick Spin Column (Qiagen Inc., Valencia, CA) and used for PCR amplification. The amplified region of PB promoter (-359 bp / -106 bp) includes ARBS-1, ARBS-2, and two FoxA1 binding sites. The amplified region of Spermine Binding Protein (SBP) promoter (-538 bp / -285 bp) includes two ARE and two FoxA1 binding sites (Gao et al., 2005a). The amplified region of PSA core enhancer (-4131 bp / -3938 bp) includes AREIII, and two FoxA1 binding sites (Gao et al., 2003). For each PCR, a distal region was amplified as a negative control.

SYBR green real-time PCR

Real-time PCR and data analysis were performed in a total volume of 25 μl using 96-well microwell plates and MyiQ Single Color Real-time PCR Detection System (BIO-RAD, Hercules, CA). 5 ul of purified DNA sample from ChIP assay, 12.5 μl SYBR green I PCR Master Mix (Applied BioSystems, Foster City, CA), and 500 nM of each primer pair (Table 1) were added to each microwell. To reach a total volume of 25 μl per well, DNase-RNase-free distilled water (Sigma-Aldrich, St. Louis, MO) was added. Following a pre-incubation of reaction mix at 95°C for 10 minutes, the following parameters were used for 40 cycles: 95° C for 45 seconds, 56° C (PB) / 56° C (SBP) / 52° C (PSA) for 1 minute, 72°C for 30 seconds, and an extension phase of 1 cycle at 72°C for 7 minutes. All reactions were performed in triplicate. The amount of DNA in each sample was calculated based on a standard curve, then each DNA sample amount was divided by input DNA amount and graphical data is depicted as the average of the normalized data from three individual reactions. cDNA from USF2 knockdown and control LNCaP cells were used for real time PCR following the same procedure as above. The amount of DNA in each sample was normalized relative to GAPDH. The DNA amount from control LNCaP cells was set as 1 and that of the USF2 knockdown samples were compared with the control.

Co-Immunoprecipitation (Co-IP)

LNCaP cells were washed three times with cold 1x PBS and lysed with 1 ml of nondenaturing lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.02% NaN_3, 50 mM NaF, 1 mM Na_3VO4, 1% NP-40, 1 mM PMSF, 0.5 mM dithiothreitol, and 1x

concentration of protease inhibitor cocktail). After sonication and centrifugation, 1 mg of total cell lysate for each reaction was incubated at 4° C for 3 h with 20 µl (dry volume) protein G-Sepharose beads (Amersham Pharmacia Biotech, Pittsburgh, PA), which were pre-conjugated with 1 µg of experimental antibody (anti-HNF-3α/β (C-20)X, Santa Cruz Biotechnology, Santa Cruz, CA) or goat serum negative control).   Beads were washed four times with lysis buffer and once with PBS for 5 minutes, followed by Western blotting analysis for USF2 (Santa Cruz Biotechnology, Santa Cruz, CA). Reciprocal IP was also performed.   For Co-IP experiments using ethidium bromide (EB) to disrupt DNA-protein interactions, 25 ug/ml and 100 ug/ml was used.

GST-USF2 fusion proteins expression, in Vitro Translation of FoxA1 Proteins, and GST Pull Down assay

GST-USF2 fusion proteins were produced by expressing USF2 fusion protein-encoding vectors in the BL21-CodonPlus(DE3)-RIL strain, which allows the inducible expression of transformed vectors by Isopropyl β-D-1-thiogalactopyranoside (IPTG) addition.   Following IPTG treatment, bacterial cells were harvested and resuspend in lysis buffer (1x PBS, 1% Triton X-100, 1x protease inhibitor), cells were sonicated 3 times for 1-3 minutes at an output level of 3-4. Samples were then centrifuged and the supernatant was transferred to pre-equilibrated glutathione agarose beads, followed by washing (1x PBS, 1x concentration of complete protease inhibitor cocktail), and stored at -80 ^{o}C without elution.

Recombinant FoxA1s and LacZ proteins labeled with a C-terminal V5-epitope were synthesized in vitro using the TNT T7 Quick Coupled Transcription/Translation System

(Promega Corp., Madison, WI)

For GST pull-assays, glutathione agarose beads conjugated GST or GST-USF2 fusion proteins (each contains 20 ug protein) were equilibrated with PBS-T binding buffer [1x PBS (pH 7.4), 1% Tween 20, and protease inhibitors] and incubated for 2 hours at $4^{o}C$ with 10 ul products from the TNT reactions. Complexes were washed four times with 1.5 ml of cold binding buffer, heated for 10 min at $70\,^{o}C$ in 1X LDS loading buffer, and separated by SDS-PAGE. Western blotting analysis with V5-horseradish peroxidase antibody or USF2 was used to detect the domain that involved in interaction.

USF2 Knockdown

LNCaP cells treated in the presence or absence of DHT for over night were transiently transfected with either ON-TARGET Plus SMART pool human USF2 siRNA or ON-TARGET Plus Non-targeting siRNA #2 (DHARMACON RNAi Technologies, Chicago, IL) at a working concentration of 100 nM. Western blotting was performed 48 h and 72 h post transfection to detect verify USF2 knockdown.

Transfection Assays

Transient transfection assays were performed using lipofectamine 2000 for LNCaP cells. The pRL-CMV containing the *Renilla* luciferase reporter gene (Promega Corp., Madison, WI) was used to optimize transfection efficiencies for each cell line. LNCaP cells were planted in 24-well plates in a density of 8-10 X 10^4/well at the first day. Cells were transfected with plasmid DNA and lipofectamine 2000 in Opti-MEM I reduced Serum Medium (Gibco) at the second day. All samples received 12.5 ng/well of

pRL-CMV and the total amount of plasmid DNA was 0.8ug/well. After 6 hours of transfection, the medium was replaced by Minimum Essential Medium (Gibco) with 5% Charcoal/Dextran Treated Fetal Bovine Serum (HyClone, Logan, UT) in the presence or absence of 10^{-8} M of DHT (same amount of 100% alcohol as control). Cells were harvested and lysed with passive lysis buffers after 24 hours of incubation.

Luciferase Assay

The luciferase activity was determined by using the dual luciferase reporter assay systerm (Promega Corp., Madison, WI) and LUMIstar (BMG lab Technologies Durham, NC). Background activity of the cell lysate with no DNA transfection was subtracted from the activities obtained from experimental group. All values were normalized by *Renilla* activity to correct for the transfection efficiency. Results are presented as relative luciferase activities. Each experiment was at least repeated three separate times.

RNA extraction and Reverse Transcriptase PCR

RNA extracted from LNCaP cells (USF2 knockdown LNCaP cells and control LNCaP cells) using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). 2 ug RNA of each sample were reverse transcribed into cDNA using Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA used for real time PCR analysis.

Enzyme-linked Immunosorbent Assays (ELISA)

The DSL-10-9700 ACTIVE PSA ELISA kit (Diagnostic Systems Laboratories, Inc. Webster, TX) was used to examine the PSA level in LNCaP cells medium supernatant. 96

microtitration wells are coated with anti-PSA antibody. Protein-based (BSA) buffer with a non-mercury preservative contains 0 ng/ml PSA is used as negative control, and Level I and II protein-based (BSA) buffer with a non-mercury preservative contain low and high concentration of PSA served as positive controls. The PSA standard curve was made by using protein-based (BSA) buffer contain 1.0, 2.0, 10.0, 25.0, 50.0, 100.0 ng/ml PSA. Samples from the LNCaP cells medium supernatant was dilute 10 times. 25 ul of the samples, standards, and controls are incubated in the 96 microtitration wells on an orbital microplate shaker for 1 hour at room temperature. After 5 times wash each well with Wash Solution using an automatic microplate washer, blot dry by inverting the plate on absorbent material. Then the wells were treated with another anti-PSA detection antibody labeled with the enzyme horseradish peroxidase by adding 100 ul of the Antibody-Enzyme Conjugate Solution to each well. Incubate the wells for 10 min at room temperature. After the washing steps, the wells were incubated with the substrate tetramethylbenzidine. An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm.

The absorbance measured is directly proportional to the concentration of PSA present in the samples. A set of PSA standards is used to plot a standard curve of absorbance versus PSA concentration from which the PSA concentrations in the samples can be calculated.

Establish HeLa-FoxA1 / HeLa-E (empty) HeLa AR-FoxA1/HeLa AR-E and LNCaP-FoxA1 / LNCaP-E cell lines

One FLAG adjacent to 6 X His tag was added in the N-terminal of human FoxA1 cDNA by PCR. The PCR products were cloned into pCR-TOPO 2.1 vector (Invitrogen, Carlsbad, CA). The sequence of tagged FoxA1 was confirmed by DNA sequencing. The retro-virus vector pLPCX (Clontech, Mountain View, CA) was kindly provided by Dr. David Cortez's lab (Biochemistry Department, Vanderbilt University Medical Center). Both pCR-TOPO 2.1-tagged-FoxA1 and pLPCX were digested with Xho I and Hind III, and the tagged FoxA1 was inserted into the pLPCX vector at Xho I/Hind III site. The Phoenix A cells were transfected with the pLPCX-FoxA1 or pLPCX empty vector to generate active viral particles. LNCaP, HeLa and HeLa AR cells were infected by the viruses that were generated by the Phoenix A cells. Selection of the infected LNCaP, HeLa and HeLa AR cells was performed by growing cells in the medium that contains puromycin. The puromycin concentration was increased from 1 ng/ml to 5 ng/ml for LNCaP FoxA1 / LNCaP E cells and 10 ng / ml for HeLa FoxA1 / HeLa E / HeLa AR FoxA1 / HeLa AR E cells.

Nuclear Extract Preparation

Twenty five 150 mm plates each of LNCaP FoxA1 and LNCaP E cells were grown to 90% confluency and were used for a single nuclear extraction experiment. Cell pellet volume (CPV) was measured and pellet was washed with 5 X CPV volume of cold 1 X PBS, and then centrifuged for 5 min at 2000 rpm at 4o C. cells were resuspended in 2 X CPV volume of Buffer A [10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM NaCl, 1mM

DTT, Protease Inhibitor]. Cells were broken with 10 strokes using pestle "A" (loose), spin for 15 min at 3000 rpm. Supernatant was decanted and spin 20 min at 12,500 rpm. Pellet was resuspended in Buffer C [20mM HEPES (pH 7.9), 1.5mM MgCl2, 420mM NaCl, 25% (v/v) glycerol, 0.2mM EDTA, 1mM PMSF, 1mM DTT, Protease Inhibitor] in 2 X CPV volume/109 cells. The nucleus was broken with 10 strokes using pestle "B" (tight), and mixed end-over-end on a rotator for 30 min at 4o C. Spin 30 min at 25,000 g. supernatant was then collected. Dialysis tube was heated with water until boiling. Supernatant was dialyzed in 50 time volume of the Buffer D [20mM HEPES (pH 7.9), 100mM NaCl, 20% (v/v) glycerol, 0.2mM EDTA, 1mM PMSF, 1mM DTT, Protease Inhibitor] for 2 hours per change of buffer, with total 3 changes. After dialysis, samples were spun at 25,000 g for 15 min. The supernatant, which is the nuclear extract, was stored at -80o C.

Tandem Affinity Purification

The nuclear extracts from 109 LNCaP FoxA1 cells / LNCaP E cells were mixed to pre-equilibrated TALON Metal Affinity Resins (Clontech, Mountain View, CA), 1 ml of nuclear extract loads to 250 ul TALON resin (dry volume). Gently agitate the mixture at 4o C degree for 4 hours, and then spin at 700 g for 5 min. Carefully remove the supernatant without disturbing the pellet and wash the resin using 10-20 volumes 1 X equilibration/wash buffer (protease inhibitors added) for 2 times. Remove and discard the supernatant after a spin in the centrifuge. Add one bed volume of the 1 X equilibration/wash buffer to the resin and resuspended by vortexing. Transfer the resin to a 2 ml gravity flow column (with end cap in place). Allow buffer to drain until it reaches

the top of the resin bed, making sure no air bubbles are trapped in the resin bed. Wash the column once with 5 bed volumes of quilibration/wash buffer. Elute the polyhistidine tagged protein by adding 2 X 0.5 ml (pH 7) + 100 mM imidazole elution buffer to the column. Collect the eluate and add to 200 ul pre-equilibrated ANTI-FLAG M2 Affinty Gel (dry volume) (Sigma, Saint Louis, MI) and agitate gently for overnight. Centrifuge the sample next morning for 30 seconds at 5000 g. Remove the supernatant with a syringe carefully and wash the resin for 4 times with 1 ml 1 X PBS (protease inhibitors added). The tagged protein complex was eluted by boiling in Laemmli sample buffer.

<u>Mass Spectrometry Analysis</u>

The eluate from the tandem affinity purification were subject to 10% SDS-PAGE followed by Collioidal Blue staining according to the manufacturer's instruction. Protein bands were visualized by Colloidal Blue staining. The gel was divided into sections for the LNCaP FoxA1 cells. The gel sections that were excised were then subjected to in-gel trypsin digestion with trypsin protease. The corresponding area in the gel of the LNCaP E cells was also processed as negative controls to identify any non-specific proteins bound during the purification. Protein bands of interest were excised and cut into 1 mm cubes and equilibrated in 100 mM NH4HCO3. Proteins were then reduced within the gel pieces with DTT (1/10 volume 45 mM DTT, 50°C for 20 min) followed by alkylation with iodoacetamide (1/10 volume 100 mM iodoacetamide, dark at room temperature for 20 minutes). The gel pieces were then dehydrated with acetonitrile and rehydrated with 15 mL 12.5 mM NH4HCO3 containing 0.01 mg/mL trypsin (Trypsin Gold, Promega), and trypsin digestion was carried out for >2 h at 37°C. Peptides were extracted with 60%

acetonitrile, 0.1% formic acid, dried by vacuum centrifugation and reconstituted in 15mL

0.1% formic acid. 5mL of peptide hydrosylate were analyzed by C18 reverse-phase

LC-MS/MS using a Thermo LTQ ion trap mass spectrometer equipped with a Thermo

MicroAS autosampler and Thermo Surveyor HPLC pump system, nanospray source, and

Xcalibur 2.0 instrument control using standard data-dependent methods. Tandem MS data

were analyzed with the Sequest algorithm against the IPI human database (135,674

entries, October 2007 release) including a concatenated reverse database for calculating

false-discovery rate.

Computational model of AR, FoxA1, and USF2 binding to PB promoter

The x-ray crystal structure of AR binding to AR consensus DNA sequence (1R4I.pdb)

is obtained from the protein data bank (http://www.rcsb.org). The only existing crystal

structure of FoxA is for the FoxA3 protein and its DNA binding sequence (pdt013),

which came from the Nucleic Acid Database (http://ndbserver.rutgers.edu). Since the

forkhead domain (DNA binding domain) of FoxA proteins are highly conserved, the

FoxA3 crystal structure is used for this study. The USF2 X-ray crystal structure has not

been published. An X-ray crystal structure of USF1 and its binding DNA sequence

(1AN4.pdb), which is obtained from the protein data bank (http://www.rcsb.org), was

substituted since USF2 and USF1 have highly conserved DNA binding domains. The

DNA structure of PB promoter is based upon James Stroud's make-na server that uses

David Case's program NAB (http://structure.usc.edu/make-na/server.html) (Macke et al.,

1998). The 3-D model is assembled manually by optimal 3-D alignment of the DNA

oligos from the crystal structure (AR, FoxA3, and USF1) with the NAB-generated

structure of the PB promoter region using the molecular visualization program called UCSF Chimera (http://www.cgl.ucsf.edu/chimera) (Pettersen et al., 2004). This 3-D model can mimic the binding of AR, FoxA1, and USF2 to PB promoter.

CHAPTER III

Upstream Stimulatory Factor 2 Interacts with FoxA1 and is Involved in Prostate

Specific Gene Expression

<u>**Introduction**</u>

The prostate is a male accessory reproductive gland in mammals. Human prostatic secretions are rich in PSA, acid phosphatase, and citric acid components that are found in semen. Androgen plays a key role in the normal development and physiology of the prostate. However, little is known about the basic molecular events that are required for organ determination and cell differentiation of the prostate. Our previous studies show that a member of the forkhead family, FoxA1, interacts with the AR, and that this interaction is required for the expression of prostate specific proteins such as the rodent probasin, Spermine Binding Protein and human PSA (Gao et al., 2003). Prostate tissue rescued from FoxA1 knockout mice exhibits abnormal prostate development, typified by the absence of differentiation markers and the inability to engage in secretion (Gao et al., 2005b). Recent studies have confirmed that FoxA1 and AR binding sites are frequently found adjacent to each other on multiple genes (Wang et al., 2007; Jia et al., 2008; Yu et al., 2005; Yu et al., 2006). Taken together, these studies indicate that FoxA1 plays an essential role in expression of androgen regulated and prostate-specific genes, as well as the normal differentiation of the prostate gland.

Originally the FoxA proteins were termed Hepatocyte Nuclear Factor 3 (HNF3) and

were discovered as liver-enriched factors because of their ability to bind the transthyretin gene promoter (Lai et al., 1990). There are three FoxA proteins, FoxA1 (HNF3α), FoxA2 (HNF3β) and FoxA3 (HNF3γ), which are encoded by different genes on different chromosomes (Kaestner et al., 1994). The FoxA proteins are involved in endoderm differentiation and also regulate gene transcription in various other endoderm-derived organs, including the lungs, pancreas, intestines and thyroid (Kaufmann and Knochel, 1996). Developmental studies have shown that FoxA proteins are expressed at a very early stage of mouse embryo development (Zaret, 1999; Sasaki and Hogan, 1993; Sasaki and Hogan, 1993) For these reasons, FoxA proteins have been referred to as "genetic potentiators", which act to enhance the activation of gene expression by facilitating the binding of other transcription factors during developmental and differentiation-associated events (Zaret, 1999). Thus, the temporal and spatial expression patterns of FoxA proteins and the ability of FoxA proteins to access and modify compacted chromatin indicate that FoxA proteins are important positive acting transcription factors that regulate organ differentiation and cell specific gene expression.

Probasin is a prostate specific protein that was first identified in rat dorsolateral prostate epithelial cells (Matuo et al., 1982; Yamamoto et al., 1989). Although the function of probasin is unknown, the prostate-specificity of this promoter (Greenberg et al., 1994) has resulted in the further development of a potent androgen and prostate specific promoter (Zhang J et al., 2000) that has been extensively characterized and utilized as a tool to target gene expression to the prostate (Wen et al., 2003; Ellwood-Yen et al., 2003; Klezovitch et al., 2004). In our previous study, two FoxA1 binding sites were

identified, which are immediately adjacent to functional AR binding sites in the PB promoter (Gao et al., 2003). A similar organization of the androgen response element adjacent to functional FoxA1 binding sites in Spermine Binding Protein (SBP), prostatic acid phosphatase, C3, and PSA gene core enhancer also were observed (Gao et al., 2003; DeGraff et al., 2009), and the close association between FoxA1 and AR binding sites has been subsequently verified at a larger scale (Jia et al., 2008). However, limited information still exists regarding corresponding co-regulators that mediate the crosstalk of the FoxA1/steroid receptor complex with the general transcription machinery. This is noteworthy because while signal dependent steroid receptors can regulate genes in a tissue-specific manner, specificity is not regulated by the steroid receptor alone. Rather, a unique complex of transcription factors must act with the receptor to restrict expression to a specific cell type (Matusik et al., 2008).

Southwestern analysis, using the androgen receptor binding site 2 of the PB promoter, identified the AR and FoxA1 as well as a third unknown 44 kDa protein binding to this DNA sequence (Gao et al., 2003). We have identified this 44 kDa protein as the Upstream Stimulatory Factor 2, an E-box binding transcription factor of the basic-helix-loop-helix-leucine-zipper (bHLH-zip) family (Sirito et al., 1992). USF2 has a weak interaction with AR, as well as with other proteins that bind to the same site to enhance the transactivation function of AR on the PB promoter (Kivinen et al., 2004). The present study reveals 1) that USF2 binds not only to the PB promoter, but also other prostate specific promoters including the SBP promoter and PSA core enhancer; 2) that a physical interaction between USF2 and FoxA1 is mediated via the DNA binding domain

of USF2 and the forkhead DNA binding domain of FoxA1; 3) that the consensus DNA sequence for the binding sites of FoxA1 and USF2 overlaps critical base pairs permitting binding to the DNA suggesting a direct interaction between these proteins at the same site; and, 4) that FoxA1 and USF2 are bound to the promoters prior to androgenic activation of AR. This data shows USF2 interacts with FoxA1 in a complex with AR to control androgen regulation of prostate specific gene expression.

Results

Analysis of USF2 expression across a panel of cell lines.

The expression level of USF2 was detected in different human prostate cancer cell lines (LNCaP, PC3, and DU145) and in mouse prostate cancer cell lines (NeoTag1, NeoTag2, and NeoTag3) and also in HeLa cells by Western Blot analysis. Figure 8 indicates that USF2 was expressed in all these cell lines. Beta-actin was used as a loading control. The expression of USF2 in LNCaP cells and NeoTag cells allows us to perform further studies of USF2 in those cells. LNCaP cells are used to study the binding of USF2 to human PSA core enhancer, and function studies of USF2 are performed in USF2 knockdown LNCaP cells. NeoTag1 cells are used to study the binding of USF2 to mouse PB and SBP promoters. The expression level of USF2 was reported to have a dramatically decrease in androgen-independent prostate cancer cell lines, PC3 and DU145, compared with androgen-dependent cell line, LNCaP (Chen et al., 2006). From our data, no obvious different of the USF2 expression level is observed among these cell lines (Figure 8). The western experiments have been repeated several times. The different results may be caused by the cell lines alteration after long-term culture.
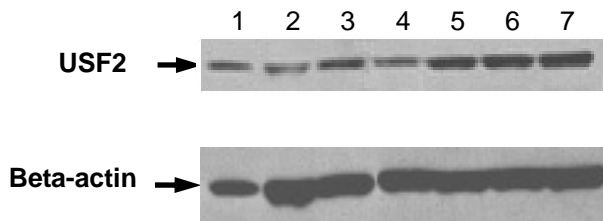
Figure 8. The expression level of USF2 protein in different cell lines by western blot analysis. 1: LNCaP; 2: PC3; 3: DU145; 4: NeoTag1; 5: NeoTag2; 6: NeoTag3; 7: HeLa. Westen blot was used to examine the USF2 expression level in human prostate cancer cells (1, 2, 3), mouse prostate tumor cells (4, 5, 6), and non-prostate cells (7) by using USF2 antibody, and beta-actin antibody was used in western blot as a loading control.

USF2 Binds to PB and SBP Promoters and the PSA Core Enhancer.

EMSA has shown that USF2 is associated with GAAAATATGATA element in prostate. This region is also a FoxA1 binding site (Gao et al., 2003). Previous studies have identified multiple FoxA1 binding sites in prostate specific gene promoters and enhancers including the PSA core enhancer and SBP promoter (Gao et al., 2003), so it is very likely that there exists an USF2 binding site in prostate specific gene enhancers and promoters. The probe PSA1 (Figure 9A) used in our EMSA experiments encompasses sequence located at -4122/-4109 bp of the PSA gene core enhancer, which contains an AR binding site and a FoxA1 binding site (Gao et al., 2003). Figure 9B shows an EMSA using LNCaP human prostate cancer cells nuclear extract, which expresses USF2. When the USF2 antibody was incubated with PSA1 probe and LNCaP cell nuclear extract, a band was supershifted when compared with the PSA1 probe and LNCaP cell nuclear

extract alone. However, no visible effect was observed when the USF1 antibody or GATA3 antibody was used. These results indicate USF2 binds the PSA core enhancer *in vitro*, while USF1 does not bind the PSA1 sequence.

A

Probe PSA1:
```
          -4163      ┌────── ARE III ──────┐
          CCTACTCTGGAGGAACATATTGTATTGATTG
                    ┌──── FoxA1 ────────▶
          TCCTTG ACAGTAAACAAATCTGTT
                                    -4105
```

B

USF1 USF2 GATA3

1 2 3 4 5

N.E: - + + + +
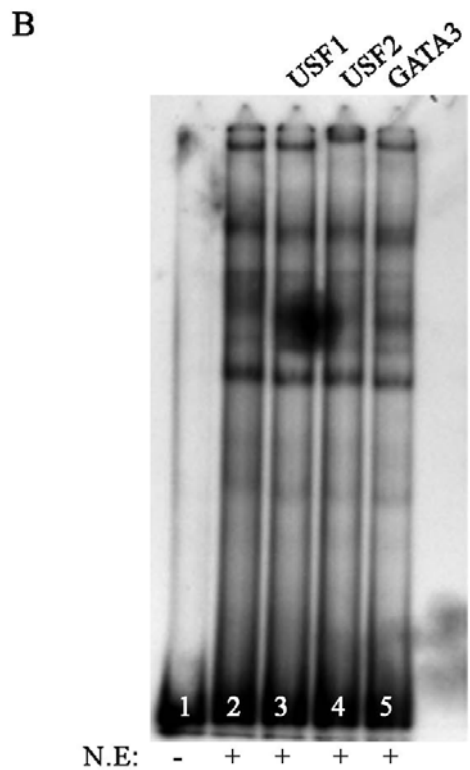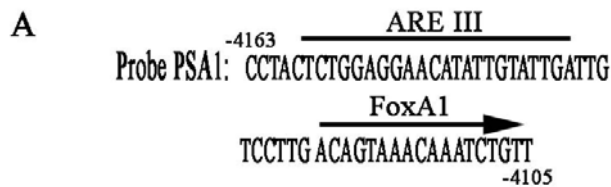
Figure 9. Identification of USF2 binding site in PSA core enhancer. A, PSA1 EMSA probe. The probe PSA1 used in our EMSA experiments encompasses sequence located at -4122/-4109 bp of the PSA gene core enhancer, inclusive of ARE III and a FoxA1 binding site, which also contains the USF2 binding site. B, EMSA. LNCaP nuclear extracts were incubated with radiolabeled PSA1 probe. Strong complexes formed in lane 2-5. The very top complex was shifted by USF2 antibody (lane 4), but not USF1 or GATA3 antibodies (lane 3 and 5).

57

To investigate the association of USF2 with a prostate specific gene promoter in cultured cells, Chromatin Immunoprecipitation (ChIP) assays were performed. Because the Neotag1 mouse cell line express the endogenous mouse PB and SBP genes, and LNCaP cells express endogenous human PSA gene, both Neotag1 and LNCaP cells were used for ChIP assays. Figure 10A is a schematic diagram of the PB, SBP, and PSA regulatory regions. Since USF2 binds to the same region as FoxA1, the regions that cover the USF2/FoxA1 binding sequences were tested in the experiments. A distal region of each promoter/enhancer (negative controls) was also tested (Figure 11). NeoTag1 and LNCaP cells were either treated with 10^{-8} M dihydrotestosterone (DHT) or maintained in androgen-depleted medium to determine the occupancy of USF2 on transcriptionally active vs. inactive PB/SBP/PSA chromatin via ChIP. As expected, DHT results in the recruitment of AR to responsive regions (Figure 10B, 10C, 10D). USF2, in contrast, was constantly bound to the promoter/enhancer as was FoxA1 (see Figure 11 for a gel of the PCR products). Only a small change in USF2 binding occurred with the binding of AR, thus the *in vivo* evidence for the binding of USF2 with PB and the SBP promoter and the PSA core enhancer shows that the binding of both USF2 and FoxA1 are largely independent of DHT treatment and AR binding. The PSA promoter contains relatively low level of USF2 as measured by real-time PCR (Fig. 10D) and the binding can be seen in the Figure 11D where a USF2 band shows up after 31 PCR cycles in the absence of androgens.
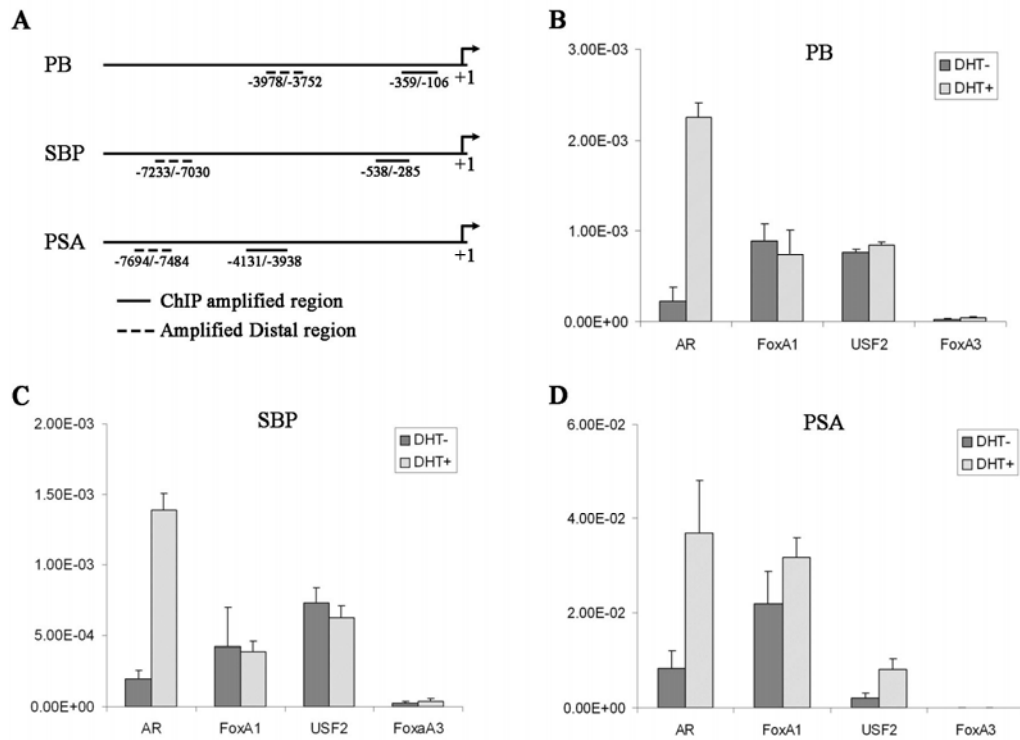
Figure 10. USF2 occupies PB promoter, SBP promoter, and PSA enhancer in vivo.
A: Schematic diagram of the 5' upstream regions of PB, SBP, and PSA genes. The diagram shows the amplified regions including distal regions (negative controls) and the USF2 binding regions. B, C, D: ChIP. After an overnight IP with indicated antibodies, formaldehyde cross-linking was reversed, and DNA fragments were extracted, followed by real-time PCR amplification. The y-axis unit is relative DNA enrichment. The amont of DNA pulled down by antibody was normalized with the amont of input DNA.
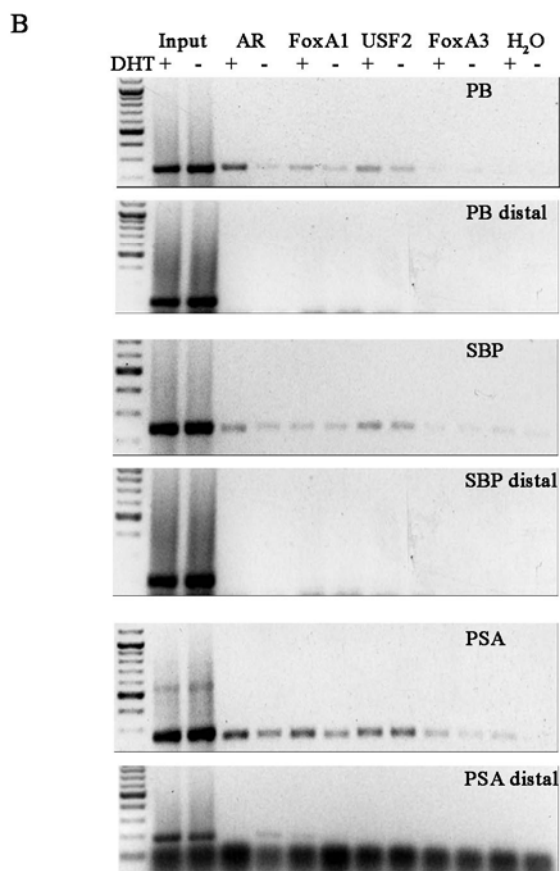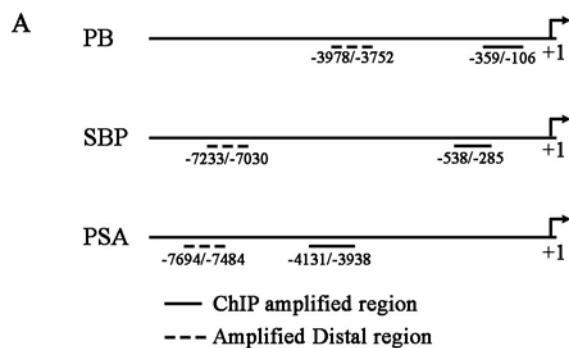
Figure 11. USF2 occupies PB promoter, SBP promoter, and PSA enhancer in vivo. A, Schematic diagram of the 5' upstream regions of PB, SBP, and PSA genes. The diagram shows the amplified regions including distal regions (negative controls) and the USF2 binding regions. B, ChIP. After an overnight IP with specific antibodies, formaldehyde cross-linking was reversed, and DNA fragments were extracted, followed by PCR amplification ("+" or "−" represents cells with or without DHT treatment).

USF2 Physically Interacts with FoxA1

The observation that overlapping binding sites for FoxA1 and USF2 exist at the androgen response regions of the PB, SBP, and PSA regulatory elements prompted us to determine if FoxA1 and USF2 physically interact. As LNCaP cells express both FoxA1 and USF2, we used this cell line to perform co-immunoprecipitations. Cell lysates immunoprecipitated with anti-FoxA1 conjugated protein G-sepharose beads followed by western blotting for USF2 (Figure 12A) indicate FoxA1 and USF2 interact. Reciprocal immunoprecipitations using anti-USF2 conjugated protein G-Sepharose beads in the presence of ethidium bromide followed by western blotting for FoxA1 confirm the FoxA1 and USF2 interaction and also indicate this interaction does not require the presence of DNA (Figure 12B). Although we previously reported a physical interaction between FoxA1 and AR (Gao et al., 2003), we did not detect an interaction between USF2 and AR (data not shown). Taken together, these results demonstrate that FoxA1 and USF2 interact.

A
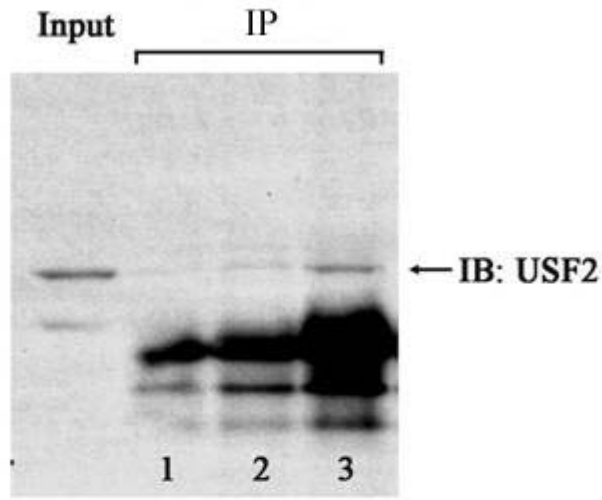
B
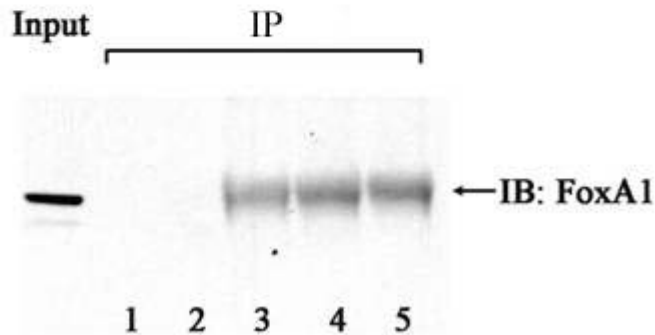
Figure 12. IP. A: LNCaP cell lysates were immunoprecipitated with FoxA1 antibody (lane 3). Water (lane 1) and goat serum (lane 2) were used as negative controls. LNCaP cell lysate (Input) served as a positive control. Western blotting was performed using USF2 antibody. B: LNCaP cell lysates were immunoprecipitated with USF2 antibody (lane 3, lane 4, and lane 5), water (lane 1), and rabbit IgG (lane 2, negative control). LNCaP cell lysate (Input) served as a positive control. Ethidium bromide (EB) was added to remove the influence of DNA presence on observed protein-protein interactions (0ug/ml EB in lane 3, 50 ug/ml EB in lane 4, 100ug/ml EB in lane 5). Western blot was performed using FoxA1 antibody.

Responsible Domains for USF2/FoxA1 Interaction

To further confirm the interaction between FoxA1 and USF2, as well as to determine the responsible domains for the USF2/FoxA1 interaction, *in vitro* GST pull-down assays was performed. Full-length FoxA1 and FoxA1 subdomain (V-5 epitope on the C-terminal) expression vectors with a T7 promoter were transcribed and translated in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega Corp., Madison, WI). (Figure 13A), and four GST-USF2 fusion proteins with different subdomains deletion were synthesized and purified (Figure 13B). *In vitro* synthesized FoxA1s and LacZ proteins labeled with a C-terminal V5-epitope were shown by western blot using anti-V5 antibody (Figure 13C) and used to map the USF2 binding regions of FoxA1. In Figure 13D, the FH domain of FoxA1 alone was sufficient to mediate binding to USF2. Furthermore, the N-terminal and C-terminal domains of FoxA1 alone did not bind to USF2. Figure 13E shows that the USF2 DNA binding domain, but not the transcription activation domain, is involved in the FoxA1/USF2 interaction. These results indicate that the C-terminal DNA binding region of USF2 and the FH domain of FoxA1 are the respective responsible regions for the interaction between USF2 and FoxA1.
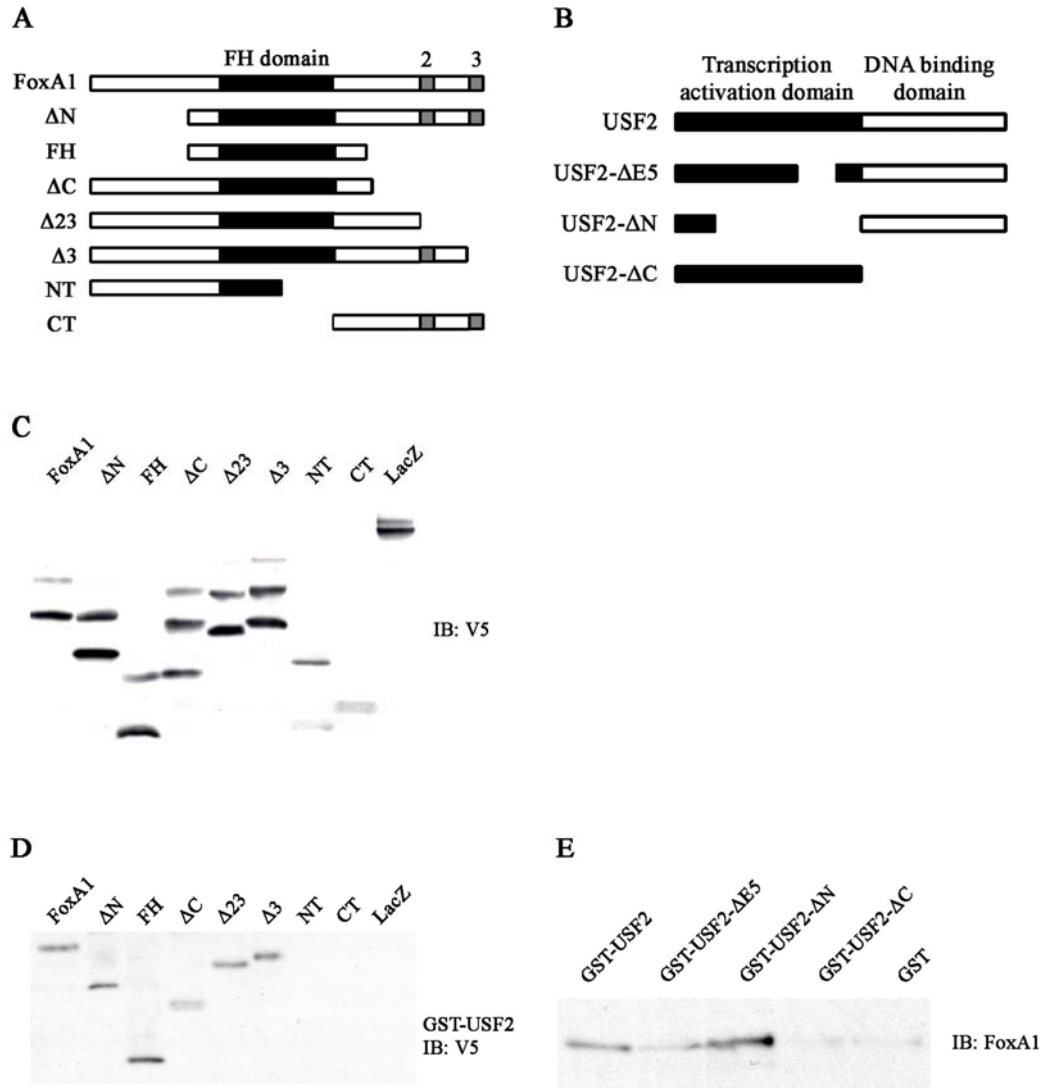
Figure 13. GST pull-down assays. A and B: Schematic diagrams showing a series of FoxA1 and USF2 subdomains used in vitro GST pull-down assays. The FoxA1 FH domain and USF2 transcription activation domain are highlighted in black. Two conserved FoxA1 C-terminal domains designated as region 2 and 3 are in grey boxes. C: Western blot using anti-V5 antibody shows eight in vitro synthesized V5-labled FoxA1 subdomains and a V5-labled LacZ protein. D: Eight V5-labeled FoxA1 subdomains as well as a LacZ protein were synthesized in vitro and incubated with the GST-bound USF2 to identify the USF2-interacting region in FoxA1. E: Four purified truncated GST-USF2 fusion protein were bound to glutathione agarose beads, followed by incubation with V5-labeled FoxA1 protein synthesized in vitro. Western blot was performed to identify the FoxA1-interacting domain in USF2.

USF2 Knockdown modestly increases PSA expression at mRNA and protein level in LNCaP human prostate cancer cells

To further examine USF2 function, USF2 was knocked down in LNCaP cells. PSA expression was evaluated at the RNA level both for USF2 knockdown LNCaP cells and control LNCaP cells. USF2 levels were decreased by 90% in LNCaP cells following 72 hours siRNA treatment (Figure 14A). Real-time PCR results (Figure 14B) indicate the PSA mRNA levels slightly increased in USF2 knocked down LNCaP cells compared with control LNCaP cells when the LNCaP cells were treated with DHT (10^{-8} M), but the change was not statistically significant. The PSA level change was very minor when the LNCaP cells were under DHT depletion condition.
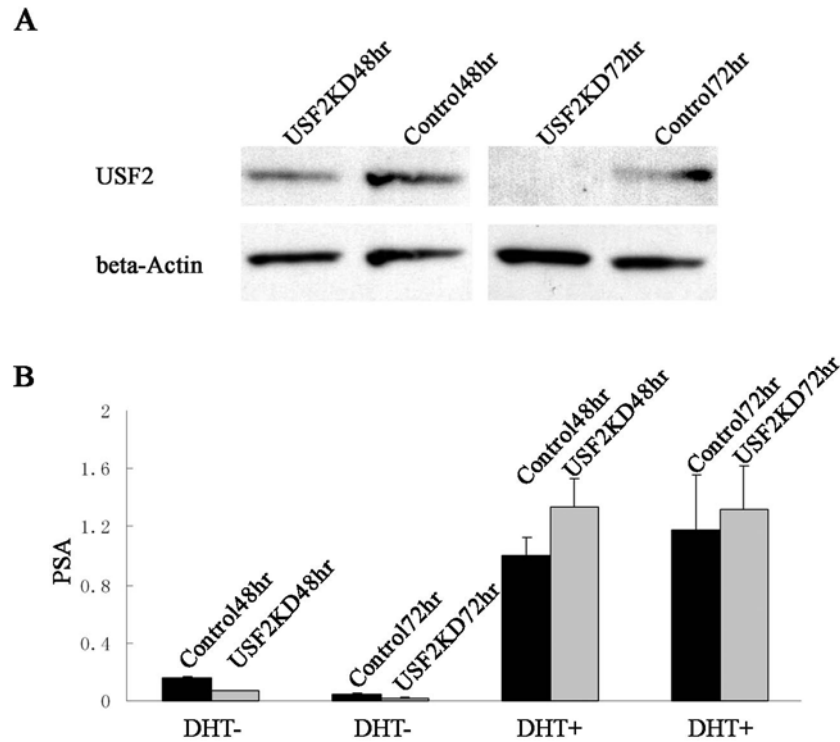
Figure 14. PSA level in USF2 knockdown LNCaP cells. A: USF2 knockdown in LNCaP cells. Western blotting shows a ~50% decrease in USF2 expression following 48 hours knockdown, and a ~95% decrease following 72 hours knockdown. B: Steady-state levels of PSA mRNA were determined following USF2 knockdown in LNCaP cells by quantitative real-time PCR. Total RNA was extracted from USF2 knockdown LNCaP cells and control LNCaP cells treated in the presence and absence of DHT. The unit of y-axis is relative fold change of PSA. Cycle number for each sample was normalized by GAPDH, and the cycle number difference between control samples and USF2 knocked down samples was converted as fold change.

Experiments were also done to examine the secreted PSA level in LNCaP cells (USF2 knockdown or control cells) medium. At intervals of 24 hours, conditioned medium was collected from control and USF2 knockdown LNCaP cells, and replenished with fresh medium allowing for the analysis of PSA levels via Enzyme-linked Immunosorbent Assays (ELISA). USF2 knockdown resulted in a significant ($p < 0.05$)

increase in the amount of secreted PSA (Figure 15). The result indicated that USF2 could

suppress the activity of PSA gene and negatively regulate the PSA expression.
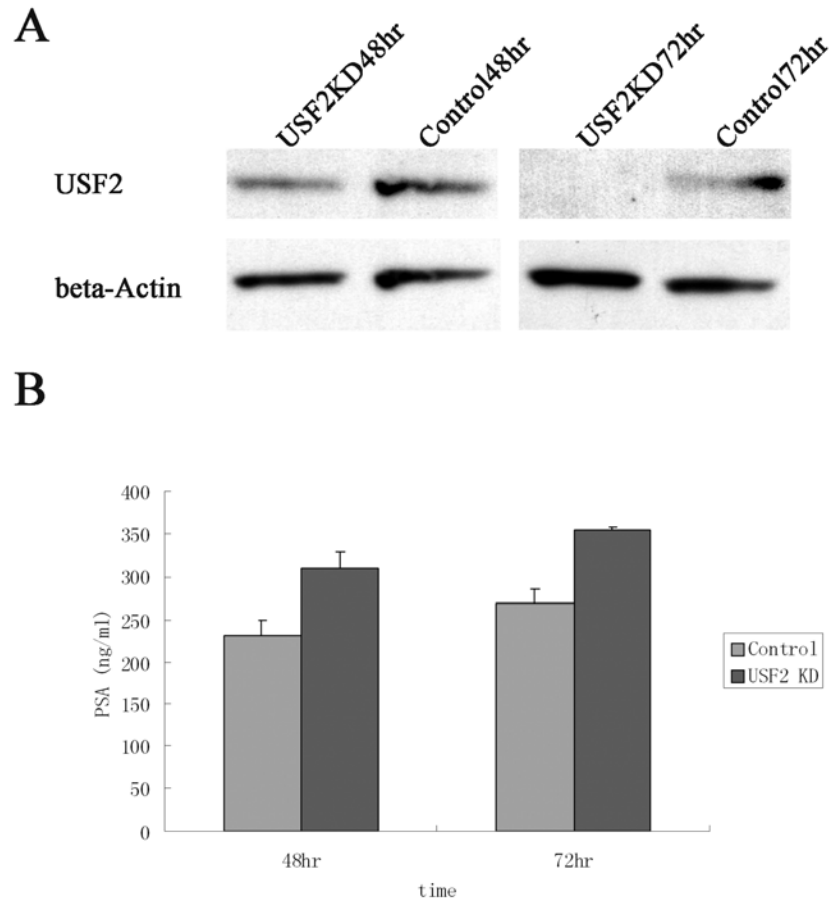
Figure 15. USF2 knockdown resulted in an increase in the amount of secreted PSA. A, USF2 knockdown in LNCaP cells. Western blotting shows a ~50% decrease in USF2 expression following 48 hours knockdown, and a ~95% decrease following 72 hours knockdown. B, The secreted PSA protein level in LNCaP cells medium following USF2 knockdown was examined by ELISA. The P value for 48 hours is 0.006269 (highly significant); P value for 72 hours is 0.003223 (highly significant).

USF2 knockdown does not cause a dramatic change in the promoters (ARR2PB Luc and PSA Luc) activity

ARR$_2$PB-Luc or PSA-Luc reporter construct was transient transfected into USF2 knockdown LNCaP cells and control LNCaP cells. Luciferase assay indicate that the activity of the promoters in USF2 knockdown cells almost in a same level as in the control cells (Figure 16). This indicates USF2 is constitutively bound to important prostate *cis* regulatory sequences.
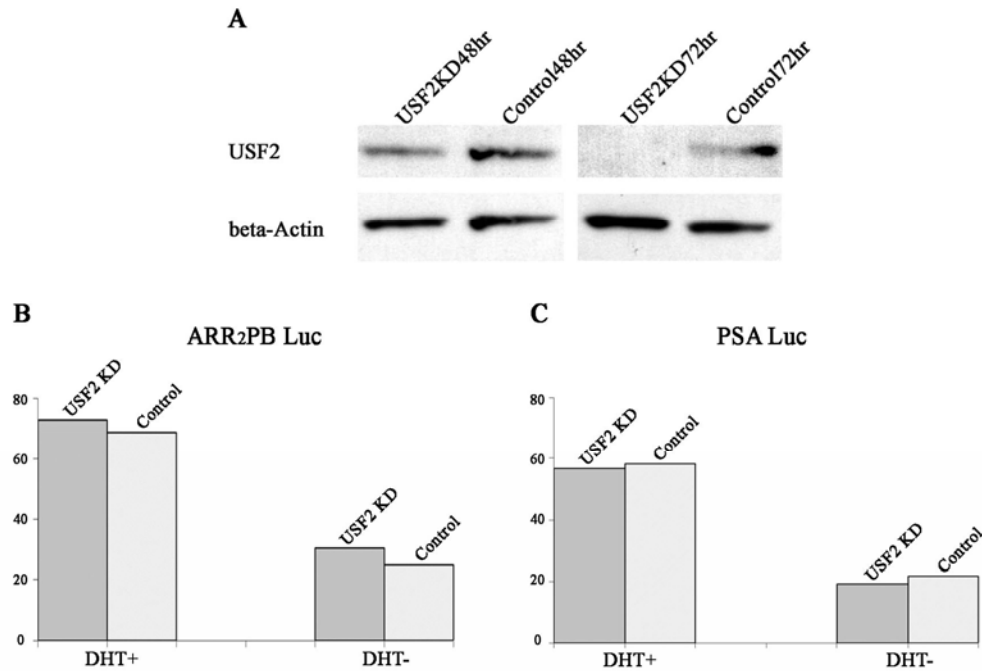
Figure 16. USF2 knockdown (72 hr) in LNCaP cells does not cause a dramatic change in the transient transfected ARR2PB promoter or PSA promoter directed Luciferase. A, 95% USF2 has been knockdown in 72 hours by siRNA compared with the control. B and C, The change of ARR$_2$PB / PSA promoter activity is very minor in USF2 knockdown (72 hr) LNCaP cells compared with the control LNCaP cells (Luciferase units expressed as Raw Luciferase Values/ mg protein/ 4 seconds).

CHAPTER IV

Identifying FoxA1 Interacting Partners

<u>Introduction</u>

Hormone ablation therapy is commonly used for most localized and metastatic prostate cancer. However, some cancer cells survive the treatment and develop into androgen-independent prostate cancer. One major reason causing this progression is due to the alteration in androgen receptor function or localization (Debes and Tindall, 2004). The molecular mechanisms of this progression are not very clear. Transcription factors that are involved in the AR activation pathway may play important roles in the development of androgen-independent cancer (Debes and Tindall, 2004). Studies on the transcription factor networks involved in androgen receptor action will help us to understand how castrate resistant prostate cancer (CRPC) develops.

AR activity is dependent on ligand binding, nuclear translocation, and association with AR coactivators or corepressors. In CRPC tumor cells, AR may contain mutations that cause the receptor to response to lower levels of androgens or antiandrogens which now enables the tumor to regrow (Feldman and Feldman, 2001). In some CRPC patient, nuclear AR levels are increased (Debes and Tindall, 2004). AR coactivators and corepressors may be implicated in all of these processes (Debes and Tindall, 2004). Studies have found that AR coregulators can increase AR sensitivity to weak androgens or antiandrogens (Debes and Tindall, 2004). AR coactivators may enhance

ligand-induced transcription of the AR by general mechanisms such as controlling nuclear translocation, bridging the AR with the basal transcriptional machinery, facilitating ligand binding, recruiting chromatin-modifying complexes, and/or enhancing DNA binding (Debes and Tindall, 2004). Thus, AR is the central factor in the progression to CRPC. Identifying AR coregulators can help us to explain how androgen-independent tumors cells develop. Targeting the transcription factors that interact with AR by inhibiting the protein-protein interaction can become a new treatment to the patients who fail hormone ablation therapy. Understanding the interaction between AR and its binding partners can provide a foundation for the development of next generation drugs.

We have reported that the forkhead protein, FoxA1, is critical for androgen regulation of prostate-specific promoters, such as human Prostate Specific Antigen, rodent probasin, and human/rodent Prostatic Acid Phosphatase (Gao et al., 2003). Our study shows that:

1. FoxA1 binding sites exist in the PB promoter. Two elements were referred to as TS1 and TS2 (Tissue Specific) (Zhang J-F et al., 2010). When TS sites are mutated, the bioactivity is reduced only in prostatic cells lines, but not in non-prostatic cells (Gao et al., 2003). DNA alignment of TS1 and TS2 revealed sequence similarity, EMSA and nuclear protein fractionation revealed nearly identical protein binding patterns of TS1 and TS2 (Gao et al., 2003). Southwestern and EMSA disclosed that both TS1 and TS2 harbor a FoxA1 binding sites (Gao et al., 2003). The FoxA1 binding sites are confirmed to be essential for maximum induction by androgens since point mutations in FoxA1 binding sites block activation of the PB promoter (Gao et al., 2003). Further, southwestern

analyses mapped at least two small proteins bound to TS2 as 52 kDa (FoxA1) and 46 kDa (USF2) as well as the large protein at 113 kDa identified as AR plus additional weaker bands (Gao et al., 2003).   The FoxA1 and AR bands have been confirmed (Gao et al., 2003). Mutation of the FoxA1 site prevents expression in transgenic mice (Zhang J-F et al., 2010). Further, the PB constructs tested in transgenic mice reveal that the fragment of DNA between -244 to -96 bp contains sufficient information to specifically target prostatic epithelium (Zhang J-F et al., 2010). This fragment of DNA contains the TS1 and TS2 elements which bind FoxA1. Our data strongly suggests that FoxA1 functions as a critical component in a TF complex that controls androgen regulated and prostate-specific expression of PB.

2. Androgen receptor interacts with FoxA1. IP was carried out using LNCaP total lysate that contained endogenous AR and FoxA1 (FoxA1 has been confirmed to present in LNCaP, and PC-3 cells). Immunoprecipitating one antigen, AR or FoxA1, with its corresponding antibody simultaneously precipitated the other factor, which was detected by western blot. Further, the same results were obtained with GST-AR and FoxA1 (Gao et al., 2003).

3. FoxA1 binding sites have been identified on other prostate specific gene promoter/enhancers. TF string search and EMSA using *in vitro* synthesized FoxA1 protein identified two FoxA1 binding sites on the PSA core enhancer region reported as the Prostate Specific Enhancer (PSE) (Cleutjens et al., 1997; Huang et al., 1999; Schuur ER et al., 1996; Wu et al., 2001) and one on the PAP proximal promoter (Virkkunen et al.,

1994). Importantly, all of three FoxA1 sites are immediately adjacent to a functional androgen response element and the PSA and PAP FoxA1 sites have been confirmed as being functional within the promoters (Gao et al., 2003).

4. FoxA1 regulates prostate development. FoxA1 is expressed throughout prostate development and maturation and is broadly expressed in almost all epithelium (Mirosevich et al., 2005b). From our FoxA1 knock-out study, we observed that structurally aberrant epithelial ducts demonstrate a hyperproliferative feature reminiscent of solid primitive epithelial cords that are surrounded by thick stromal layer. The abnormal ductal phenotype of FoxA1-deficent prostate is accompanied by a series of alterations in some early signaling molecules (Gao et al., 2005a). We confirmed that the activation of Sonic hedgehog (Shh) and elevated FoxA2 and Notch1 in FoxA1-deficient prostate (Gao et al., 2005a). The prostatic protein Nkx3.1 was down regulated in the null epithelium (Gao et al., 2005a). Further, the siRNA to FoxA1 in LNCaP cell will cause LNCaP cells to die (unpublished data from our laboratory).

These data show that FoxA1 plays a pivotal role in controlling prostate morphogenesis, cell differentiation, and proliferation. Here I propose to identify other transcription factors that interact with FoxA1 and function in a complex with androgen receptor. Androgen activates the androgen receptor to increase transcription. In order to do so, the spatial order and the temporal addition of proteins to the promoter must occur to create the TF complex. Through protein-protein and protein-DNA interactions, the complex is stabilized permitting the initiation of transcription. Using yeast two-hybrid

system, extensive research has been conducted to identify TFs that interact with the AR (Yeh and Chang, 1996; Fujimoto et al., 1999; Kang et al., 1999; Wang et al., 2001). However, by this method, no one has ever detected the interaction between FoxA1 and AR, indicating that this approach has missed an important TF involved in steroid receptor action. Since our publication on AR/FoxA1 interactions (Gao et al., 2003), others have implicated FoxA1 with estrogen receptor action (Carroll et al., 2005) and confirmed our findings with AR (Jia et al., 2008; Lupien and Brown, 2009). Recently, an estrogen receptor (ER) negative subset of breast cancer cells was shown to be androgen responsive and FoxA1 positive (Doane et al., 2006). These data suggest that FoxA1 and its binding partners play a significant role in hormonal regulation of not only prostate but also breast cancer. Thus, understanding the essential mechanism of androgen regulation can give insights into hormonal regulation of both prostate and breast cancer.

In summary, we believe that FoxA1 and its binding partners form a protein complex with AR and other TFs that now direct prostate-specific gene expression. We believe that the same TFs complex with the AR to control PSA also will play a critical role in the androgen regulation of tumor growth. Purification and identification of the interacting partners of FoxA1 will facilitate our understanding of the role of these TFs to interact with the AR to regulate tumor growth.

Results

Cloning of FLAG, 6His tagged human FoxA1 into retroviral vector pLPCX.

A FLAG-6His tag was added to human FoxA1 N-terminal by PCR. The PCR products were cloned into pCR-TOPO 2.1 vector, then the tagged-FoxA1 was inserted into retroviral vector-pLPCX in frame using restriction enzymes. The sequence was confirmed by DNA sequencing (Figure 17).
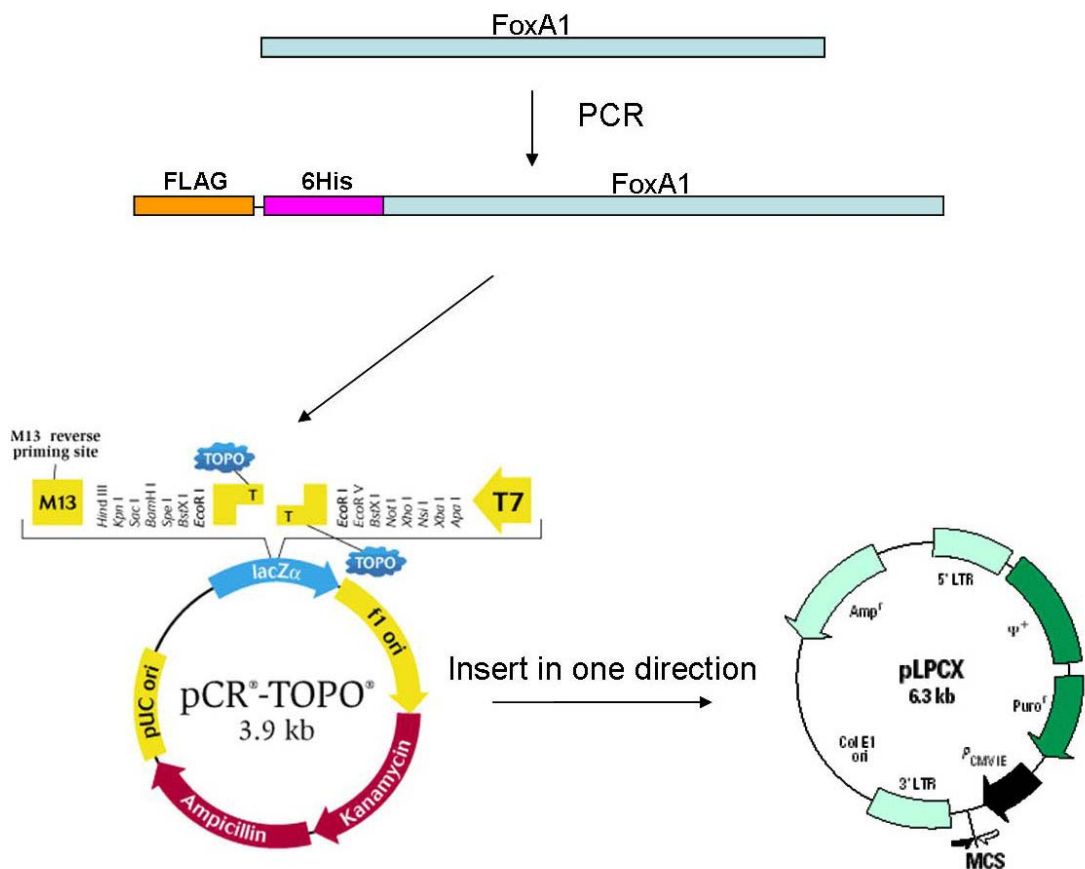
Figure 17. The procedure of cloning tagged-FoxA1 into a retroviral vector-pLPCX. A FLAG tag and 6 Histidine tag were cloned in the N-terminal of human FoxA1 cDNA by PCR. The PCR products were inserted into pCR-TOPO 2.1 vector (Invitrogen, Carlsbad, CA) and then cloned into retroviral vector pLPCX in frame (Clontech, Mountain View, CA).

Transiently Transfect HeLa cells with pLPCX-FoxA1

pLPCX-FoxA1 was first transiently transfected into HeLa cells. HeLa cells do not expression endogenous FoxA1. A western blot using anti-FoxA1 antibody was done to examine whether tagged Foxa1 can be properly expressed in HeLa cells. LNCaP cells were used as a positive control (Figure 18). The result ensures that tagged FoxA1 is inserted in the vector in frame and can be expressed. The quantitative expression level of tagged FoxA1 is measured in Figure 20.
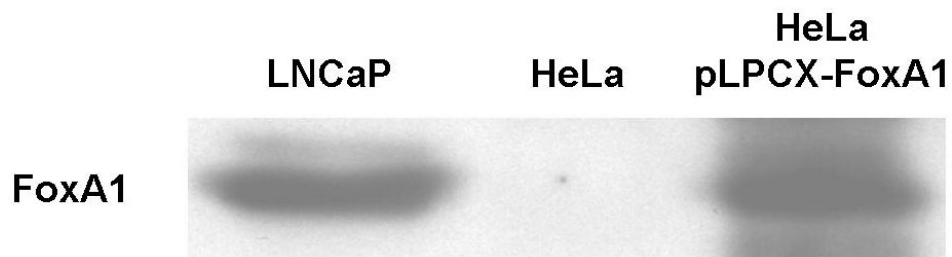
Figure 18. The expression of tagged-FoxA1 in HeLa cells. Western blot using FoxA1 antibody to confirm the tagged-FoxA1 expression in transient transfected pLPCX-FoxA1 HeLa cells. HeLa cells do not contain endogenous FoxA1; LNCaP cells have endogenous FoxA1 served as positive control.

Establish FLAG-6His FoxA1 stably expressing LNCaP, HeLa, and HeLa AR cell lines and determining the level of expression.

HeLa, HeLa AR, and LNCaP cell lines were infected with pLPCX-FoxA1 vector or pLPCX empty vector that would serve as the control cells. Cells were first selected by 1 ug/ml puromycin. The concentration of puromycin was then increased to a final

contraction 10 ug/ml for HeLa and HeLa AR cells, 5 ug/ml for LNCaP cells (Figure 19).
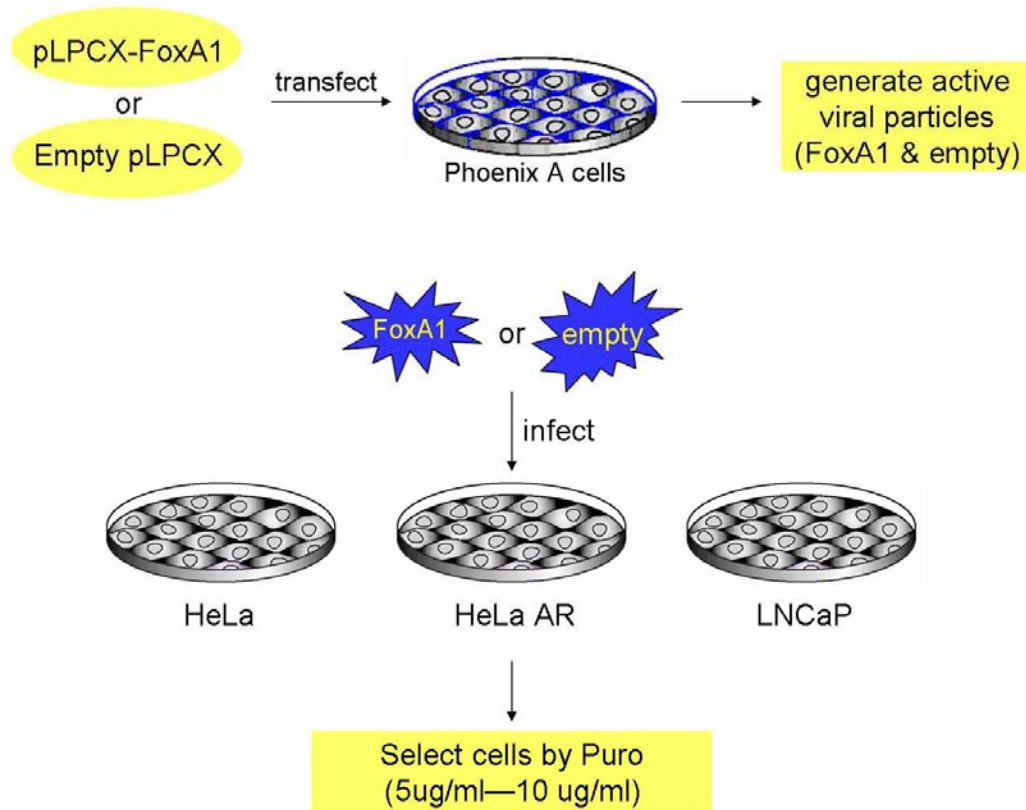
Figure 19. The process of establishing the stable tagged FoxA1 expression cells lines. pLPCX-FoxA1 or empty pLPXC vectors are used to transfect Phoenix A cells to generate active FoxA1 or empty viral particles which are used to infect HeLa, HeLa AR, and LNCaP cells to generate tagged FoxA1 stable expressing cell lines and control cell lines. Each cell line was grown under puromycin selection.

The expression level of tagged FoxA1 in each cell line was examined by a Western blot using both FoxA1 antibody and Anti-His antibody. The tagged FoxA1 level in LNCaP FoxA1 cell line is much higher than the endogenous FoxA1 level in LNCaP with empty pLPCX vector (Figure 20). Since the high level of tagged FoxA1 expression may not reflect the true FoxA1 level in biological system, some false positive binding partners

may be identified due to the high level expression of tagged FoxA1. To avoid the false

positive binding, the identified FoxA1 binding proteins need to be subjected for further

studies, such as IP or GST-pull down, to confirm the binding with FoxA1. HeLa FoxA1

and HeLa cells with both AR and FoxA1 always had very low levels of FoxA1

expression even after repeating the infection multiple times. The reasons for the low level

of FoxA1 expression in HeLa and HeLa AR cells are unknown.
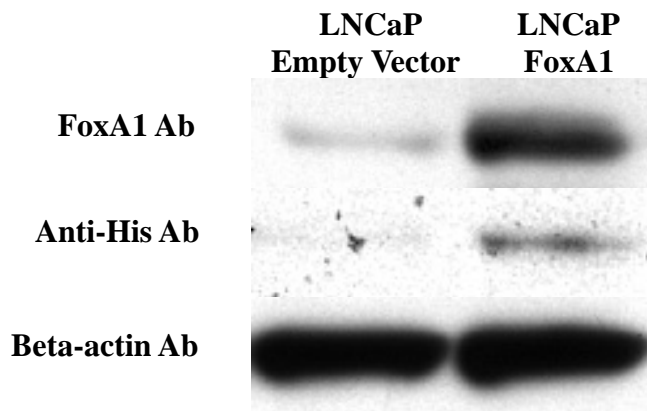
Figure 20. The tagged FoxA1 expression level in LNCap FoxA1 and control cell lines. In both LNCaP FoxA1 and LNCaP control cell lines, FoxA1 antibody was used to indicate the expression level of FoxA1, anti-Histidine antibody was used to indicate the expression of tagged FoxA1 in western blot. Beta-actin antibody was used as a loading control.

Tandem Affinity Purification of tagged-FoxA1 protein complex from LNCaP FoxA1 and LNCaP E cells.

We have established FLAG-6His-FoxA1 stably expressing LNCaP cell line and a

LNCaP cell line with the empty vector as the control. Both the cells were harvested at

about 1×10^9 cells. Harvested cells were lysated and the nuclear extracts were prepared for

tandem purification. The BD TALON Single Step Column, which binds the His-tagged protein, was used to perform the first step purification. In the second step, ANTI-FLAG M2 Affinity Gel, which specifically binds to FLAG fusion proteins, was used to further purify the eluate from the first step (Figure 21).
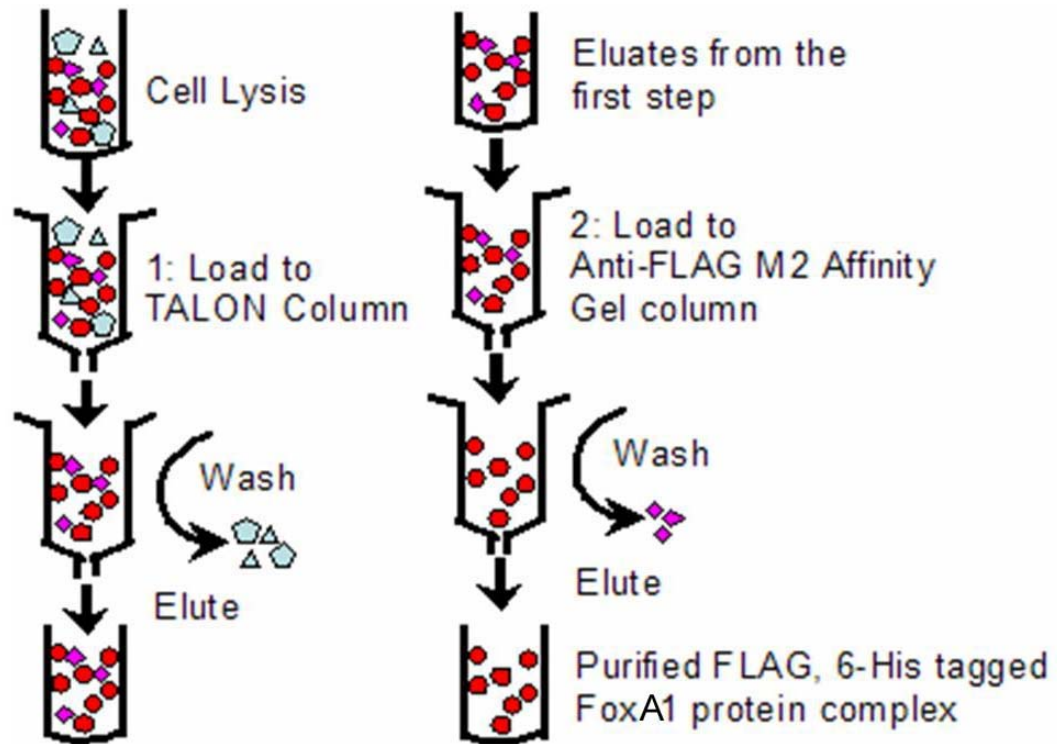
Figure 21. The process of the Tandem Affinity Purification of tagged-FoxA1 protein complex from LNCaP FoxA1 and LNCaP E cell lysates. Total cells (LNCaP FoxA1 cells and LNCaP control cells) of 10^9 were harvested and nuclear extract of the cells were prepared. The nuclear extracts were purified by using TALON column and anti-FLAG M2 affinity gel column respectively. The final eluated from the purification were collected for LC-MS/MS analysis.

Examination of the tagged-FoxA1 expression level in the elutes of LNCaP FoxA1 cells and LNCaP E cells.

During tandem affinity purification, each portion from every purification step was collected for western blot analysis. Tagged-FoxA1 expression levels need to be examined in the final elutes before the liquid chromatography and tandem mass spectrometry analysis. In Figure 22, the flow through from the first step purification (TALON column) is named as "supernatant from TALON"; the flow through from the second step

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purification (FLAG affinity gel) is named as "supernatant from Flag". Western blot results indicate the flow through of LNCaP E cells from TALON column contains more FoxA1 than that of LNCaP FoxA1 cells, because the tagged-FoxA1 from LNCaP FoxA1 cells is bound to the TALON column. There is no His tag detected in the first step flow through from both LNCaP E and LNCaP FoxA cells. The flow through of LNCaP E and LNCaP FoxA1 cells from the second step does not contain FoxA1, but both contain His tag. The final elute of LNCaP FoxA1 cells contains both high level FoxA1 and His Tag, but the eluate from LNCaP E cells contains neither of them. The western blot confirms the high level of tagged-FoxA1 in LNCaP FoxA1 cells eluate, but not in the control LNCaP E cells eluate.

Figure 22. Western blot analysis for each portion from the tandem affinity purification. Western blot using FoxA1 antibody to detect the expression of FoxA1 and using anti-Histidine antibody to detect the expression of tagged FoxA1. The lanes in A are the flow through of LNCaP FoxA1 cells and control cells from the first step purification. The lanes in B are the flow through of LNCaP FoxA1 cells and control cells from the second step purification. The lanes in C are the final eluate from LNCaP FoxA1 cells and control cells.

AR is identified in the final elute of LNCaP FoxA1 cells by western blot

An earlier study has shown FoxA1 and AR can physically interact with each other, so AR can serve as a positive control during identifying the FoxA1 binding partner. AR is detected in the LNCaP FoxA1 cell final elute, but not in the control LNCaP E cell elute by western blot using AR antibody (Figure 23). The results tell us that AR can be pulled down together with tagged-FoxA1 during the purification.
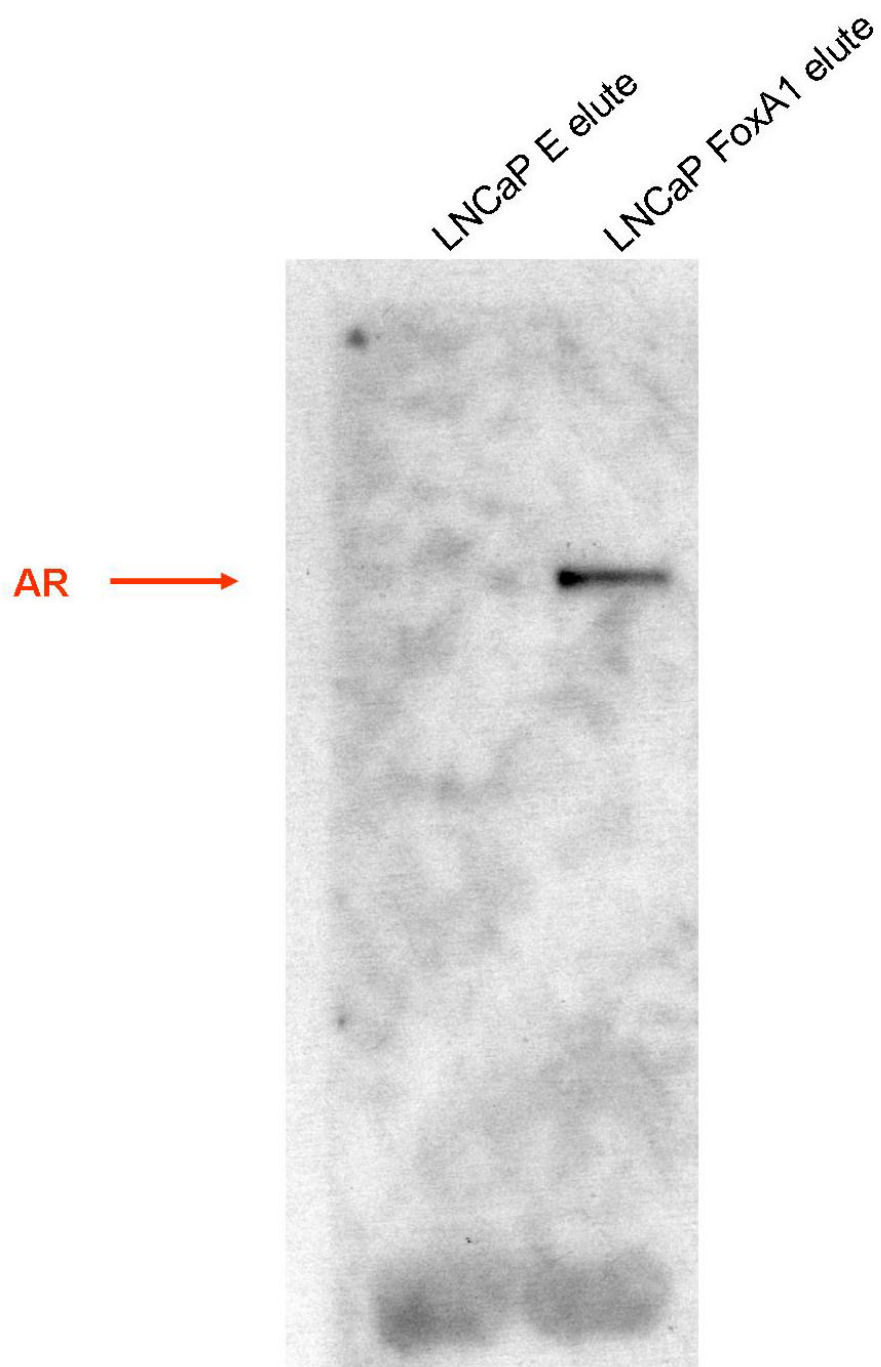
Figure 23. Western blot using AR antibody indicates that AR is pulled down together with tagged-FoxA1 from the LNCaP FoxA1 cells. Final eluates from LNCaP Foxa1 cells and control cells were subject for a SDS-PAGE gel and using AR antibody to detect whether AR was pulled down together with tagged FoxA1.

The SDS-PAGE analysis for the final elutes following with Colloidal Blue staining

In Figure 25, the elutes from the second step purification of both LNCaP FoxA1 cells and LNCaP E cells were subject to SDS-PAGE and colloidal blue staining. Select protein bands from the LNCaP FoxA1 eluate as well as the same region of the control gel were cut from the gel and the protein digested with trypsin (Figure 24). Peptide hydrosylate were analyzed by C18 reverse-phase LC-MS/MS using a Thermo LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump system, nanospray source, and Xcalibur 2.0 instrument control using standard data-dependent methods. Tandem MS data were analyzed with the Sequest algorithm against the IPI human database (135,674 entries, October 2007 release) including a concatenated reverse database for calculating false-discovery rate.
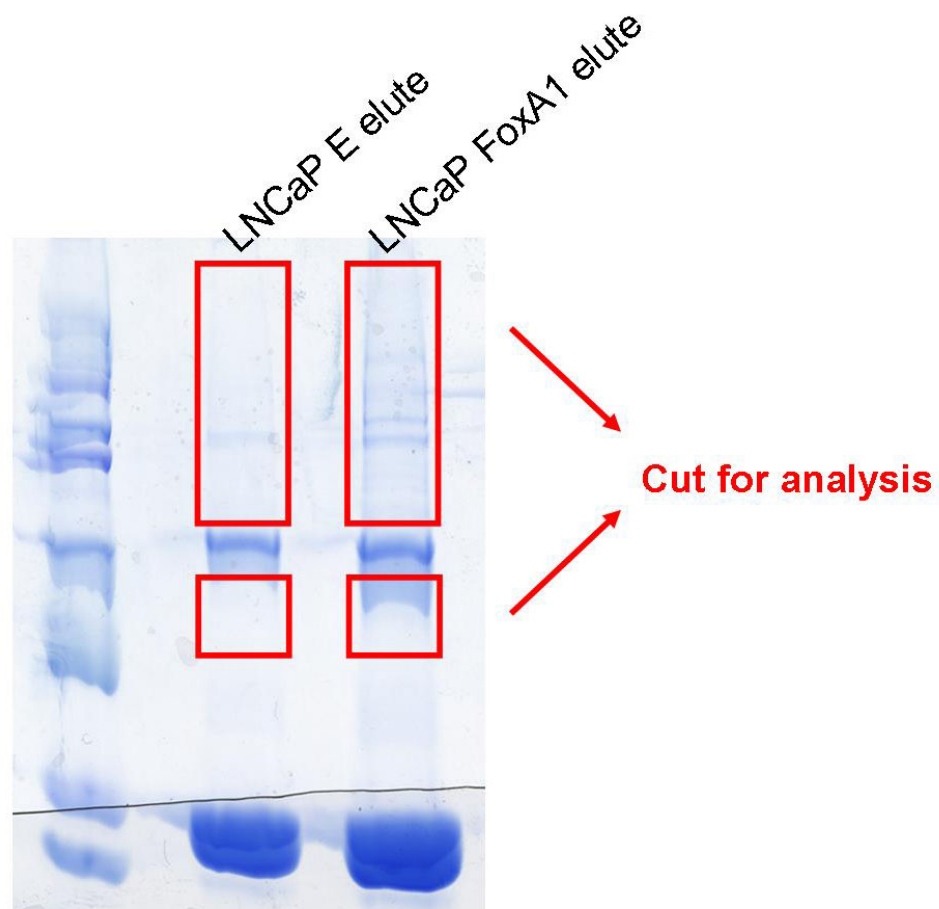
Figure 24. The SDS-PAGE analysis for the final elautes from the purification followed by Colloidal Blue staining. The final eluates from LNCaP FoxA1 cells and LNCaP control cells were separated in 10% SDS-PAGE gel. The protein bands were visualized by Colloidal blue staining. Protein bands from LNCaP FoxA1 cells were cut for trypsin digestion and LC-MS/MS analysis. The corresponding regions from control cells were also processed to identify non-specific bindings.

Identify FoxA1 binding partners by LC-MS/MS analysis

The proteins from the complex have been identified according to the established procedures of the Vanderbilt MSRC. At least two peptides from two separate runs were used as the criteria for a positive hit. After screening and elimination of false positives, we identified a total of 16 proteins that appear to interact with Foxa1 (Table 2). Of 16 proteins meeting the "hit" criteria, 7 have been previously reported to directly interact with AR, or an indirect link can be established. Three groups of nuclear proteins that were identified: 1) alter AR activity (7 proteins) while no reports exist on DNA binding protein, TOX HMG family # 4; 2) control protein structure/stability (2 proteins); and 3) involved in RNA processing (6 proteins). None of these nuclear proteins were reported by Kivinen et al., when they did affinity purification of nuclear proteins using a fragment of the probasin TS-1 sequence (Kivinen et al., 2004).

The Nuclear Factor I (NFI) family of TFs contains four genes (NFIa, NFIb, NFIc, NFIx), that bind to the consensus DNA sequesnce TTGGCN5GCCAA (Green et al., 1998). Knockout studies of individual NFI genes in mice result in a non-redundant phenotypes implying that NFI TFs are involved in tissue-specific expression (Wong et al., 2007; Grunder et al., 2002; Steele-Perkins et al., 2003; Driller et al., 2007; Campbell et al., 2008). NFIx knockout mice display an agenesis of corpus callosum and a hydrocephalus and normally die 21 to 28 days after birth (Driller et al., 2007; Campbell et al., 2008). A deformation of the spine is also observed in NFIx-/- mice (Driller et al., 2007). Embryonically, the hindgut gives rise to the prostate, bladder, rectum, and intestine. Since a phenotype in the intestine is reported in the NFIx-/-, it may be expected

that the loss of NFIx will show phenotypes in these embryonically related organs. Interestingly, each isoform of NFI has different specificity for estrogen receptor regulation of mammary gland specific differentiation markers (Murtagh et al., 2003), but no one has reported on the examination of the individual isoforms in terms of AR action.

Nucleostemin (NS) expression is largely restricted to nucleoli of pluripotent embryonic stem cells, tissue stem cells, and a number of cancers including prostate cancer, but it absent in most adult tissues (Tsai and McKay, 2002; Liu et al., 2008). Traditionally, the nucleolus is recognized as the site of rRNA synthesis but now the nucleolus has also been linked to expression of tumor suppressor and oncogenes, cell senescence, and the modulation of telomerase function (Fox et al., 2002). NS is found to interact with Nucleophosmin (NPM) and NPM has been shown to interact with AR (Ma and Pederson, 2008; Leotoing et al., 2008). Guanine nucleotide binding protein-like 3-like (GNL3L), a member of NS family, interacts with ERRgamma and can inhibit the transcriptional activities of ERR genes by reducing the steroid receptor coactivator binding (Rosen et al., 1980). An independent study recently shows that the inhibition of ERRgamma activity promotes growth of DU145 prostate cancer cells (Yu et al., 2007), and knockdown of NS in PC-3 cells decreased xenograft growth (Liu et al., 2008), suggesting a role of NS in prostate cancer progression. Although NS is absence in most adult differentiated cells, we find that NS is expressed in the normal adult mouse prostate tissue (unpublished data).

Paraspeckle Protein 1 (PSP1) is an identified component protein of paraspeckles, a

nuclear body (Myojin et al., 2004). The protein contains a Drosophila Behavior Human Splicing (DBHS) motif that is also found in polypyrimidine tract-binding protein-associated splicing factor (PSF), p54nrb, and Non-POU-domain-containing octamer binding protein (NonO) (Myojin et al., 2004). The DBHS-containing proteins were known to play important roles in DNA replication, transcription, and mRNA processing (Myojin et al., 2004). PSP1 and PSF are reported to interact with AR and functions as AR coactivators to activate androgen receptor-mediated transcription together with other DBHS-containing proteins (Ishitani et al., 2003; Kuwahara et al., 2006). The inner nuclear matrix structural protein Matrin 3 is found to form a complex with p54nrb and PSF and to play a role in controlling the export of edited mRNAs (Zhang and Carmichael, 2001). By interacting with PSF, Matrin 3 may indirectly interact with AR.

DNA-dependent protein kinase (DNADPK), a member of the phosphatidylinositol 3-kinase family, can phosphorylate the glucocorticoid receptor (Giffin et al., 1996; Giffin et al., 1997). The Ku subunits of DNADPK are reported to bind to PSA promoter acting as an AR coactivator (Mayeur et al., 2005). DNADPK is also found to regulate AR export through phosphorylation (Shank et al., 2008). Importantly, DNADPK has been shown to phosphorylate FoxA2, a forkhead protein closely related to FoxA1 (Nock et al., 2009). The phosphorylation of FoxA2 enhances its ability to bind to DNA (Nock et al., 2009). It would be reasonable to postulate that DNADPK then functions to downregulate AR binding to the DNA but to enhance binding of FoxA1. By enhancing FoxA1, this would allow the chromatin confirmation to remain accessible for re-activation by the AR.

Two of the proteins identified in Table 2, NFIx and NS, have been priorized for further study.

Table 2: FOXA1 binding partners identified by tandem affinity purification followed by LC-MS/MS				
Protein	kDa	Function	Accession #	Comment
Known or Potential AR Co-Regulators				
◊ Nuclear factor Ix (NFIx)	50	Transcription Factor	EAW84339	Tissue specific & NFI acts as AR co-regulator (Murtagh et al., 2003; Driller et al., 2007; Campbell et al., 2008; Driller et al., 2007; Campbell et al., 2008)
*Para-speckle protein 1 α	60	Located at nuclear para-speckles	NP_001035879	Interacts with AR(Ishitani et al., 2003) Enhances AR action (Kuwahara et al., 2006)
◊ Nucleostemin (NS)	70	Cell cycle	AAV74413	Stem cell marker expressed in PCa (Tsai and McKay, 2002; Liu et al., 2008; Liu et al., 2008)
TOX HMG family # 4	70	DNA binding protein	NP_055643	High mobility group protein
* PSF	75	Co-activator/repressor	NM_005066	Interacts with AR (Dong et al., 2007). AR co-regulator (Kuwahara et al., 2006). Interacts with Matrin-3 (Zhang and Carmichael, 2001)
◊ Matrin-3	95	Nuclear matrix	NP_061322	Interacts with PSF (Zhang and

				Carmichael, 2001)
*BRG-1 associated factor	180	DNA access	Q86U86	Allows for AR access (Lemon et al., 2001)
* DNADPK	360	Kinase	NP_001075109	Phosphorylates AR (Shank et al., 2008)

Protein Stability

PPCTI-4	57	PPIase	EAW47800	Protein folding
Heat shock cognate	70	Protein stability	BAD96505	Chaperone

RNA processing

Splicing factor 3b	25	RNA splicing	Q6NT18	Nuclear protein
HNRP	60	RNA splicing	P14866	Nuclear protein
CPASF-6	68	RNA processing	Q16630	Nuclear protein
DEAD/H BOX 15	90	RNA splicing	EAW92821	Nuclear protein
RNA binding protein 10	100	Unknown	P98175	Nuclear protein
TAF4 RNA pol II	90	Transcription	NM_001358.2	Nuclear protein

Table Key: * = Identified protein directly interacts with AR; ◊ =Potential of direct or indirect interaction with AR based upon the literature. Thus, 7/16 nuclear factors identified can be implicated in AR action.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Discussion

The promoter of the rat PB gene has been extensively characterized and a small fragment of the PB promoter (-426 to +28 bp) has been shown to specifically target transgenes to mouse prostate (Greenberg et al., 1994). The testing of deletion constructs of the promoter fragment showed that an androgen response region (ARR: -244 to -96 bp) resided in this promoter (Zhang J et al., 2000). The ARR contains two strong AR binding sites termed ARBS1 (-236 to -223 bp) and ARBS2 (-140 to -117 bp) that function together in a co-operative manner (Kasper et al., 1994). Using the -244 to -96 bp fragment placed adjacent to -286 to +28 bp PB promoter, a new construct (ARR$_2$PB) was shown to target high levels of androgen regulated gene expression specifically to the prostate (Zhang J et al., 2000). These studies indicated that the -286/+28 sequences contain the information necessary to regulate prostate-specific gene expression (Zhang J et al., 2000). In order to define the minimal PB DNA sequences necessary for prostate-specific gene expression, a series of deletion constructs were tested in transgenic mice. These experiments defined that the sequences between -244 to -96 bp contained the necessary information to target transgene expression to the prostate (Zhang J-F et al., 2010). Using promoter mutagenesis analysis, EMSA assays, and transgenic mouse studies, we have identified key *cis*-acting elements in this region, including several transcriptional factors (Oct-1, c-jun, and NFI) and two tissue-specific elements (TS1 and

TS2) that bind AR, FoxA1 and USF2, which are critical for PB tissue-specific expression (Zhang J-F et al., 2010).

Oct-1 has been reported to play an important role in androgen-regulated SLP gene expression *in vivo* (Scarlett and Robins, 1995; Scheller et al., 1996) and to be required for mouse mammary tumor virus (MMTV) transactivation (Prefontaine et al., 1998). Oct-1 can directly interact with steroid receptors through its conserved POU domain (Prefontaine et al., 1999; Kallio et al., 1995) suggesting a conserved functional mechanism of action. The binding site of Oct-1 (-218/-206 bp) is located immediately downstream to ARBS-1 (Zhang J-F et al., 2010). The interaction between Oct-1 and AR on the SLP gene may occur on the PB promoter in a similar manner. Mutagenesis studies indicated that binding of Oct-1 in the PB promoter is important for promoter activation both in prostatic and and non-prostatic cells (Zhang J-F et al., 2010).

Studies have demonstrated that c-jun can positively regulate AR transactivation in a dose-dependent manner through a direct interaction with the AR DNA-binding domain/hinge region (Bubulya et al., 1996). Further, c-jun promotes AR activity by enhancing AR DNA-binding and facilitating the ligand-dependent interaction between the AR amino- and carboxyl-terminals regions (Bubulya et al., 1996; Bubulya et al., 2001; Wise et al., 1998). On the PB promoter, the c-jun binding site (-108/-94 bp) is adjacent to the ARBS2 binding site (-140/-117 bp) (Zhang J-F et al., 2010). Disruption of c-jun DNA-binding site dramatically decreases the PB promoter activity (Zhang J-F et al., 2010), which suggests the transcriptional activity is modulated by cooperation between

AR and c-jun.

The NFI family of proteins (Murtagh et al., 2003) are known to be involved in the regulation of several androgen dependent genes (Darne et al., 1997; Verrijdt et al., 1999). The *in vivo* glucocorticoid receptor (GR) induction of MMTV promoter involves a functional co-regulation between GR and NFI (Chavez and Beato, 1997). More recently, an unidentified NFI family member was shown to be essential for both GR-induced MMTV expression on naked DNA, and the recruitment of the chromatin remodeling enzyme BRG-1 (Hebbar and Archer, 2003) to regulatory sequence. A recent report indicates that NFI binding sites are significantly associated with FoxA1 binding sites in regions of acetylated DNA, which correlates with our finding that NFIx and FoxA1 are binding partners (Table 2). Linker mutagenesis, ChIP, and DNA sequence analysis revealed a critical NFI binding site (-88/-70 bp) in PB promoter (Zhang J-F et al., 2010). Mutation in NFI binding site results in a significant loss of PB promoter activity (Zhang J-F et al., 2010), which is consistent with a previous report showing that the binding of NFI to PB promoter is androgen-independent prostate cell specific (Yeung et al., 2003). However, our transgenic studies demonstrated that -244 to -96 bp of PB fragment is sufficient for the prostate specific gene expression, suggesting that the promoter binding by NFI is required for optimal PB gene activation in prostate cells or tissues, but not absolutely required for the prostatic-specific expression of the PB gene.

Using linker mutation and transgenic studies, two tissue-specific DNA elements have been identified, which are fundamentally important for androgen-regulated

prostatic-specific PB promoter activation (Zhang J-F et al., 2010). The transgenic mice experiments demonstrated that mutation of TS1 dramatically decreased the transgene expression in prostate lobes whereas mutation of TS2 almost completely abolished the transgene activities in the transgenic mouse prostate (Zhang J-F et al., 2010). These data suggests that TS1 and TS2 work cooperatively to control PB gene expression in the prostate. Both TS1 and TS2 are adjacently positioned to ARBS1 and ARBS2 (Zhang J-F et al., 2010), respectively, suggesting that a fixed binding site organization maybe conserved due to functionally importance. The ARR_2PB promoter duplicates the ARR region, containing four ARBS and three tissue-specific elements (one TS1 plus two TS2) that function cooperatively as a strong prostatic-specific promoter *in vitro* and *in vivo* (Farmer et al., 2001). Interestingly, TS1 and TS2 are interchangeable in biological function further suggesting the bind either identical or very similar factors (Zhang J-F et al., 2010). Southwestern experiments using $2 \times TS2$ as probes identified two proteins that bind at this region (Gao et al., 2003). One protein is about 52 kDa, another is about 45 kDa (Gao et al., 2003). The 52 kDa protein has been confirmed as FoxA1, a forkhead transcription factor (Gao et al., 2003). FoxA1 and AR have been identified to bind adjacent to each other at both ARBS1 and ARBS2 (Gao et al., 2003). FoxA1 is expressed in human and mouse prostate epithelium, and FoxA1 expression is important not only in prostate-specific gene regulation but also in prostate development (Gao et al., 2003; Gao et al., 2005b). The prostates rescued from FoxA1-deficient mice display a severely disrupted ductal pattern resembling primitive epithelial cords, with an expansion of the basal cell population, and a lack of epithelial differentiation and PB expression, as well as other molecular alterations (Gao et al., 2005b). Interestingly, adjacently positioned

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forkhead and AREs have also been identified in several key regulatory regions of other prostatic genes, including PSA core enhancer, SBP promoter, PAP promoter, etc., suggesting a fundamental role for FoxA1 in prostatic-specific gene expression (Gao et al., 2003). A direct interaction between FoxA1 and AR has been observed in these promoters, indicating that both transcription factors cooperatively participate in the expression of these androgen-regulated prostate specific genes (Gao et al., 2005a).

FoxA proteins activate transcription by displacing linker histones from nucleosomes and opening chromatin, making the local DNA region more accessible for other transcription factors (Cirillo et al., 1998). However, the molecular mechanisms by which FoxA proteins control transcription has only been partially characterized and little information exists on corresponding co-regulators that mediate the crosstalk of FoxA1 with components of the general transcription machinery. FoxA1 binding sites are associated with glucocorticoid receptor binding sites (O'Brien et al., 1995) and are now recognized to be a common co-regulator with androgen receptor and estrogen receptor regulated genes (Gao et al., 2003; Laganiere et al., 2005; Carroll et al., 2005; DeGraff et al., 2009; Yu et al., 2005; Yu et al., 2006; Wang et al., 2007; Jia et al., 2008). Chromosome wide mapping analysis has shown that GATA2 and Oct-1 binding sites are also commonly associated with binding sites for AR and FoxA1 (Wang et al., 2007), and more recent data has identified that NFI binding sites are also commonly associated in this complex (Jia et al., 2008). The fact that FoxA binding sites are found adjacent to these other transcription factor binding sites suggests that FoxA proteins are capable of interacting with these proteins to control the activity of *cis* regulatory elements.

FoxA1 expression is detected early in prostate development and FoxA1 gene expression is necessary for normal prostate development as well as being implicated in the androgen regulation of prostate cancer (DeGraff et al., 2009). Thus, FoxA1 must regulate genes involved in development and differentiation of the prostatic luminal cells and androgen regulation of the growth of prostate cancer. Recent publications have shown that AR and adjacent FoxA1 sites are found on multiple promoters in prostatic cells (Jia et al., 2008; Lupien and Brown, 2009). Identifying the intermolecular interactions between FoxA1 and AR as well as other transcription factors as they regulate prostatic gene expression is necessary to understand the mechanism that results in failure of prostate cancer patients to androgen ablation therapy.

One of the two proteins, which were identified from the Southwestern analysis with molecular weight about 45 kDa, we have confirmed to be USF2 protein (Sun et al., 2009). The present study has also identified USF2 as a novel FoxA1 interacting partner (Sun et al., 2009). USF2 is an E-box site binding protein which belongs to the basic-helix-loop-helix-leucine-zipper (bHLH-zip) family of transcription factors (Sirito et al., 1992). Studies suggest that USF2 functions as a tumor suppressor and can down regulate c-myc expression (Chen et al., 2006; Moriuchi et al., 1999; Luo and Sawadogo, 1996). USF2 knockout mice demonstrate prostate hyperplasia indicating that USF2 plays an important role in prostate differentiation (Chen et al., 2006).

Using the probasin GAAAATATGATA (-250 to -239 bp) sequence that overlaps with

ARBS1, affinity purification of transcription factors was performed and USF2 was shown to bind within this sequence (Kivinen et al., 2004). We confirm that USF2 does indeed bind to the PB promoter and that the DNA binding domains of USF2 and FoxA1 directly interact (Sun et al., 2009). FoxA1 binding sites exist on the PSA and SBP promoters, and we also show that USF2 binds to the PSA and SBP promoters (Sun et al., 2009). This indicates that functional binding sites for FoxA1 and USF2 are co-localized on these prostate-specific promoters (Sun et al., 2009). Whole-genomic mapping studies for USF1 and USF2 binding sites show that these two transcription factors have distinct determinants for binding to DNA (Rada-Iglesias et al., 2008). Further, the analysis showed that a subset of 240 regions bound only USF2 (Rada-Iglesias et al., 2008). Consensus FoxA binding sites were the most abundant sites associated with these USF2 unique sites, and FoxA binding sites were not significantly associated with USF1 binding sites (Rada-Iglesias et al., 2008). Furthermore, seven out of eight regions that were perfect matches for FoxA sites were confirmed to bind both FoxA and USF2 (Rada-Iglesias et al., 2008). In addition, a recent study using a oligonucleotide-based assay to identify novel factors that bind to the ARE1 oligonucleotide from the PSA gene promoter indicate both FoxA1 and USF2 are bound to the region (Wong et al., 2009). In the present study, EMSA experiments confirmed the binding site of USF2 in the PSA core enhancer and show that USF1 fails to bind this region (Sun et al., 2009), in spite of the fact that USF2 and USF1 share a highly conserved C-terminal DNA binding domain (Sirito et al., 1994). These results indicate that the binding of USF2 in the PSA core enhancer is specific. Furthermore, USF2 binding sites were confirmed by ChIP assay on PB, SBP, and PSA regulatory regions, suggesting USF2 plays an important role in

prostate-specific promoters (Sun et al., 2009). Our studies and others (Rada-Iglesias et al., 2008) now confirm that FoxA1 and USF2 binding sites often co-exist on multiple promoters.

While the AR requires the presence of hormone to bind to functional *cis* regulatory sequence, FoxA1 and USF2 bind to the PB and SBP promoter both in the presence and absence of androgen (Sun et al., 2009). The PSA promoter contains low levels of USF2 as measured by real-time PCR and the binding can be seen in Figure 11, where the USF2 band shows up after 31 PCR cycles in the absence of androgens (Sun et al., 2009). The USF2 consensus DNA sequence is CANNTG and the matches found in PB is CAATTT, SBP is CAAAGT, and PSA is CAAATC. These differences between the three promoters may cause the differences in binding affinity of USF2. FoxA1 plays an essential role in the "pioneering" of gene regulatory elements, allowing for the recruitment of additional factors required for gene regulation (Cirillo et al., 1998), and our data suggests that USF2 cooperates with FoxA1 to regulate promoter activity. Changes in USF2 binding with or without AR activation were minimal. Thus, our results could not determine if USF2 association is changed or unchanged by the binding of AR. As USF2 knockout mice develop prostate hyperplasia (Chen et al., 2006), and our data indicates that USF2 cooperates with FoxA1 to regulate gene expression that is associated with normal differentiation in the prostate.

As previously reported, down-regulation of FoxA1 had a limited effect on promoter activity following transient transfection (Carroll et al., 2005). This appears to be true for

USF2 as well, since down regulation USF2 expression in LNCaP cells failed to cause a dramatic change in ARR_2PB or PSA promoter activity following transient transfection (Sun et al., 2009). This may be explained by the fact that in transient transfected DNA studies, the DNA remains naked and normal nucleosome structures do not form (Cirillo et al., 1998). Therefore, even though FoxA1 and USF2 knockout mice exhibit significant prostate phenotypes, it is reasonable to expect a limited impact of FoxA1 and USF2 knockdown on promoter activity following transient transfection. However, the endogenous PSA mRNA levels slightly increased in LNCaP cells when USF2 levels were decreased. Even though the change of the PSA level was not statistically significant, it may suggest that USF2 is affecting chromatin structure of the PSA promoter that is absent on transient transfected DNA.

Our previous studies have shown that androgen induced AR binding is adjacent to bound FoxA1 and that the DNA binding domain of the AR interacts with the DNA binding domain of FoxA1 (Gao et al., 2003). We show that USF2 is bound in a complex on DNA; whether FoxA1 and USF2 are bound to the same promoter at the same time or independent of each other we could not determined. Since FoxA1 has been reported as a common co-regulator of AR and ER (Gao et al., 2003; Laganiere et al., 2005; Carroll et al., 2005; DeGraff et al., 2009; Yu et al., 2005; Yu et al., 2006; Wang et al., 2007; Jia et al., 2008) and USF2 can bind to the same/similar DNA sequence as FoxA1 (Rada-Iglesias et al., 2008), (Zhao et al., 2001) (Schuur et al., 2001; Kivinen et al., 2004)it is highly likely that our observation that USF2 is a co-regulator for these three androgen regulated and prostate-specific genes will also extend to a larger list of AR/FoxA1 (DeGraff et al., 2009)

as well as ER/FoxA1 regulated genes. We show that USF2 interacts with FoxA1 (Sun et al., 2009), and although a weak interaction of USF2 with AR has been reported (Kivinen et al., 2004), we could not detect the interaction of USF2 with AR (data not shown). Thus, USF2 may exist in a complex with the AR and other steroid receptors, but it is likely that the main interaction of USF2 is with FoxA1, suggesting FoxA1 acts as a bridge between USF2 and the AR, as well as other steroid hormone receptors. By ChIP assays, it is possible that we are detecting promoter sequences either bound with FoxA1 or USF2 but not both FoxA1 and USF2 at the same time. Also, it seems contrary that two transcription factors can bind to the DNA at the same site at the same time but it is possible to envision that the DNA is sandwiched between the these two factors. Since FoxA1 and USF2 directly interact at the protein level via their DNA binding domains, we would expect protein to protein contact as well as protein to DNA contact would occur with both factors bound to the DNA at the same time. We have used ChIP on ChIP assay to examine whether the two proteins bind to the DNA the same time or not, the method could not be established due to the poor quality of the USF2 antibody.

Working with Dr. Jonathan H Sheehan and Dr. Jarrod A Smith (Vanderbilt University), we have generated a computational model of protein-protein interactions that predicts the interaction of AR, FoxA1, and USF2 bound to probasin, a prostate specific promoter (Figure 25). The 3-D model is assembled manually by optimal 3-D alignment of the DNA oligos from the protein X-ray crystal structures with the NAB-generated structure of the PB promoter region using the molecular visualization program called UCSF Chimera (http://www.cgl.ucsf.edu/chimera) (Pettersen et al., 2004). This 3-D

model can mimic the binding of AR, FoxA1, and USF2 to PB promoter. Our data

demonstrated that AR and FoxA1 bound to DNA at adjacent sites and the USF2 bound to

the same binding site as FoxA1. Using existing X-ray crystal structures of the DNA

binding region of AR, FoxA3, and USF1 (the X-ray crystal structure of FoxA1 and USF2

is not available) bound to DNA, Dr. Sheehan and Smith created a model that predicted an

interface between the AR DNA binding domain and the FoxA1 DNA binding domain.

USF2 is predicted to binding at the same DNA site as FoxA1 but forming a clothes pin

structure that helps secure both FoxA1 and AR to their binding sites.
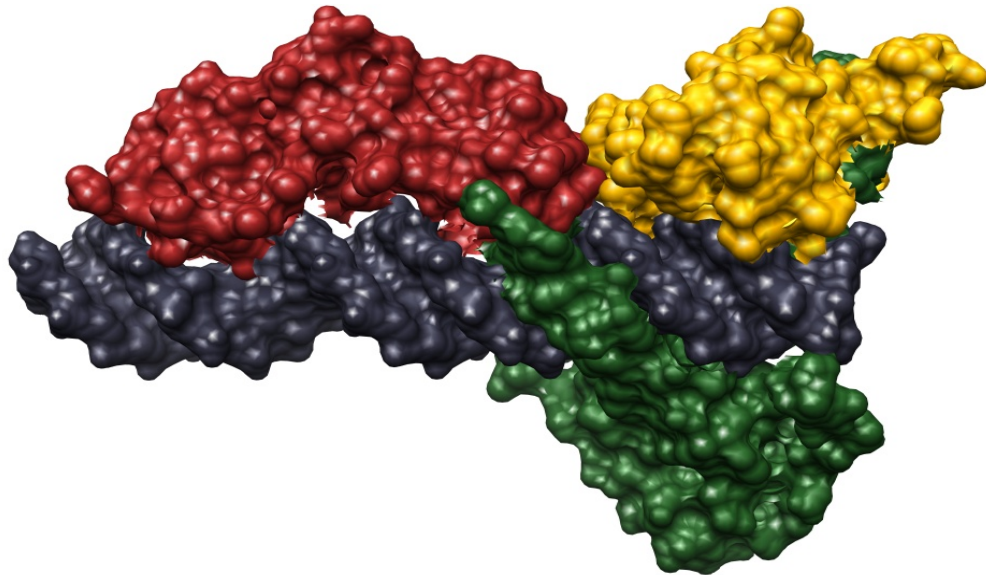
Figure 25. Computational modeling of AR, FoxA1, and USF2 protein interacting with PB promoter. The blue DNA represents the PB sequences of the prostate specific promoter. The red, yellow, and green proteins represent AR, FoxA1, and USF2 respectively (see details in material and methods).

Using the 3-D molecular model, which is based on the experimental X-ray crystal structures of AR and FoxA3 bound to DNA, we have identified the interface between these two proteins. Within seven animo acids in the DNA binding domain of AR interface identified, point mutations of two amino acids (L574P and T575A) occurs in castrate-resistant prostate cancer and point mutation of two amino acids occurs in androgen insensitivity syndrome (AIS). Our lab has demonstrated that the two mutations found in AIS inactivate the AR. However, DHT induced AR activity was enhanced only in the presents of FoxA1 by 32% with the L574P mutation and 72% with the T575A mutation (DeGraff, unpublished data). Since this increase in AR activity only occurred when FoxA1 was expressed, this suggests that an increase in binding between AR and FoxA1 fosters an increased sensitivity to low dose androgens. This could account for continued AR action even when androgens are blocked in the CRPC patients. Thus, designing drugs to interfere with AR activity by blocking AR-FoxA1 protein interactions provides an achievable and unique approach to inhibit the growth of prostate cancer. Since we have shown that USF2 interacts with FoxA1 and binds to the same DNA site (Sun et al., 2009), a drug that targeted AR-FoxA1 likely would disruption of the AR/FoxA1/USF2 complex. In addition to AR/FoxA1/USF2, we have shown that Oct-1, c-jun, NFI, and other unidentified TFs are bind to the proximal PB DNA region (Zhang J-F et al., 2010), suggesting a large protein complex forms together to regulate prostate-specific gene expression. Identifying additional proteins that may also enter into the transcriptional complex with FoxA1 and AR can help us to discover the critical transcription factors that control cell type specific gene expression and provide a more complete understanding of prostate development. New drugs and therapies for prostate

cancer patients could be developed to target the key transcription factors that control AR activity.

To identify novel FoxA1 binding partners, FLAG and 6-His tagged human FoxA1 protein stably expressed in the LNCaP cell line was established. The LNCaP cell line is androgen-dependent and AR positive cell line that contains endogenous FoxA1. This cell line serves as a good model to study FoxA1 binding partners. The tagged FoxA1 was highly expressed in LNCaP cells when compared to the endogenous level of FoxA1 (Figure 20). The high expression level of the tagged FoxA1 can reduce the DNA binding or protein-protein binding of the endogenous FoxA1. However, the high level of tagged FoxA1 expression may not reflect the true FoxA1 level in biological systems; therefore, some false positive binding partners may be pulled down. To avoid the false positive binding, the identified FoxA1 binding proteins need to be subjected for further protein-protein interaction studies to confirm the interactions are real.

After the tandem purification of the tagged FoxA1 protein complex, LC-MS/MS analysis has detected 16 proteins that appear to interact with FoxA1. AR was shown to be pulled down together with FoxA1 (Figure 23) by western blot, but AR was not identified by LC-MS/MS analysis, which may be due to the low sensitivity of LC-MS/MS compared with western blot or immunoprecipitation. USF2 could not be identified either by this study, this may be due to the relatively low expression level of USF2 or low binding affinity with FoxA1. Of the 16 identified proteins, 7 have been previously reported to directly interact with AR, or an indirect link can be established. Three groups

of nuclear proteins that were identified: 1) alter AR activity (7 proteins) while no reports exist on DNA binding protein, TOX HMG family # 4; 2) control protein structure/stability (2 proteins); and 3) involved in RNA processing (6 proteins). None of these nuclear proteins were reported by Kivinen et al., when they did affinity purification of nuclear proteins using a fragment of the probasin that contains the TS-1 sequence and the AR/FoxA1 binding site (Kivinen et al., 2004).

The seven proteins that co purified with FoxA1 that have been shown to associate with AR are most interesting. NFI TFs are involved in tissue-specific expression (Wong et al., 2007; Grunder et al., 2002; Steele-Perkins et al., 2003; Driller et al., 2007; Campbell et al., 2008), which may act in concert with AR and FoxA1 to modulate prostate-specific gene expression. NS is reported to involved in tumorigenesis and cell senescence (Fox et al., 2002) and is absent in most adult differentiated cells (Tsai and McKay, 2002; Liu et al., 2008); however, NS is expressed in the normal adult mouse prostate tissue (unpublished data), which indicates that interaction of NS with FoxA1 and AR suggests that NS is involved in the regulation of prostate specific gene expression. PSP1 is known to play important roles in DNA replication, transcription, and mRNA processing, suggesting that the interaction of this protein with FoxA1 may have several important consequences (Myojin et al., 2004). Most interestingly, Matrin 3 has been shown to be a major component of the nuclear matrix, suggesting that FoxA1 subnuclear localization is maintained by this important protein. The fact that Matrin 3 has also been shown to play a role in controlling the export of edited mRNAs (Zhang and Carmichael, 2001), suggests that the interaction of FoxA1 with Matrin 3 may have important

consequences for the splicing of FoxA1 regulated transcripts. It is also important to note that the DNADPK that co-purified with FoxA1 has been shown to phosphorylate FoxA2, a forkhead protein closely related to FoxA1 (Nock et al., 2009). The phosphorylation of FoxA2 enhances its ability to bind to DNA (Nock et al., 2009). It would be reasonable to postulate that DNADPK then functions to downregulate AR binding to the DNA but to enhance binding of FoxA1. By enhancing FoxA1 binding, this would allow the chromatin confirmation to remain accessible for re-activation by the AR.

In summary, we report a novel FoxA1 interacting partner USF2 which shares the same binding sites as FoxA1 in multiple prostate specific promoters. Since FoxA1 plays a major role in modifying chromatin structure and USF2 interacts with FoxA1 at the same DNA binding site, we would predict the USF2 is involved in FoxA1 regulation of chromatin structure. Whole-genomic mapping studies for USF1 and USF2 binding sites show that USF2 binding sites, but not USF1, are frequently associated with consensus FoxA binding sites (Rada-Iglesias et al., 2008). This supports the conclusion that the functional interaction between USF2 and FoxA1 is not limited to AR regulated genes and that this interaction play a fundamental role in FoxA1 role as a "pioneer factor".

FoxA1 functions in a cell type-specific manner to attract lineage-specific transcription factors. Together with the methylation of histone H3, FoxA1 forms part of the epigenetic signature that alters chromatin structure allowing for lineage-specific gene regulation (Lupien et al., 2008). This is consistent with the role of FoxA1 to serve as a "pioneer factor" that displaces linker histones from nucleosomes, causing an unfolding of

chromatin and allowing the accessibility of DNA regulation region to other TFs (Cirillo et al., 2002). This fundamental function of FoxA1 should play a critical role in chromosome organization in response to AR signaling. Prostate epithelium requires AR for differentiation, FoxA1 interacts directly with AR, and prostate tissue rescued from FoxA1 null mice exhibits abnormal histology and fails to express differentiation markers such as probasin (DeGraff et al., 2009). This indicated that FoxA1 expression is required for AR action.

Transcriptional regulation not only depends on the interactions between DNA and binding partners, but also on the interactions among the protein-protein interactions. 16 FoxA1 binding partners have been identified by using tandem purification followed by LC-MS/MS analysis. Transcriptional factor NFIx is shown to also interact with AR (DeGraff, unpulished data) and NS is reported to indirectly interact with AR. Both of the proteins are involved in prostate development and prostate cancer. A recent study using formaldehyde assisted isolation of regulatory elements monitored the chromatin structure at FoxA1 binding sites chromosome-wide and found out that a significant portion of FoxA1-bound sites represent a relatively closed chromatin conformation and are not correlated with gene expression in a certain cell type (Eeckhoute et al., 2009). Interestingly, the same FoxA1-bound regulatory sites are inactive in one cell type, but functional in another cellular context, suggesting that FoxA1 binding is required, but not sufficient, for a functional activity of bound enhancers in a given cell type (Eeckhoute et al., 2009). These findings provide evidence that other interacting partners of FoxA1 are necessary to define the cell-type-specific expression.

Future Direction

We have identified some of the binding partners of FoxA1 in LNCaP cells. The same experimental approach will be applied to HeLa FoxA1 / HeLa E cells and HeLa AR FoxA1 / HeLa AR E cells. Comparing the binding partners for FoxA1 in LNCaP cells with that from HeLa cells will reveal which binding partners are important in prostate cancer cells and which are common to a non-prostatic cells. Also, by using HeLa-AR cells, the purification of proteins by a tagged FoxA1 may form a complex with AR that will identify transcription factors not seen in HeLa cells alone.

Further study on the identified FoxA1 binding transcription factors will be performed:

1. To confirm the binding between FoxA1 and the binding partners which have been identified by LC-MS/MS. After the identification of the FoxA1 binding partners, they will be assigned into classes of function, if possible. Only transcription factors identified as FoxA1 binding partners will be pursued at this time. Clones will be obtained, if necessary by PCR cloning. Confirmation of the binding between FoxA1 and these binding partners will be performed to avoid the false positive results by the following approaches:

a) *In vitro* GST pull-down assay or co-IP followed with Western blot will be used to confirm the binding.

b) Confocal fluorescent microscopy will be performed for colocalization, if

antibodies are available.

2. Function analysis of the FoxA1 binding partners. After the confirmation of the binding between FoxA1 and other transcription factors, we will further analyze the function of this complex to androgen regulated gene expression. The identified transcription factors will be placed in an expression vector. The following experiments will be performed:

a) Using the PSA-Luc and PB-Luc reporter described above, transient transfection with FoxA1, AR, and expression vectors for the identified transcription factors will be performed to test the biological activity of the FoxA1 binding partners.

b) Since FoxA1 interacts in a complex of proteins, the newly identified binding partners will be characterized not only for there ability to bind to FoxA1 but also to AR and USF2.

c) EMSA and supershifts using specific antibodies against the transcription factors will be performed using a radiolabeled oligonucleotide containing both FoxA1 and ARE of the human PSA core enhancer (5'-CCTACTCTGGAGGAACATATTGTATTGATTGTCCTTGACAGTAAACAAATCT GTT-3', 55bp). These experiments will test if the transcription factor binds to the DNA and interact with FoxA1 or if they bind by protein-protein interactions.

d) ChIP will be performed to investigate the *in vivo* association of the transcription factors with human PSA enhancer and PB promoter. Specific antibodies against the transcription factors, FoxA1, and AR will be used for immunoprecipitation of antigen-bound transcription factors to genomic DNA fragments from LNCaP cell lysate

(treated with DHT or maintained in androgen-depleted medium). The DNA precipitated down by the antibodies will be amplified by PCR using specific primers spanning the tested regions.

e) Transcription factors identified to have the greatest effect on FoxA1 activity will be up or down regulated by siRNA in LNCaP cells and androgen regulation of both PSA by Western and RT-PCR will be monitored. Further, cell proliferation will be monitored.

In summary, by a combination of approaches, the functional importance in AR action of the transcription factors identified as FoxA1 binding partners will be determined. This information will help decipher the mechanism of AR action in controlling prostate-specific gene expression as well as proliferation of prostatic cells.

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