# ANDROGEN RECEPTOR AND INTEGRIN REGULATION OF PROSTATE TUMOR SURVIVAL AND INVASION

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## ABSTRACT

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The human prostate gland is dependent on circulating testosterone for growth, development, and maintenance in men throughout their lifespan. In early stages of prostate cancer, the prostate epithelial cells express androgen receptor (AR) and rely on testosterone for growth and proliferation. At this stage, the cancer is localized and can be treated easily. One of the most common treatments that has been used for over 50 years is androgen deprivation therapy (ADT), which kills epithelial cells that express AR, giving the patient a very high regression rate. Unfortunately, this disease relapses several years later and when this occurs, the disease is non-responsive to ADT and termed castration-resistant prostate cancer (CRPC). Interestingly, a large percentage of these tumor cells express a mutated form of AR or have higher expression of AR and still depend of AR for survival. Furthermore, this is complemented with increased expression of transmembrane heterodimeric adhesion proteins called integrins and migratory proteins that lead to prostate cancer metastasis, for which there is no cure. Recent studies showing that depletion of AR in metastatic prostate cancer cells can lead to the death of these prostate tumor cells suggest that AR is vital for the survival of even CRPC. To build upon this finding and establish a prostate cancer model that closely resembles what is observed in the metastatic disease, we generated several cell line models in which AR expression in a metastatic prostate cancer cell line causes the cells to behave like hormone-refractory tumors in that they do not respond to androgen.

Within this model there was a drastic increase in androgen-independent, but AR-dependent, tumor cell survival and a drastic increase in integrin α6β1 with a concomitant decrease in β4 integrin, all of which is observed clinically in patients with CRPC. We also observed an AR-dependent increase in the level of non-receptor tyrosine kinase Src activity as well as its downstream effector proteins and proteases that are crucial in tumor cell migration and tumor cell escape respectively. With these findings, we hypothesized that AR plays an essential role in both the survival and invasiveness of prostate cancer. We propose that tumor survival is driven through a classical nuclear regulation pathway and a novel intracellular and non-nuclear signaling mechanism promotes the aberrant cellular behavior leading to enhanced migration and invasion. In this dissertation I will test this idea in our cell models to establish the importance of these novel pathways.

Prostate cancer is a disease that afflicts our fathers, uncles, brothers, nephews, cousins, neighbors, and loved ones. Usually, these men when over the age of 50 will be affected. As stated above, the metastatic and castration-resistant disease is untreatable and will ultimately lead to death. Since it is the metastatic and lethal disease that ultimately leads to death, the proposed studies will potentially help all of these men that suffer from lethal disease by targeting AR, Src, and integrin α6β1 with therapeutic inhibitors that may suppress prostate cancer tumors from surviving and migrating. With the understanding of how these signaling proteins are increased during prostate cancer progression and eventual metastasis, these studies will be applicable to treat disease using drugs that will target these signaling proteins.

In loving memory of my Grandparents, Mrs. Estella Louise Dabney (1929-2009) and Mr. Clarence Dabney (1929-2012) who both passed away as I pursued this degree. Both inspired and encouraged me to always strive for the very best. Grandma, you prayed for this.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## KEY TO SYMBOLS OR ABBREVIATIONS

| Abbreviation/Symbol | Definition |
|---|---|
| 3βHSD1 | 3β-hydroxysteroid dehydrogenase type 1 |
| A | adenine or alanine |
| α | Alpha |
| a2 | integrin α2 |
| a3 | integrin α3 |
| a5 | integrin α5 |
| a6 | integrin α6 |
| ABI | Applied Biosystems |
| Act D | Actinomycin D |
| ABL | Abelson murine leukemia viral oncogene homolog 1 |
| ADT | androgen deprivation therapy |
| AF-1 | activation function domain 1 |
| AF-2 | activation function domain 2 |
| AKR1C3 | Aldo-keto reductase family 1 member C3 |
| Akt | protein kinase B |
| APC8015 | Sipuleucel-T |
| AR | androgen receptor |
| AR1 | PC3-AR-1 |
| AR2 | PC3-AR-2 |
| ARE | Androgen Response Element |

| | |
|---|---|
| AR-V | Androgen Receptor Variant |
| ATCC | American Type Culture Collection |
| AZ | Arizona |
| β | Beta |
| b1 | integrin β1 |
| b4 | integrin β4 |
| Bcl2L1 | Bcl-xL |
| Bcl-xL | BCL2-like 1 |
| BCR | breakpoint cluster region |
| BPE | bovine pituitary extract |
| BPH | benign prostate hyperplasia |
| BSA | bovine serum albumin |
| C | Cytosine |
| $[Ca^{2+}]_i$ | intracellular $Ca^{2+}$ concentration |
| CaCl | calcium chloride |
| Caso | Casodex |
| CDCP1 | cub domain containing protein1 |
| cDNA | complementary DNA |
| ChIP | chromatin immunoprecipitation |
| CK | cytokeratin |
| CL | collagen |
| cm | centimeter |
| CMB | Cell and Molecular Biology |

| | |
|---|---|
| $CO_2$ | carbon dioxide |
| CRPC | castration-resistant prostate cancer |
| Csk | c-terminal Src kinase |
| CSS | charcoal stripped serum |
| Ct | count |
| CYP11A1 | Desmolase |
| CYP17A1 | 17 α-hydroxylase/C17,20 lyase |
| D | DMSO |
| DAP3 | death-associated protein 3 |
| Das | Dasatinib |
| DBD | DNA binding domain |
| DHEA | dehydroepiandrosterone |
| DHT | dihydrotestosterone |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DNAse | deoxyribonuclease |
| Dox | doxycycline |
| DR | death receptor |
| ECD | extracellular domain |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| e.g. | exempli gratia |

| | |
|---|---|
| EGF | epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| EMT | epithelial-mesenchymal transition |
| ER | estrogen receptor |
| ERE | estrogen-response element |
| Erg | ETS-related gene |
| ERK | extracellular signal-regulated kinase |
| ETS | E-twenty six family of transcription factors |
| et al. | et alii (and others) |
| ETV | ets variant |
| F | Phenylalanine |
| FACS | Fluorescence Activated Cell Sorting |
| F-actin | filamentous polymers of actin |
| FAK | focal adhesion kinase |
| FBS | fetal bovine serum |
| FISH | fluorescent in situ hybridization |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| FOXO | forkhead box-O transcription factors |
| γ | gamma |
| G | guanine |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| h | Hour |

| | |
|---|---|
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HI-FBS | heat inactivated fetal bovine serum |
| HGPIN | high grade PIN |
| Hsp | heat shock protein |
| hygro | hygromycin |
| IAP | inhibitor of apoptosis |
| IF | Immunofluorescence |
| i.e. | id est (that is) |
| IGF-1 | insulin growth factor-1 |
| IGFR | Insulin-like growth factor receptor |
| IgG | immunoglobulin G |
| IHC | immunohistochemistry |
| IP | immunoprecipitation |
| ITG | integrin |
| ITGA6 | integrin α6 |
| JNK | jun n-terminal kinase |
| k | kappa |
| K | keratin or lysine |
| KLK | kallikrein |
| LBD | ligand binding domain |
| LBD18 | PC3-ΔLBD-AR-18 |
| LBD28 | PC3-ΔLBD-AR-28 |
| LDLRA | lipoprotein receptor class A |

| | |
|---|---|
| LHRH | luteinizing-hormone releasing hormones |
| LM | laminin |
| LN | LNCaP |
| LY | LY294002 |
| LY294002 | 5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione |
| M | methionine |
| MAPK | Mitogen-activated protein kinase |
| MEM | Minimum Essential Medium |
| MI | Michigan |
| μL | microliter |
| mL | milliliter |
| μM | micromolar |
| mM | millimolar |
| MMP | matrix metalloproteinases |
| MNAR | modulator of non-genomic activity of ER |
| Mptase | matriptase |
| MSU | Michigan State University |
| mTOR | mammalian target of rapamycin |
| MUC-1 | Mucin 1, cell surface associated |
| N | any nucleotide or the number of experimental replicates |
| $Na_3VO_4$ | sodium orthovanadate |
| NaCl | sodium chloride |
| NaDOC | sodium deoxycholate |

| | |
|---|---|
| NaF | sodium fluoride |
| NF-kB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| Nkx3.1 | NK-3 transcription factor, locus 1 |
| NLS | nuclear localization signal |
| NLS30 | PC3-ΔNLS-AR-30 |
| NLS4 | PC3-ΔNLS-AR-4 |
| nM | nanomolar |
| NR13 | anti-apoptotic protein NR13 |
| NT | not treated |
| OD | optical density |
| P | phosphorylation or proline or PBS |
| PAP | prostatic acid phosphatase |
| PBS | phosphate buffered saline |
| PDGF | platelet-derived growth factor receptor |
| PDK1 | phosphatidylinositol-dependent kinase 1 |
| PhD | Doctor of Philosophy |
| phospho | phosphorylation |
| PI | propidium iodide |
| PI3-K | Phosphoinositide-3 kinase |
| PIN | prostatic intraepithelial neoplasia |
| $PIP_2$ | phosphatidylinositol (4,5)-bisphosphate |
| $PIP_3$ | phosphatidylinositol (3,4,5)-trisphosphate |
| PKC | protein kinase c |

| | |
|---|---|
| PL | PC3-pLKO |
| PMSF | phenylmethylsulphonyl fluoride |
| PP | PC3-puro |
| PR | progesterone receptor |
| PSA | prostate specific antigen |
| PTEN | Phosphatase and Tensin Homolog |
| puro | puromycin |
| PVDF | polyvinylidene difluoride membrane |
| PY416 | anti-phoshotyrosine 416 antibody for Src |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| R | adenine or guanine |
| R1881 | Metribolone |
| RACK1 | receptor for activated C kinase 1 |
| RGD | Arginylglycylaspartic acid |
| RIPA | Radio Immunoprecipitation Assay |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RPMI | Roswell Park Memorial Institute |
| rRNA | Ribosomal RNA |
| RTK | receptor tyrosine kinase |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| RU486 | Mifepristone |
| scr | scram |

| | |
|---|---|
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis |
| SEA | sperm protein, enteropeptidase and adrin |
| Ser | serine |
| SFM | serum free media |
| SH1 | src homology domain 1 |
| SH2 | src homology domain 2 |
| SH3 | src homology domain 3 |
| SH4 | src homology domain 4 |
| shRNA | short hairpin RNA |
| siA6 | siRNA against A6 |
| siAR | siRNA against AR |
| siRNA | small interfering RNA |
| sSrc/siSrc | siRNA against Src |
| SNP | single-nucleotide polymorphism |
| SP | serine protease |
| SPOP | speckle-type poxvirus and zinc finger (POZ) domain protein |
| Src | sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog |
| SRC-1/NCOA1 | Nuclear receptor coactivator 1 |
| SRC-2/NCOA2 | Nuclear receptor coactivator 2 |
| SRC-3/NCOA3 | Nuclear receptor coactivator 3 |
| SFKs | src family kinases |

| | |
|---|---|
| SRS | src response signatures |
| starv. | starvation |
| STAT-3 | signal transducer and activator of transcription 3 |
| SYBR green | N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine |
| T | thymine |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline containing 0.1% Tween 20 |
| TetON | tetracycline inducible shRNA |
| TFIIB | Transcription Factor II B |
| TFIID | Transcription Factor II D |
| TFIIE | Transcription Factor II E |
| TFIIF | Transcription Factor II F |
| TFIIH | Transcription Factor II H |
| TGFβ | transforming growth factor β |
| Thr | Transmembrane protease, serine 2 |
| TMP | TMPRSS2 |
| TMPRSS2 | transmembrane protease, serine 2 |
| TRAIL | TNF-related apoptosis-inducing ligand |
| Tub | tubulin |
| U | Units |
| Uegfr | urchin embryonic growth factor |
| UV | ultraviolet |

| | |
|---|---|
| VAI | Van Andel Institute |
| VARI | Van Andel Research Institute |
| Veh/Vec | vehicle |
| W | adenine or thymine |
| WCL | whole cell lysate |
| X | any amino acid |
| Y | cytosine or thymine, or tyrosine |

# CHAPTER ONE:
# INTRODUCTION

## Introduction

Prostate cancer is a leading cause of cancer death in men in the U.S., and in the past year, over 186,000 men were diagnosed and more than 28,000 died from this disease (Siegel et al., 2013). Prostate cancer is usually indolent, strongly correlates with age, and usually is detected in men sixty or older (Bubendorf et al., 2000; Sakr et al., 1994). Several post-mortem studies suggest that up to 80% of 80 year-old men have prostate cancer and some experts predict that all men would develop prostate cancer if they lived long enough (Bubendorf et al., 2000; Sakr et al., 1994). Given that prostate cancer is a relatively slow growing disease, the majority of men diagnosed with prostate cancer may actually die from other causes (Abate-Shen and Shen, 2000). This disease also affects some ethnic groups, particularly men of African descent, at earlier ages disproportionately (Agoulnik and Weigel, 2006; Culig et al., 1994). While primary prostate cancer is highly treatable by surgical resection and radiation, prostate cancer that has metastasized is not. This is reflected by a five-year survival rate of 100% for local and regional prostate cancer, and 31% for metastatic prostate cancer (Horner et al., 2009). When the disease is at a small focus in the gland, usually in its androgen-dependent stage, survival rates are at their highest. Treatments include chemotherapy, hormonal therapy, tumor removal, implantation of radiation seeds, androgen deprivation therapy (ADT), and radical prostatectomy. However, after ADT, the disease is still able to convert low levels of testosterone to dihydrotestosterone (DHT; a more potent ligand for AR), to activate AR for growth, and elude therapeutic approaches. After this, the disease is able to progress to a metastatic androgen-insensitive stage, which is practically incurable.

## The Prostate Gland

The prostate is a small, walnut-sized exocrine gland weighing an average of 11 grams that surrounds the bladder and neck of the urethra in men (Abate-Shen and Shen, 2000; Cunha et al., 1987; Leissner and Tisell, 1979). During the fetal stages and during the lifespan of men, prostate gland growth is dependent on a secreted sex hormone, testosterone. The prostate gland functions to secrete alkaline proteins that nourishes the sperm (Abate-Shen and Shen, 2000). This seminal fluid is important for sexual reproduction.

The human prostate gland is composed of a simple stratified epithelium containing two layers: a basal layer and a secretory layer. The basal layer is composed of basal cells which express cytokeratins 5 and 14 and do not produce secretory prostatic proteins (Abate-Shen and Shen, 2000). Neuroendocrine cells are also dispersed sparsely within the basal cell layer and express neuroendocrine markers such as chromogranin A and synaptophysin (Abate-Shen and Shen, 2000). The second layer is the secretory layer, which is where the luminal cells are located. These cells are columnar in shape and express cytokeratins 8 and 18 (Brawer et al., 1985; Nagle et al., 1987). The prostatic stroma, which surrounds the epithelial layer, consists of fibroblasts, smooth muscle cells, mast cells, dendritic cells, and endothelial cells (Abate-Shen and Shen, 2000; Feldman and Feldman, 2001). These cells provide growth factors such as andromedins for the growth and differentiation of the epithelial cells in the gland (Agoulnik and Weigel, 2006). The basal epithelial cells, which the secretory cells rest upon, do not express AR, but express integrins α2β1, α3β1, and α6β4. The basal cells adhere via these integrins to a basement membrane rich in extracellular

matrix (ECM) proteins laminin 5, laminin 10, collagen IV and collagen VII. The luminal cells (also called secretory cells) are positioned atop the basal cells bound to the extracellular matrix. It is to be noted that within the prostate epithelium, stem cells are present but rare, and are located within the basal layer. Less than 1% of all basal cells are stem cells (Richardson et al., 2004). The luminal cells express AR and secrete prostate specific antigen (PSA) into the lumen of prostate glands (Abate-Shen and Shen, 2000; Agoulnik and Weigel, 2006; Feldman and Feldman, 2001). PSA is secreted into the lumen to degrade proteins that are produced in the seminal vesicles to attenuate clotting of the semen (Lilja, 1985). AR is expressed only in the differentiated secretory cells and not in the basal cells; however, AR is also expressed in the stroma (Abate-Shen and Shen, 2000; Cunha et al., 1987). Tissue recombination studies over the years have demonstrated that AR is required in the prostate stroma, but not in the prostate epithelium, for prostate duct development (Cunha, 1996; Cunha et al., 1987; Hayward et al., 1997). However, androgen signaling in the epithelium is required for prostate secretory function and the ablation of androgen leads to apoptosis of the secretory layer (Arnold and Isaacs, 2002). Androgen signaling in the luminal cells also drives AR-mediated transcription of proteins, such as prostate specific antigen (PSA/KLK3), which are secreted into the lumen of the prostate. Within the normal prostate gland, adhesion to matrix and expression of AR are mutually exclusive (Lamb et al., 2010).

## Prostate Cancer and Prostate Cancer Progression

Prostate cancer typically arises from the epithelial cellular compartment and appears to be associated with an initial dysplastic lesion referred to as prostatic intra-

epithelial neoplasia (PIN). PIN has three stages: low-grade PIN, PIN, and high grade PIN (Abate-Shen and Shen, 2000). At the low grade PIN stage, abnormal AR positive cells are observed in the lumen (Bostwick et al., 2004). This occurrence is hypothesized to be due to a loss in cellular polarity (Webber et al., 1997; Webber et al., 2001). However, as PIN progresses to high grade PIN (HGPIN), there is a loss of basal cells allowing the AR positive carcinoma cells to adhere to matrix (Bostwick et al., 2004). The loss of the basal cells is hypothesized to be due to invasion or an over-proliferation of the carcinoma cells into the basal cell layer, or due to death of the basal cells themselves (Bonkhoff, 1996; Yu et al., 2004). High grade PIN (HGPIN) precedes the appearance of prostate cancer usually by five to ten years (Bostwick et al., 2004). These lesions are heterogenic and multifocal; PIN can be immediately adjacent to and within the same acini structure as normal epithelium and PIN fails to permeate into the stroma (Bostwick et al., 2004).

Although the cell of origin for prostate cancer remains a controversial topic in the field of prostate cancer biology (Craft et al., 1999a; Garber, 2010; Goldstein et al., 2010; Isaacs, 1999; Wang et al., 2009), several lines of evidence strongly support the idea that cancer arises from a luminal or luminal precursor cell type. Firstly, all primary prostate cancer is positive for AR, suggesting it must arise from AR positive cells. Also, AR expression is observed in a significant amount of metastatic tissues obtained at autopsy from patients who underwent ADT (Shah et al., 2004). Furthermore, secretory cells first appear during male sexual maturity when there is a dramatic and permanent increase in circulating testosterone levels within the blood. Lastly, no diagnosis of

prostate cancer has been made in pre-pubescent males or before the appearance of prostate secretory cells (Bostwick et al., 2004; Sakr et al., 1994).

While the cell of origin is still debated, the location of where prostate cancer begins appears to be more lucid. The prostate gland is divided into five lobes: anterior, posterior, median and two lateral lobes. The gland is divided up into distinct morphological zones based on histological three dimensional studies (McNeal, 1968, 1969; McNeal and Bostwick, 1984). These zones are central, anterior fibromuscular stroma, peripheral, and transitional zones (Cunha et al., 1987; McNeal, 1968, 1969). These histological studies have found that almost all prostate cancers originate from the peripheral zone of the prostate. Another prostate disorder called benign prostate hyperplasia (BPH), originates almost exclusively from the transition zone and the periurethral glands (McNeal, 1984).

Unlike other glandular solid tumors, the vast majority of primary prostate cancer grow slowly, is asymptomatic, and may take years to develop. This is evidenced by elder patients choosing the "watchful waiting" option rather than prostatectomy or radiation. A vast majority of prostate cancer tumors metastasize to the bone (usually the spine and ribs) and this is often the only clinically detectable site of metastasis (Logothetis and Lin, 2005). Unlike most other cancers such as breast cancers which metastasize to bone, prostate cancer is osteoblastic (bone-forming) rather than osteolytic (bone lysing). This is often very painful to the patient and the pain has been reported to be a side-effect of the osteolytic process which occurs in the background of osteoblast differentiation and activation (Logothetis and Lin, 2005; Mantyh et al., 2002; Vessella and Corey, 2006). These metastatic lesions of prostate cancer to the bone

often lead to leukoerythroblastic anemia, nerve-compression syndromes, hypercalcemia, and pathological fractures which all reduce the overall quality of life (Coleman, 1997). Prostate cancer can also metastasize to the lymph nodes, brain, and lungs, and to other secondary sites such as the bone marrow in which they can become dormant and resistant to therapeutic agents (Morgan et al., 2009; Townson and Chambers, 2006).

Androgen Receptor (AR) in the Prostate Gland and in Prostate Cancer

For the growth of the prostate in both normal and disease states, it has been well established that sex steroids, and especially androgens, with the help of their receptor, androgen receptor (AR) are the driving forces of the gland during the life span of men. Although other steroids are secreted in the male, the main androgen involved in driving this process is testosterone which is produced in the leydig cells of the testes. Once delivered, testosterone is modified intracellularly by 5-α reductase into dihydro-testosterone (DHT), which is ten times more potent ligand for AR than testosterone (Dehm and Tindall, 2006; Deslypere et al., 1992; Titus et al., 2005a; Titus et al., 2005b).

The androgen receptor (AR) is a nuclear steroid receptor which belongs to the family of steroid receptors (Lamb et al., 2001). The AR gene is located on the X-chromosome, specifically Xq11.2. Like other members of the nuclear receptor family, AR is characterized by four functional domains. From N to C terminus AR is composed of an N-terminal trans-activating domain, a DNA binding domain (DBD) that has 2 zinc fingers motifs, a hinge region, and a ligand binding domain (LBD) (Agoulnik and Weigel, 2006; Dehm and Tindall, 2007; Litvinov et al., 2003). AR has two activation domains. They are called activation domain 1 (AF-1) and activation domain 2 (AF-2). AF-1 is

located in the N-terminus and is involved in transcriptional activation and AF-2 which is located in the LBD, is involved in protein-protein interactions that aid in recruitment of co-activators that contain LXXLL motifs (Heery et al., 1997). AR also has an N-terminal poly-glutamine tract at residues 448-472 that varies in length from individual to individual (Heemers and Tindall, 2007). This poly-glutamine stretch normally ranges from 8-31 repeats in normal individuals. However, expansion of this poly-glutamine stretch has been reported to cause spinal and bulbar muscular atrophy (also called Kennedy's Disease) (La Spada et al., 1991). Conversely, if the poly-glutamine stretch is shortened, AR has been reported to be more transcriptionally active (Giovannucci et al., 1997). The DBD has two zinc finger motifs that allow for DNA recognition and allow AR homo-dimerization respectively. The hinge region of AR was once thought to only link the DBD and LBD together. However, studies have shown that the hinge region possesses a putative nuclear localization sequence (NLS) (Jenster et al., 1993; Zhou et al., 1994). This suggests that the hinge region is vital in AR translocation. Finally, the LBD is composed of twelve alpha-helices and upon androgen binding, helix 12 becomes stabilized. This leads to the formation of a hydrophobic binding pocket for ligands such as androgen or other proteins that have the preferred FXXLF motif (Heemers and Tindall, 2007). The AR-LBD also aides in keeping AR localized within the cytosol.

Within the prostate epithelial cell, AR is kept in a deactivated conformation by heat shock proteins (Hsp) such as Hsp 90, Hsp 70, Hsp 56, and p23. Circulating testosterone is delivered to AR positive cells and reduced intracellularly to DHT. When DHT binds to the LBD of the AR, it causes displacement of its Hsp chaperone. AR then

homodimerizes, is phosphorylated, undergoes a conformational change which exposes the nuclear localization sequence (NLS), and translocates to the nucleus. AR can be phosphorylated at serine residues 16, 81, 94, 256, 308, 424, and 650 and the action of phosphorylation appears to be a late event (Gioeli et al., 2002). AR translocation can occur within 15-60 minutes after androgen stimulation.

Once in the nucleus, activated and dimeric AR binds to DNA sequences called androgen response elements (AREs) that are in the promoter or enhancer region of target genes. These ARE sequences are usually 6 base pair long “half site” direct or inverted repeats and usually separated by 3 base pairs (Claessens et al., 2001; Dehm and Tindall, 2007; Reid et al., 2001). There appear to be two classes of AREs in which class I possess typical guanine residues and class II has atypical sequences and features that allow AR to have synergistic transcriptional activity (Reid et al., 2001). Interestingly, AR has also been reported to have the ability to bind upstream of promoters and enhancer regions of genes that do not possess a putative ARE (Massie et al., 2007; Waghray et al., 2001). Once AR is bound to AREs, AR then recruits co-activators, co-repressors, and other regulatory components of the pre-initiation complex. This complex is assembled first by TFIID, within the vicinity of the transcription start site. TFIIB is then recruited to TFIID at the Tata-binding protein motif and recruits TFIIF and RNA Polymerase II. TFIIE and TFIIH are then recruited to aide in DNA melting to allow transcription initiation (Heemers and Tindall, 2007; Roeder, 1996). This can either activate or repress transcription of target genes. With the help of co-activators such as p300 and members of the p160 family, these events lead to nuclear transcription of the AR gene as well as other androgen-regulated genes. Widely known AR transcriptional

targets include NKX3.1, PSA/KLK3, and transmembrane protease, serine 2 (TMPRSS2) (Feldman and Feldman, 2001; Heemers and Tindall, 2007; Murtha et al., 1993; Young et al., 1992). NKX3.1 is found to be reduced in focal prostate atrophy and PIN tissues from humans (Bethel et al., 2006). However, both PSA and TMPRSS2 are often increased during prostate cancer progression (Nam et al., 2007). Currently, PSA detection is frequently used as a diagnostic bio-marker for not only prostate abnormalities (prostatitis and BPH), but also for prostate cancer screening, and for monitoring the recurrence after ADT. However, the PSA test, which measures PSA within the blood, has its limitations. These include its lack of specificity for predicting prostate cancer, its elevation in benign tissue, its elevation after a recent ejaculation, its reduction in obese males (Banez et al., 2007), its inability to detect PSA-negative tumors and importantly, the inability to distinguish indolent tumors from the ones that will become aggressive. A study assessing cancer cell stem-ness demonstrated that PSA null or low (PSA-/lo) cells had higher regenerative capacity, were castration resistant, and displayed tumor-propagating capacity in castrated mice whereas PSA positive cells had a limited propagating capacity in castrated hosts and were sensitive to castration (Qin et al., 2012). Also, these PSA (-/lo) cells underwent asymmetric cell division which gave rise to PSA positive cells (Qin et al., 2012). This limited predictive value has caused the United States Preventative Task Force in 2012 to recommend against using the PSA test stating that PSA testing may lead to over-diagnosis and over-treatment of the malady (Moyer, 2012). Altogether, this makes the understanding and discovery of improved biomarkers critical in the field of prostate cancer research.

AR can also be post-translationally modified causing AR to possess non-genomic signaling activity that is independent of its transcriptional activity, which may potentiate hormone-refractory disease (Arnold and Isaacs; Craft et al.; Li et al., 2007; Linja et al.; Migliaccio et al., 2007). Additionally, post-translational modification of AR can affect AR stability, transcriptional activity, protein-protein interactions, or ligand binding affinity. Many of these modifications have been observed using *in vitro* cell line models following stimulation of AR. Although the latency period of transcription initiated by sex steroids is as fast as 7.5 minutes, it takes several hours for transcriptional out-put to reach its peak (Groner et al., 1983). After that, it takes additional time for the mRNA to be processed and to be translated. While AR is still in the cytosol, it can undergo several protein-protein interactions within seconds to minutes after androgen stimulation. One of these post-translational pathways involves poly-ubiquitylation by E3 ligase. Once AR is bound to androgen, a portion of AR is sent to the 26S proteasome while the remaining ligand bound AR translocates to the nucleus. Inhibition of the 26S proteasome using the proteasome inhibitor, MG132, in LNCaP cells show that AR is not only unable to translocate to the nucleus but dramatically attenuates PSA levels (Lin et al., 2002). This data suggests that the proteasome plays an important role in AR regulation. Additionally, there have been numerous reports of E3 ligases that act as either co-activators or co-repressors of AR. These include E3 ligases such as Mdm2 (Gaughan et al., 2005), E6-AP (Khan et al., 2006), Chip (He et al., 2004), SNURF/RNF4 (Moilanen et al., 1998; Poukka et al., 2000), and RNF6 (Xu et al., 2009).

Another well studied mechanism of AR non-nuclear signaling is evidenced in several studies that analyze androgen stimulation and its effects on second messenger

pathways. These studies have been carried out in several cell types such as murine macrophages, rat sertoli cells, cardiac myocytes, and LNCaP cells. This pathway appears to have a general signaling mechanism in increasing intracellular calcium concentrations. First androgen binds to AR, which then leads to activation of L-type calcium channels. This then leads to an increased activation of both protein kinase C and protein kinase A to have a positive transcriptional effect on downstream gene targets (Foradori et al., 2008). In LNCaP cells, stimulation of AR using non-aromatizable DHT resulted in an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). This was blocked by using flutamide suggestive that this pathway occurs via androgen binding to AR (Foradori et al., 2007; Steinsapir et al., 1991). Interestingly, $[Ca^{2+}]_i$ can also regulate the binding affinity of androgen for AR in platelets (Cabeza et al., 2004). Additionally, inhibitors of $Ca^{2+}$-ATPase have been shown to decrease AR expression levels in LNCaP cells (Gong et al., 1995).

In addition to calcium, circulating androgens have also been reported to rapidly activate second messenger pathways. In the presence of low levels of circulating androgens, receptor tyrosine kinases (RTKs) are able to activate AR (Craft et al.). Also, non-receptor tyrosine kinase Src can phosphorylate AR at Ser-650 which is within the hinge region of AR and this phosphorylation increases AR translocation to the nucleus (Gioeli et al., 2002; Zhu et al., 2001). AR has also been reported to form a tertiary complex with modulator of nongenotropic activity of estrogen receptor (MNAR) and Src. Initially, Src is inactive within this complex. However, when AR binds Src, this leads to the activation of Src in this complex (AR/MNAR/Src) and the subsequent activation of a downstream effector, MEK. This complex is androgen dependent in LNCaP cells, but is

constitutively active in a castration-resistant LNCaP derivative cell line, C42. The activation of this pathway stimulates prostate tumor cell proliferation and survival (Unni et al., 2004). Additionally, a report from Migliaccio et al. has shown that Src can form a complex with AR upon androgen stimulation via the SH3 domain of Src (Kousteni et al., 2001; Migliaccio et al., 2000). These studies show that AR can form a complex with Src in a non-nuclear fashion as well as lead to the activation of Src. However, the role that AR non-nuclear signaling may have on other tumor cell behavior such as migration and invasion is unresolved and has not been thoroughly explored. Thus, multiple mechanisms for AR activation in hormone-refractory tumors are possible in the presence or absence of androgen.

Other known mechanisms to positively regulate AR include genetic over-expression of AR, intragenic deletions within AR, and gain-of-function mutations in AR and in other genes that regulate androgen synthesis. Patients that have undergone surgical removal of the prostate and/or ADT (i.e. luteinizing-hormone releasing hormones (LHRH)) still have very low levels of androgen within the circulation and within the tissue perhaps due to production of androgen and androgen analogs by the adrenal glands (Titus et al., 2005a; Titus et al., 2005b). In any case, AR is still able to capitalize on these low levels of circulating androgen and become activated due to elevated expression of both type 1 and 2 5α reductase (Titus et al., 2005a). Under normal conditions, type 1 5α reductase is expressed in various cell types such as fibroblasts and skin cells while type 2 5α reductase is expressed in prostate (Titus et al., 2005a). However, both isozymes of 5α reductase have been found to be expressed in prostate cancer (Xu et al., 2006). Furthermore, AR is found to be amplified and over-

expressed in 20-30% of castration-resistant prostate cancer (CRPC) cases (Chen et al., 2004; Gelmann, 2002; Visakorpi et al., 1995). Recent studies have also shown that over expression of AR sensitizes prostate cancer cells to low levels of androgen (Waltering et al., 2009). Altogether, the low levels of androgens present in tissues may be sufficient for AR function and is a phenomenon that is clinically relevant.

Intragenic deletions that produce AR splice variants have also been hypothesized to give rise to the de novo resistance to ADT. The intragenic deletion mechanism that leads to this is caused by deletions of exons 5 to 7 (Li et al., 2011). The translation of the remaining exons produces an AR-variant (AR-V), which lacks a LBD but is capable of nuclear translocation, ARE binding, and is able to activate AR target genes in the absence of androgen. Ectopic expression of AR-V has been shown to be dependent on wild-type full-length AR activity (Watson et al., 2010). It has also been recently demonstrated *in vitro* that 22Rv1 and CWR-R1 cell lines co-express both the full length AR and AR-V; however, recent studies suggest that full-length AR function may not be a requirement for AR-V function in CRPC (Li et al., 2011; Li et al., 2012). Proof of principle studies have seen this in the LuCaP 86.2 line, which was derived from a CRPC bladder metastatic tumor, favorably expresses the AR-V (Li et al., 2011). Further proof-of-principle studies have also demonstrated that the AR-V is expressed in metastatic CRPC, is resistant to enzalutamide, and expression of this splice variant is associated with shorter patient survival rates (Guo et al., 2009; Hornberg et al., 2011; Li et al., 2013; Zhang et al., 2011). Altogether, there are no current antagonists that target the AR-V and this area of study demands attention given that it is one avenue that promotes resistance to current therapeutics.

The other well investigated mechanism is gain-of-function mutations in AR. This yields the ability of sex steroids (i.e. corticosteroids, estrogen) and even anti-androgens (i.e. hydroxyflutamide and cyproterone acetate) to bind to the LBD of AR and activate it. This mutation found in the LBD of AR replaces threonine 877 with alanine (T877A) (Taplin et al., 1995; Veldscholte et al., 1990). These mutations in AR are generally not observed in prostate cancer until after ADT and relapse of the disease. AR mutations are observed in approximately 10-25% of CRPC (Gaddipati et al., 1994; Taplin et al., 2003). A recent study has identified another mutation that is localized to the LBD of AR which is homozygous to the T877A. This mutation replaces phenylalanine with leucine (F876L). The mutation allows the novel AR antagonist enzalutamide (discussed later) to act as an agonist for AR in tumor cell lines and for tumor cells to be dependent on enzalutamide for in vivo growth under androgen deprived conditions (Korpal et al., 2013). A long-standing hypothesis that CRPC tumor cells may be able to make their own androgen (Abate-Shen and Shen, 2000) has also been recently demonstrated. This was recently found to be caused by a mutation in the androgen-synthesizing enzyme, 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1) at residue N367T, which confers resistance to poly-ubiquitylation which leads to accumulation of DHT (Chang et al., 2013). These mutations allow CRPC tumors to elude therapeutic insults, and to synthesize DHT intratumorally thus, promoting CRPC growth and proliferation.

Several AR co-activators such as p300-CBP and p160 have been demonstrated to enhance AR activity (Culig et al., 2004; Heemers et al., 2007). They are also seen to be over-expressed in prostate cancer clinically. These co-activators can increase the likelihood of AR being in its active conformation in the absence of androgen and thus

increase its transcriptional activity. The p160 steroid receptor co-activators (SRC-1/NCOA1, SRC-2/NCOA2, and SRC-3/NCOA3) have recently become of therapeutic interest. SRC-3 specifically is degraded by the E3 ubiquitin ligase adaptor speckle-type poxvirus and zinc finger (POZ) domain protein (SPOP) (Geng et al., 2013). However, SPOP has missense mutations found within its substrate binding domain causing it not bind to SRC-3 (Barbieri et al., 2012; Lindberg et al., 2013). These SPOP mutations dramatically decrease its tumor suppressor activity which leads to increased AR signaling making SRC-3 a potential therapeutic target in prostate cancer. Genetic alterations in AR co-regulators such as insulin-like growth factor-1 (IGF-1) can also contribute to CRPC by de-phosphorylating AR at S650 and thus allowing AR translocation and increasing PSA levels (Wu et al., 2006). AR interaction with scaffold proteins can lead to crosstalk with other pathways and can also lead to activation of AR in the absence of ligand (Scher and Sawyers, 2005). One scaffold that has been demonstrated to do so is receptor for activated C kinase 1 (RACK1), a known protein kinase C (PKC) anchoring protein. It was also discovered to bind to Src and regulate cell protrusion and chemotactic cell migration (Cox et al., 2003). However, it has also been demonstrated, by using a yeast two hybrid screen, to be an AR interacting protein (Rigas et al., 2003). The authors also demonstrated *in vitro* that RACK1 promotes AR nuclear translocation upon activation of PKC in the absence of androgen (Rigas et al., 2003).

While AR is important, so is its ligand, androgen. ADT depletes nearly all circulating androgens; however, the adrenal glands remain unaffected. The adrenal glands are still able to produce androgens and androgen precursors within their inner

cortex and these can be delivered to CRPC cells (Yamaoka et al., 2010). Another mechanism in which androgens can also be synthesized is by *de novo* synthesis from cholesterol via enzymatic steps that are catalyzed by cytochrome P450 (CYP) members (Rainey and Nakamura, 2008; Yamaoka et al., 2010). Cholesterol undergoes a cleavage reaction by the enzyme desmolase (CYP11A1) to convert it into pregnenolone which can be further converted into progesterone by 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1) (Yamaoka et al., 2010). After these steps, pregnenolone or progesterone can be further converted into 17-OH pregnenolone or 17-OH progesterone by CYP17A1 (Rainey and Nakamura, 2008). These are then further converted into the metabolic intermediates dehydroepiandrosterone (DHEA) or androstenedione respectively, which are converted to testosterone and then reduced to DHT by 5α reductase (Rainey and Nakamura, 2008; Yamaoka et al., 2010).

In summation, in normal prostate luminal cells, AR function is dependent on androgen and this interaction drives differentiation of these cells. AR signaling under normal conditions also suppresses growth of these cells creating balance for the gland (Sensibar, 1995). However, in prostate cancer, this balance goes awry, and drives proliferation, survival, and promotes cancer growth. In cancer, AR signaling is able to occur despite low to no physiological levels of circulating androgens. While ADT is usually successful after initial diagnosis, the cancer typically will recur shortly which leads to metastasis, typically to the bone, which has no proven cure. Recurrence may be due to several factors: hypersensitivity to low amounts of androgen due to 5α reductase isozymes or other sex steroids, AR non-nuclear signaling, AR amplification or amplification of its co-regulators, AR gain-in-function mutations, mutations in the

androgen synthesis pathway, and crosstalk with other pathways that are also mis-regulated in prostate cancer. Many of these pathways demand attention of prostate cancer researchers and clinicians.

Prostate Cancer Treatments and Novel AR antagonists

Current ADTs are designed to prevent AR signaling by blocking androgen binding to AR. This is done primarily through two mechanisms. The first is to reduce circulating androgen levels in the body using chemical castration agents such as LHRH analogs (i.e. leuprolide), by abiraterone acetate and TAK-700 which all inhibit 17 α-hydroxylase/C17,20 lyase (CYP17A), an enzyme which is expressed in testicular, adrenal, and prostatic tumor tissue and is responsible for converting pregnenolone into androgen (Attard et al., 2005; Hara et al., 2013; Soifer et al., 2012; Yamaoka et al., 2012). TOK-001 is a dual AR antagonist and CYP17A1 inhibitor (Brawer, 2008). The mechanism in which TOK-001 and abiraterone acetate both work is by first binding to the active site of the CYP17A1 enzyme and then coordinating the heme iron by its pyrimidine nitrogen. This allows these pharmacological inhibitors to mimic its substrate and reduce the levels of circulating androgens (DeVore and Scott, 2012). Given its novelty as a dual inhibitor of both AR and CYP17A1, TOK-001 is currently in phase II clinical trials and highlights the need of targeting more than one pathway in CRPC. Another part of the androgen synthesis pathway is the conversion of androstenedione, a relatively weak androgen, into testosterone (Yamaoka et al., 2010). This reaction is catalyzed by a reductase aldo-keto reductase family 1 member 3 (AKR1C3) (also known as HSD17β) (Yamaoka et al., 2010). One group using high throughput screening has identified a compound called compound 17 (chemical name 2-methyl-1-

{1-[(5-methyl-1H-indol-2-yl)carbonyl]piperidin-4-yl}propan-2-ol) that has specificity for AKR1C3 (Watanabe et al., 2013). The authors demonstrated its inhibitory capabilities by using androgen insensitive prostate cancer cells CWR22R cells, which have high levels of AKR1C3 as well as orchidectomized nude mice that were implanted with these cells. In cell culture the $IC_{50}$ was 1.9 nM for compound 17 and in the xenograph studies, 10mg/kg completely ablated the production of testosterone (Watanabe et al., 2013). These studies highlight the importance of not only targeting AR but also of the importance of inhibiting intratumoral production of androgen through the *de novo* androgen synthesis pathway during CRPC.

Another mechanism is to target 5α reductase by using pharmacological inhibitors finasteride and dutasteride. Each of these agents has been effective in rodent and human tissues in preventing the intracellular conversion of testosterone to DHT, which is a more potent ligand for AR (Deslypere et al., 1992; Xu et al., 2006). Other strategies that have been proposed and tested *in vitro* include targeting Hsp90 which leads to the degradation of AR (Solit et al., 2002) and by microinjection of an AR mRNA hammerhead ribozyme (Zegarra-Moro et al., 2002). Altogether, these findings demonstrate that after ADT, residual levels of circulating androgen still need pharmacological inhibition.

The third mechanism is by direct competition using competitive antagonists for AR binding (i.e. bicalutamide, flutamide). It has been reported consistently that these inhibitors have limitations. These include a 30-fold weaker binding affinity for AR LBD than DHT (Kolvenbag et al., 1998). Also, the T887A mutation allows these antagonists to act as agonist, thus they do not prevent AR nuclear translocation (Mohler et al., 2004;

Schuurmans et al., 1991; Veldscholte et al., 1992; Veldscholte et al., 1990). Both of these strategies, targeting circulating androgens and use of competitive antagonists, are prescribed in combination during ADT as adjuvant therapy. ADT generally causes prostate cancer remission in 80-90% of patients, resulting in a median progression-free survival of 2.5-3 years (Bracarda et al., 2005; Hellerstedt and Pienta, 2002; Pienta and Smith, 2005; Shah et al., 2004). However, after this remission period, the cancer becomes castration resistant (Pienta and Smith, 2005). The median survival is 23–37 months from the time of initiation of ADT (Hellerstedt and Pienta, 2002; Shah et al., 2004). However, AR is still found to be persistently active during this phase of the disease. Given this notion of AR still being active and their ability to elude current competitive antagonists, next-generation AR antagonists that are non-competitive or have a greater AR affinity, have been developed (Jones and Diamond, 2008; Tran et al., 2009). One of these next-generation antagonists is enzalutamide (also known as MDV3100) which, unlike bicalutamide, prevents AR from translocation and decreases PSA and TMPRSS2 levels (Tran et al., 2009). This compound displayed effectiveness initially; however, many initial responders later developed resistance after 47 weeks (Scher et al., 2010) and one mechanism of MDV3100 resistance is due to a mutation in the LBD of AR which replaces phenylalanine 876 to leucine (F876L) (Korpal et al., 2013). Other novel AR antagonists that have been recently reported on include ARN-509 (Clegg et al., 2012), MEL-3 (Helsen et al., 2012), Sintokamide A and EPI-001 both of which target the AF-1 domain of AR in the N-terminus and prevents AR from binding to AREs (Andersen et al., 2010; Sadar et al., 2008). Each of these pharmacological

agents show promising results *in vitro* and/or *in vivo* by repressing androgen/AR induced transcription. Currently, ARN-509 is in phase-III clinical trials.

Sipuleucel-T (APC8015) is an novel immunostimulant manufactured by Dendreon Corporation to treat CRPC (Plosker, 2011). This drug is administered to the patient in three steps. First, the patients' own leukocytes are extracted and then the blood is incubated with recombinant fusion protein PA2024 (which is prostatic acid phosphatase (PAP) and an immune signaling factor (granulocyte-macrophage colony stimulating factor) (Kantoff et al., 2010). The blood is then re-infused into the patient with the activated product (APC8015) and causes an immune response against tumor cells that express the PAP antigen, which is found to be elevated in metastatic prostate cancer cells (Kantoff et al., 2010; Small et al., 2006). Treatments are given three times a month to patients (2006). This method has been reported to be effective and prolongs the life of the patient for just over four months (Kantoff et al., 2010). Due to the clinically effectiveness of APC8015, this drug was FDA approved in 2010. Perhaps usage of this immunotherapy could be combined with AR antagonism or with antagonism of the androgen synthesis pathway.

Molecular and Genetic Changes Associated with Prostate Cancer Progression

Prostate cancer is largely a heterogeneous malady (Abate-Shen and Shen, 2000; Tomlins et al., 2006). Given the diversity in foci within the prostatic tissues, there has not been a single causative mutation, genetic inactivation, or common molecular change to which prostate cancer can be attributed (Tomlins et al., 2006). This even includes different foci within the same patient often whom may have different genetic alterations. Altogether, this suggests that prostate cancer can develop through a slew

of mechanisms (Abate-Shen and Shen, 2000; Bostwick et al., 1998). This also highlights the molecular heterogeneity between tumors and may be one explanation as to why the majority of prostate cancers remain indolent for a period of years while a subset of prostate cancer may progress to an aggressive disease resulting in patient demise. This makes prostate cancer difficult to manage and exposes the need for tumor molecular classification based on the distinct genetic abnormalities. Recently, a group performed RNA-seq and Sanger sequencing on 300 primary and metastatic prostate cancer tumors. The authors discovered that the most commonly mutated gene is a substrate-binding subunit of the Cullin-based E3 ubiquitin ligases, *SPOP*. *SPOP* was found mutated in 6-13% of primary tumors and was also found mutated in 14.5% of metastatic tumors (Barbieri et al., 2012). Tumors with *SPOP* mutations lacked the TMPRSS2/Erg fusion and other ETS rearrangements commonly seen in prostate cancer (Barbieri et al., 2012). This finding may aide in sub-type classification of the disease $Ets^+$ and $Ets^-$ prostate tumors (Barbieri and Tomlins, 2014).

Prostate cancer is also largely an age-associated disease. Other risk factors include obesity, family history of the disease, genetics and ethnic make-up. Here in the United States, prostate cancer more frequently affects African-American men than Caucasian or Hispanic men, and is also more deadly among African-Americans (Hoffman et al., 2001). Several single nucleotide poly morphisms (SNPs) have been identified in African-American men making them at least 1.6 times more likely to develop prostate cancer than their European counterparts (Eeles et al., 2008; Thomas et al., 2008). Several genetic loci have been identified as risk alleles. These include 17q21-22 and 8q24 which raises the question of whether or not the ancestry of men is a

better predictor of disease (Chen et al., 2010; Lange et al., 2003; Robbins et al., 2007). Although there is no one single genetic insult that leads to prostate cancer, there have been several studies that have identified some candidate genes that may be involved in disease development and subsequent metastasis. Clinically, prostate cancers display several of the molecular changes that are discussed below while others that are discussed are still not lucid, are under investigation and will be discussed in the chapters to follow.

TMPRSS2-ERG signaling

Gene fusions have been observed (*BCR-ABL*) in chronic myelogenous leukemia whose function has been demonstrated to be vital for proliferation (Szczylik et al., 1991). The first known fusion identified in prostate cancer was discovered in 2005 which involves the fusion of the 5' untranslated region of transmembrane protease, serine 2 (*TMPRSS2*) fused to the coding sequence of erythroblastosis virus E26 (*Ets*) family members such as Erg, *ETV1*, *ETV4*, *ETV5* and *FLI1* (Barbieri and Tomlins, 2014; Tomlins et al., 2005). The ETS domain serves a DNA binding recognition site function and commonly interacts with other transcription factors (Carrere et al., 1998; Verger and Duterque-Coquillaud, 2002). Therefore, the fusion of these two genes can lead to the production of Ets factors under the control of the *TMPRSS2* promoter, which is androgen sensitive, and thus result in the over-expression of *ETS* family transcription factor members.

ETS family member rearrangements have been detected using modern techniques such as fluorescent in situ hybridization (FISH) as well as immunohistochemistry (IHC) (Barbieri and Tomlins, 2014; Rubin et al., 2011; Tomlins et

al., 2009). In HGPIN, the ERG oncoprotein can be readily detected using IHC and ERG may correlate with disease progression (Furusato et al., 2010; Tomlins et al., 2012; Young et al., 2012). Recent studies have shown that ERG staining in atypical glands that were suspected of cancer helped to correctly diagnose prostate cancer in 28% of cases that would have not been diagnosed based on other cell markers (Shah et al., 2013).

The fusion is also associated with metastasis and death in population based studies (Attard et al., 2010; Demichelis et al., 2007). While several groups have reported that fusion is associated with aggressive disease, Gleason grade, and poor prognosis (Darnel et al., 2009; FitzGerald et al., 2008), other groups have shown that is associated with more indolent diseases and favorable outcomes (Saramaki et al., 2008; Winnes et al., 2007). Thus, there are a number of conflicting reports over the years on whether or not *TMPRSS2-ERG* is a predictor of clinical outcomes (St John et al., 2012). These conflicting reports may reflect the multi-focality and the molecular heterogeneity of the disease or simply reflect the sampling techniques of the tissues used. Furthermore, the lack of *TMPRSS2-ERG* fusions found in specific tumors may reflect the data that shows that ETS-negative tumors possess *SPOP* mutations, also have deletions in both chromodomain helicase *CHD1* and *PTEN* but have an over-expression in secreted protease SPINK1, thus there may be two sub-classes of prostate cancers: $ETS^+$ and $ETS^-$ which have two different sets of driver mutations (Barbieri et al., 2012; Grasso et al., 2012; Lindberg et al., 2013; Tomlins et al., 2008).

Phosphatase and tensin homolog (PTEN) and Phosphatidylinositol 3-kinase (PI3-K)

Phosphatase and tensin homolog (PTEN) is a lipid phosphatase located on chromosome 10 that was discovered (then called MMAC1) in 1997 as a tumor suppressor gene (Steck et al., 1997). PTEN functions to dephosphorylate phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$) which converts it back to phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) (Cully et al., 2006). With this simple signaling mechanism, PTEN negatively regulates the lipid kinase phosphatidylinositol 3-kinase (PI3-K) signaling by converting $PIP_3$ back to $PIP_2$ (Cully et al., 2006; Maehama and Dixon, 1999). Class IA PI3-K is activated by RTKs upstream and in its active form PI3-K is composed of a regulatory subunit p85 and a catalytic subunit p110 (Cully et al., 2006). There are five variants of p85 (p85α, p55α, p50α, p85β, and p85γ) with p85α being the most highly expressed regulatory subunit (Cully et al., 2006). There are four variants in the p110 catalytic subunits (p110α, p110β, p110γ, and p110δ) with both p110 α and β subunits being most commonly found in cells. PI3-K signaling acts through the downstream $PIP_3$ target which recruits phosphatidylinositol-dependent kinase 1 (PDK1) and Akt (also known as protein kinase B (PKB)) to the membrane. PDK1 then phosphorylates and activates Akt (Cully et al., 2006). This activation of Akt by PI3-K has been shown consistently to promote tumor cell survival, proliferation, adhesion, and cell spreading (Cully et al., 2006). Thus, the up-regulation and alterations in PI3-K/Akt signaling can provide numerous growth and proliferative advantages for tumor cells. Several of these mechanisms include the phosphorylation and inhibition of pro-apoptotic proteins Bax, Bad, Forkhead box-O transcription factors (FOXO) 1, 4, 3A, death-associated protein 3 (DAP3), and pro-caspase 9, through

increased expression of the anti-apoptotic protein survivin, and by regulating mTOR signaling (Cully et al., 2006). PTEN can act as a tumor suppressor by not only regulating the PI3-K/Akt pathway but by also regulating tumor glutamine and glucose uptake, thus reversing tumor metabolism to an anti-Warburg state (Cantley and Neel, 1999; Garcia-Cao et al., 2012; Maehama and Dixon, 1999). However, the mis-regulation of PTEN (via inactivating mutations or loss of heterozygosity) is common in many cancers including glioblastoma, breast, ovarian, colon, and endometrial cancer (Cantley and Neel, 1999; Cully et al., 2006). Several functional studies validate how crucial the *PTEN* tumor suppressive activity is in prostate cancer (Carver et al., 2009; Chen et al., 2011; Trotman et al., 2003).

*PTEN* locus deletions are seen in nearly 40% of primary prostate cancers and in ~60% of metastatic cancers, resulting in constitutive activation of PI3-K/Akt signaling (Barbieri and Tomlins, 2014; Beltran et al., 2013; Dong et al., 2001). The deletion of *PTEN* deletion has also been reported to be common in advanced disease and has been demonstrated to increase likelihood of disease relapse after ADT, higher Gleason grade, and patient demise (Attard et al., 2009; Barbieri and Tomlins, 2014; Cairns et al., 1998; Choucair et al., 2012; Krohn et al., 2012; McMenamin et al., 1999; Reid et al., 2010). While Akt itself is typically not amplified or overexpressed, point mutations and amplifications in *PIK3CA*, which encodes the catalytic subunit of PI3-K (p110α), have been reported to occur in nearly 25% prostate cancers with activating point mutations making up 5% (Barbieri et al., 2012; Barbieri and Tomlins, 2014; Sun et al., 2009). This mutation can lead to the hyper-activation of the PI3-K/Akt pathway. While these deletions of *PTEN* and point mutations in *PIK3CA* have been reported in prostate

cancer, they appear to be mutually exclusive of each other with the former being a more common mechanism of action (Barbieri and Tomlins, 2014). In *in vivo* studies, *PTEN* null mice develop HGPIN but do not progress to aggressive and invasive prostate cancer suggestive that PTEN loss alone is not sufficient for development of prostate cancer (Kwabi-Addo et al., 2001). The findings from the Kwabi-Addo *et al*. study highlights that prostate cancer development may be dependent on several genetic insults. Such insults are the over-expression of Akt concomitant with AR over-expression, which has been demonstrated to be sufficient to initiate and drive castration-resistant prostate cancer progression using a mice model (Xin et al., 2006). Another genetic insult involves the AR regulated target, NKX3.1. Loss of NKX3.1 has been demonstrated to inhibit Akt activation and blocks prostate cancer that is caused by PTEN loss (Lei et al., 2006). However, NKX3.1 is lost during PIN as stated earlier which further highlights the many genetic mis-regulations that can lead to cancer development (Bethel et al., 2006). Thus, these observations suggest a strong connection between AR, PTEN, and the PI3-K/Akt pathway in prostate cancer.

The PI3-K/Akt pathway has been reported to interact with AR signaling by both direct and indirect mechanisms (Wang et al., 2008). This relationship has been also been demonstrated outside of the prostate in human sperm cells where AR is also important (Aquila et al., 2007). It has been demonstrated that AR can activate the PI3-K/Akt pathway (Cinar et al., 2007; Wang et al., 2007). Also, Akt can regulate AR in several ways. One mechanism includes Akt regulation of AR transcriptional output and expression (Manin et al., 2002; Sharma et al., 2002). Other mechanisms include Akt regulation of post-transcriptional modifications (Reddy et al., 2006). However, contrary

to many reports, Akt does not appear to directly phosphorylate AR *in vivo* (Gioeli et al., 2002).

Many prostate tumor cells and cell lines rely heavily on PI3-K/Akt signaling for survival (Edick et al., 2007). This has also been demonstrated in the androgen sensitive cell line LNCaP (Carson et al., 1999). These cells experience cell death within 24 hours of inhibiting PI3-K/Akt using pharmacological inhibitors; however, the addition of androgen can rescue cell survival (Carson et al., 1999; Li et al., 2001). Moreover, long term androgen ablation in LNCaP cells causes resistance to pharmacological inhibition of the PI3-K/Akt pathway (Carson et al., 1999). This may partly explain the findings that Akt activity is significantly increased in C4-2 which are androgen-independent derivative of LNCaP (Murillo et al., 2001). Using *in vivo* prostate regeneration models, Xin *et al.* nicely demonstrated that AR and Akt signaling can promote tumor formation in the absence of androgen (Xin et al., 2006). This suggests that AR signaling may promote tumor survival independent of the PI3-K pathway (discussed in chapter 2), or that AR along with PI3-K/Akt signaling may cooperate during the progression of prostate cancer.

p53

p53 (*TP53*) located on the short arm of chromosome 17, is a widely known tumor suppressor gene and is the most commonly mutated gene in human cancers (Hollstein et al., 1991; Levine and Oren, 2009; Matlashewski et al., 1984; McBride et al., 1986; Olivier et al., 2002). TP53 can become activated due a myriad of stressors such as DNA damage, oxidative stress, and mis-regulation of oncogenes. It has been demonstrated that 25-30% of localized prostate tumors harbor TP53 mutations (Barbieri

et al., 2012). Also, deletions within the TP53 locus have also been recently observed in prostate cancer samples (Barbieri and Tomlins, 2014). Within the prostate cancer field since 1993, it has been reported that mutation rate of p53 is relatively low in primary prostate cancer, that p53 mutation is a late event in the progression of prostate cancer, is associated with advanced (metastatic) stage, loss of differentiation, the transition from androgen-dependent to androgen-independent growth, and its over-expression is associated with poor disease outcome (Aprikian et al., 1994; Bauer et al., 1995; Bookstein et al., 1993; Brewster et al., 1999; Eastham et al., 1995; Effert et al., 1993; Heidenberg et al., 1995; Henke et al., 1994; Matsushima et al., 1997; Moul et al., 1996; Navone et al., 1993; Prendergast et al., 1996; Shurbaji et al., 1995; Stackhouse et al., 1999; Theodorescu et al., 1997; Thomas et al., 1993; Voeller et al., 1994). However, recent whole genome sequencing has found that p53 mutations may occur earlier on in the disease, and not later on in the path of the disease (i.e. after ADT) or recurrence of the disease as has been previously reported by several independent groups (Baca et al., 2013).

Integrins

Adhesion proteins such as integrins have also been shown to be crucial in both normal and malignant states of the gland. Integrins are heterodimeric transmembrane proteins that attach to molecules in the extracellular matrix (ECM) (Hynes, 2002; Miranti and Brugge, 2002). There have been 18 alpha and 8 beta subunits identified, which when dimerized yield over 20 combinations of integrins, each dependent on specific extracellular matrix ligands (Hynes, 2002). Once bound to their respective ECMs, integrins can regulate a slew of cellular functions such as migration, proliferation and

cell survival (Knudsen and Miranti, 2006). Integrins can receive signals from outside the cell to the inside of the cell (termed outside in signaling) or, signals can arise intracellularly and get transmitted to the integrin to effect its function outside of the cell (inside out signaling) (Miranti and Brugge, 2002).

In the normal prostate cells, integrins α6β4, α2β1, and α3β1 are expressed on the basal cells and no integrins are expressed on the secretory cells. During prostate cancer initiation and PIN, cells begin to crowd the lumen due to loss of cell polarity (Webber et al., 1997; Webber et al., 2001). In the latter stages of PIN termed high grade PIN (HGPIN), there is a loss of cell-cell adhesion and cell-membrane adhesion (Litvinov et al., 2003). There are also alterations in ECM ligands during disease progression. The changes in ECM environment, particularly a loss of both laminin V and collagen VII, loss of integrins β4 and α3 causing retention of integrin α6β1, loss of basal cells, expression of integrins on the secretory cells and thus attachment of these secretory cells to the basement membrane. All of these changes can markedly increase in human prostate tumors, promoting tumor survival and proliferation (Koivisto et al., 1998).

The impact that AR may have on cellular adhesion, particularly on integrins, has been studied. PC-3 cells are derived from a bone metastasis of grade IV from a Caucasian male (Kaighn et al., 1979). However, these cells do not express detectable levels of AR nor PSA. Since AR is expressed in nearly every step of the disease, several groups have stably re-introduced human full length AR into these cells. In one *in vitro* study, stable expression of wild type human AR into PC3 cells and stimulation with DHT led to cellular detachment and programmed cell death (Heisler et al., 1997).

This cellular detachment led to the hypothesis that AR could perhaps regulate de-adhesion, which is seen during the early stages of metastasis. Further studies analyzing the high metastatic potential and integrin expression of these cells have been carried out. *In vitro* experiments using PC3 cells and osteoblast-like cells, U2OS show that PC3 cells are able to adhere to these bone cells which is observed clinically in metastatic disease (Kostenuik et al., 1996). The mechanism of this adhesion was inhibited by using these two approaches: (1) antibodies against integrin α2 or β1 collagen receptor subunits and (2) usage of Arg-Gly-Asp (RGD) peptides. This group also used transforming growth factor-β (TGF-β), which is up-regulated in many human cancerous cells. Adhesion of PC3 cells to osteoblasts appeared to be mediated by integrin α2β1. Treatment with TGF-β increased this adhesion 2-fold which was suggestive that this may act along with adhesion of PC3 cells to increase skeletal localization (Kostenuik et al., 1996). Conversely, stable expression of AR in PC3 cells decreased integrin α2β1 but increased MUC-1, a glycoprotein that is over-expressed in several human cancers (Evangelou et al., 2002). In 2000, Bonaccorsi *et al*. demonstrated that when human AR cDNA was re-expressed in PC3 cells it decreased α6β4 integrin, a known hemidesmosome protein, thus further suggesting that AR and integrins have a regulatory relationship (Bonaccorsi et al., 2000). With these changes in the ECM microenvironment, the increase in the expression of integrin α6β1, enhanced proliferation and survival, along with over expression of AR led us to hypothesize that AR and integrins interact with each other to promote prostate cancer progression. We tested this hypothesis by assessing the role of AR signaling in integrin-mediated

survival *in vitro*. This hypothesis was tested empirically and will be further discussed in chapter two.

Src and Src Family Kinases (SFKs)

Src is the oldest known human oncogene discovered by pathologist and Nobel Laureate, Dr. Peyton Rous in the early 1900s (Rous, 1910, 1911; Rous and Murphy, 1914). Its gene encodes the first reported tyrosine kinase, termed non-receptor tyrosine kinase (Courtneidge, 2002). The over-expression and hyper-activation of Src and members of its family, SFKs which consists of 8 members, play a crucial role in a diverse number of human cancers particularly colorectal, breast, and prostate cancer. Among the SFKs, the activation of Src, its prototypical member has been frequently implicated in cancer progression and metastasis. Src is a 60,000 Dalton protein composed of seven functional domains: (1) an amino-terminal region that contains a myristic acid moiety, (2) an unique domain specific to each SFK member, (3) a Src homology domain 3 (SH3), (4) Src homology domain 2 (SH2), (5) a linker region, (6) a catalytic domain (SH1), and (7) a COOH-terminal tail (Guarino, 2010; Yeatman, 2004). The myristic acid moiety located within the N-terminus allows Src to localize to the inner membrane. The SH2 domain binds to phosphorylated tyrosines on itself or other proteins and the SH3 domain binds to PXXP sequences to mediate inter- or intra-molecular interactions (Guarino, 2010). The linker region contains PXXP sequences and allows Src to maintain its “folded” conformation so intermolecular SH3 interactions can occur. The catalytic domain contains the “activation loop” which is the location of the auto-phosphorylation site Y419 (in humans). When this site is phosphorylated, Src undergoes a conformation change, and possesses its maximal kinase activity. The c-

terminal tail contains Y530 (in humans) that when phosphorylated, allows Src to fold and interact with its SH2 domain. This “folded” or “closed” conformation masks the activation loop and renders Src inactive. However, when Y530 is dephosphorylated by protein tyrosine phosphatases, Src forms an “unfolded” conformation which un-masks the activation loop allowing it to become phosphorylated and activated. *In vitro* studies in colon cancer have demonstrated the phosphatase responsible for dephophosphorylating the Y530 site is PTP1B and it is also found to be over-expressed, thus allowing Src to be hyperactive to promote tumorigenic properties (Zhu et al., 2007). Once Src is active, it can phosphorylate its many substrates such as STAT3, Shc, FAK, Ras, JNK, p130Cas, paxillin, p190 RhoGAP, AR, and CDCP1 (discussed later) (Spassov et al., 2011b; Summy and Gallick, 2003). Tyrosine Y419 in the activation loop can be dephosphorylated by phosphatase, PEP (Ingley, 2008). Conversely, C-terminal Src Kinase (Csk) can phosphorylate the C-terminus of Src and attenuate the Src activity as well as the activity of other SFKs (Chong et al., 2006; Chong et al., 2005a; Chong et al., 2005b; Nada et al., 1991; Nada et al., 1993). Cells that lack Csk display constitutively active Src (Nada et al., 1993). Src can exert its effects on cell motility by promoting the ubiquitinylation and endocytosis of E-cadherin and also by its phosphorylation of FAK, RRAS, p120ctn, STAT3, and cortactin (Yeatman, 2004). The viral oncogenic version of this protein, termed v-Src, contains several mutations throughout the protein and importantly, it does not contain a negative regulatory c-terminus allowing it to remain constitutively active (Yeatman, 2004).

Although studies on Src began over a century ago, we still do not fully understand much about how it becomes mis-regulated in cancer. Src has been shown

to be endogenously over-expressed in prostate cancer cell lines such as the LNCaP derivative, C4-2 and enhances AR function in the absence of androgen (Asim et al., 2008; Castoria et al., 2003). *In vitro* experiments have shown that Src can form a complex with AR as well as the estrogen receptor to impact prostate tumor cell proliferation (Migliaccio et al., 2005). AR can also form a complex with Src/MNAR and can further activate Src while in this complex (discussed in chapter 3). Inhibition of Src activity *in vitro* decreases prostate cancer cell adhesion, migration, and invasion (Nam et al., 2005; Recchia et al., 2003; Slack et al., 2001). Inhibition of Src activity also decreases androgen independent cell proliferation *in vitro* (Lee et al., 2004). Additionally in orthotopic athymic nude mice with PC3MM2GL cells, dasatinib (a potent inhibitor of Src/Abl activity) treatment prevented lymph node metastasis compared to the vehicle control (Park et al., 2008). Lastly, in tissue obtained from patients with castration resistant disease, the SFK member FGR was found to be significantly up-regulated (Edwards et al., 2003). This is suggestive that Src and SFKs have an important role in a number of prostate cancer signaling mechanisms and in several stages of the disease.

Clinically, Src has been implicated in driving tumor metastasis to the bone. *In vivo* studies using murine models of breast cancer, inhibition of Src activation decreased the size of metastatic tumors and also delayed the appearance of bone metastasis (Myoui et al., 2003; Rucci et al., 2006). Another group demonstrated that a majority of breast cancer that metastasized to the bone possessed active Src (Planas-Silva et al., 2006). Given these previous studies, Zhang et al. used gene expression signatures to denote activation of Src in $ER^-$ and $ER^+$ breast cancer tumors called Src

response signatures (SRS). They demonstrated that tumors that were positive for SRS were associated with ER+ tumor relapse, bone metastatic tumors were Src-dependent, Src was required for CXCL12 activation of Akt, and conferred resistance to pro-apoptotic ligand TRAIL (Zhang et al., 2009). In CRPC, one group analyzed the kinase:substrate relationship and observed that the tyrosine kinases Src and Abl have elevated tyrosine phosphorylation (Drake et al., 2012). This study highlights the effects of an increase in global tyrosine without any ectopic expression or gain of function mutations in Src and that there may be alternative pathways in which Src and other tyrosines become activated. One mechanism in which Src may become activated independent of gain of function mutations will be discussed in chapter 3.

Matriptase

Serine proteases are enzymes that can hydrolytically cleave peptide bonds within proteins in which the amino acid serine serves as the enzymes' active site (Hedstrom, 2002). Matriptase is a type II serine protease that was isolated in 1993 from the medium of breast cancer cells (Shi et al., 1993). Since its discovery, it has been found to be expressed in a variety of epithelial cells and epithelial-derived cancer cell lines. Matriptase has also been reported to be expressed in neutrophils, mast cells and neural progenitor cells (Cheng et al., 2007; Fang et al., 2011; Oberst et al., 2003). Its importance in epithelial development and integrity has been demonstrated in mice. Mice with conditional depletion of Matriptase developed severe organ dysfunction, loss of tight junction, and epithelial demise (List et al., 2009). Another study demonstrated that Matriptase is required for hair follicle development as well as epidermal barrier

function (List et al., 2002). These findings are suggestive that Matriptase has vital function in specialized tissues.

Matriptase is encoded by the *ST14* gene which is located on chromosome 11q24-25, and once transcribed and translated it is made up of 855 amino acid residues (Zhang et al., 1998). It is composed of an extracellular domain within the amino-terminus, an intracellular domain, and a COOH-domain. The extracellular region is composed of a sperm protein, enteropeptidase and adrin (SEA) domain, two complement protein subcomponents C1rs./C1s, urchin embryonic growth factor (Uegfr), bone morphogenetic protein 1 (CUB) domains, and four low density lipoprotein receptor class A (LDLRA) domains. The SEA allows the protease to undergo posttranslational self-cleavage and the two CUB domains and the LDLRA domains play important roles in cell localization, protein-protein interactions, and zymogen activation (Cho et al., 2001; Levitin et al., 2005). The intracellular domain of the protease has a consensus sequence of 54 amino acids that can be phosphorylated by PKC. The COOH-terminal end has high homology to other type II serine protease members. It has a conserved H/D/S (HDS) triad that is crucial for its proteolytic activity (Chen, Y.-W., 2012).

Matriptase is initially synthesized as a pro-enzyme serine protease with a molecular mass of around 95-kD; however, this protease is cleaved into its shorter active form via N-terminal processing (Uhland, 2006). This cleavage converts the single chain zymogen into a disulfide-linked two chain active enzyme (Uhland, 2006). This two chain active Matriptase has a 45-kD non-catalytic region and a 25-kD serine protease region (Benaud et al., 2001). The active Matriptase, which is now 70-kD, is then shed

into the extracellular milieu of cells. The shedding of active Matriptase requires another cleavage occurring at either K204-T205 or K189-S190 sites (Benaud et al., 2001).

Recent studies show that Matriptase has been linked to initiation and promotion of epidermal carcinogenesis in mouse models, suggesting that its mis-regulation in epithelia can contribute to transformation. In the human prostate cancer cell line LNCaP, Matriptase activation was demonstrated to be up-regulated after 6 hours of androgen stimulation (Kiyomiya et al., 2006). This active form of Matriptase has been reported to correlate with the cleavage of laminin 5 matrix, which is lost during the progression of normal prostatic tissue to prostate cancer. Matriptase expression was reported to increase with a decrease hepatocyte growth factor activator type 1 with increasing prostate cancer tumor grades (Saleem et al., 2006; Tripathi et al., 2011). Thus, high Matriptase expression may correlate with prostate cancer progression. The regulation of Matriptase by AR in a non-nuclear fashion is further investigated and discussed in chapter 3.

Cub Domain Containing Protein 1 (CDCP1)

Cub Domain Containing Protein 1 (CDCP1) is a transmembrane glycoprotein that has been previously studied in a number of tissues by several groups under the name of gp140, SIMA35, and Trask for its role in cell adhesion (Brown et al., 2004; Carter, 1984; Hooper et al., 2003; Scherl-Mostageer et al., 2001). CDCP1 is a protein that is comprised of approximately 836 amino acid residues (Kollmorgen et al., 2012). These residues make up the following domains: a 29 amino acid signal peptide, an extracellular domain (ECD), a transmembrane domain, and an intracellular domain. The ECD of CDCP1 consists of three CUB (complement protein subcomponents

C1rs./C1s, urchin embryonic growth factor (Uegfr) bone morphogenetic protein 1) domains. These CUB domains are largely responsible for protein-protein interactions and play critical roles in developmental processes such as organogenesis and embryogenesis. Its cytoplasmic domain has five intracellular tyrosine residues, PXXP stretches, and 14 *N*-glycosylation sites.

*In vitro* studies have demonstrated that the CUB1 domain of CDCP1 is cleaved extracellularly in a number of cancer cell lines. CDCP1 can be cleaved by trypsin, plasmin, or serine proteases such as Matriptase at R368 and K369 sites which generatesthe active 70-kD fragment of CDCP1. After this cleavage, CDCP1 can be phosphorylated at the intracellular tyrosines by Src (at Y734) and PKCδ (Alvares et al., 2008; He et al., 2010). However, a fairly recent study has demonstrated that neither cleavage nor the ECD of CDCP1 is required for its ability to be tyrosine phosphorylated by Src or to suppress adhesion (Spassov et al., 2011a); but phosphorylation is necessary for it to be functionally active. Under normal conditions, CDCP1 has been demonstrated *in vitro* to be expressed in many normal human tissues and is a marker for cell types such as hematopoietic stem cells and neuronal progenitor cells (Buhring et al., 2004; Conze et al., 2003; Kimura et al., 2006). However, there is mounting evidence that functional CDCP1 is highly expressed in many human cancers, is a HIF-2α target that aides in promoting tumor escape, stromal invasion, and trans-endothelial migration (Dong et al., 2012; Emerling et al., 2013; Hooper et al., 2003; Ikeda et al., 2009; Liu et al., 2011; McGovern et al., 2013; Miyazawa et al., 2013; Perry et al., 2007; Scherl-Mostageer et al., 2001; Seidel et al., 2011). Known metastasis suppressor, *KAI1/CD82* was demonstrated to down-regulate CDCP1-enhanced Src activation which

may be one mechanism by which CD82 prevents metastasis in prostate cancer (Park et al., 2012). Increased levels of CDCP1 correlate with poor prognosis and relapse of disease making it a potential therapeutic target (Kollmorgen et al., 2013; Siva et al., 2008). Our studies to further understand the role of CDCP1 in prostate cancer tumor cell invasion will be explored in chapter 3.

Framework of Dissertation

Given the items discussed above, we set out to develop clinically relevant prostate cancer models. Through these models, our hope was then to be able to generate insightful and potential strategies that would be effective in therapeutic targeting of CRPC. To execute these tasks, it is first imperative to understand some of the molecular mechanisms that control tumor cell survival in CRPC cells. These mechanisms have remained poorly understood, in part due to the limitations in current cell lines. The overall objective of this thesis was to 1) generate better models to study survival of CRPC, and 2) to determine how survival prostate cancer cells was being mediated in the context of AR-integrin signaling and AR non-nuclear signaling.

In interrogating these pathways, we first sought to investigate the integrin-regulated survival pathways in prostate tumor cells PC3, which do not express detectable levels of AR, are of human origin, and are derived from a prostate brain metastasis (Kaighn et al., 1979). Although these cells do not express AR, in murine models these cells are highly metastatic to the lung and lymph nodes. Since AR is important in every step of the disease and because we wanted to generate relevant models of CRPC, wild type AR or AR mutants were stably re-expressed into PC3 cells. In addition, we further validated our findings in the LNCaP and C4-2 cell CRPC

progression model, which expresses endogenous AR. Using these models, we first investigated the downstream transcriptional targets of AR that regulate tumor cell survival when seeded on laminin matrix independent of PI3-K (see Chapter 2). Lastly, we investigated the role of AR non-nuclear signaling in our models and in other cell lines to understand what role(s) AR non-nuclear signaling had on cell movement, invasion, Src activation, protease activation, and pathways that led to tumor escape (see Chapter 3).

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# CHAPTER TWO:
# SUPPRESSION OF AR DECREASES α6β1 INTEGRIN, BCL-XL EXPRESSION AND PROSTATE TUMOR SURIVIVAL ON LAMININ MATRIX

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## Introduction

Androgen, acting through the androgen receptor (AR), is required for prostate cancer growth and survival. Therefore, chemical castration is initially an effective treatment option for advanced prostate cancer. However, patients ultimately relapse with castration-resistant tumors for which there are no effective curative treatments. Nonetheless, castration-resistant tumor cells are still dependent on AR, as inhibition of AR expression leads to cell death (Cohen and Rokhlin, 2009; Liao et al., 2005a; Yang et al., 2005). Exactly how AR regulates survival of castration-resistant tumor cells is also poorly understood.

Several mechanisms have been proposed for how AR, independent of androgen, can become active to regulate tumor growth and survival in hormone-refractory disease (Arnold and Isaacs; Craft et al.; Feldman and Feldman; Li et al., 2007; Murillo et al.; Tindall). Once prostate cancer begins to metastasize and becomes hormone-refractory, there are obvious changes in AR expression. For instance, in hormone-refractory prostate cancer, AR is often either over-expressed and/or mutated. Mutations, such as the one seen in the metastatic lymph node cell line LNCaP in which a point mutation of threonine to alanine occurs at amino acid position 877 (T877A), allow AR to become responsive to other steroids (Feldman and Feldman; Koivisto et al.; Linja et al.). Alternatively, amplification of the AR gene, seen in 20-30% of patients (Agoulnik and Weigel; Koivisto et al.; Linja et al.) may allow AR to be responsive to very low levels of circulating androgen. AR has also been reported to have non-genomic effects independent of its transcriptional activity, which may potentiate hormone-refractory disease (Arnold and Isaacs; Craft et al.; Li et al., 2007; Linja et al.; Migliaccio et al.,

2007). In the presence of low levels of circulating androgens, receptor tyrosine kinases (RTKs) are able to activate AR (Craft et al.). Thus multiple mechanisms for AR activation in hormone-refractory tumors are possible. Elucidation of the currently accepted mechanisms has relied primarily on LNCaP cells as a model. The reliance on one cell type can be misleading, thus other models are needed to fully understand the mechanisms of hormone-refractory disease.

Integrins are heterodimeric transmembrane cell surface receptors that mediate cell survival through adhesion to extracellular matrix (Hynes, 2002; Miranti and Brugge, 2002). Integrin signaling through various pathways regulates pro-survival and pro-death molecules and matrix detachment induces cell death (Reddig and Juliano, 2005). Integrin expression and signaling is aberrant in many cancers, including prostate cancer. In the normal human prostate, basal epithelial cells express two integrins, α6β4 and α3β1, which promote basal cell survival through adhesion to laminin 5 in the basement membrane (Edick et al., 2007; Knudsen and Miranti, 2006). Basal epithelial cells do not express AR but differentiate into AR-expressing secretory cells which down-regulate integrins and no longer adhere to the basement membrane (Lamb et al., 2010). Thus, integrin and AR expression are mutually exclusive in normal prostate epithelium. However, in prostate cancer the AR-expressing tumor cells exclusively express integrin α6β1 and adhere to a remodeled matrix containing the α6β1-specific substrate laminin 10 (Bonkhoff et al., 1993; Cress et al., 1995). The predilection for α6β1 expression is preserved in lymph node metastases (Pontes-Junior et al., 2009). Constitutive AR expression in immortalized prostate epithelial cells increases integrin α6 (Whitacre et al., 2002), suggesting that AR could be responsible for maintaining α6 expression in the

cancer cells. In addition, the α6 promoter contains a steroid response element capable of stimulating α6 expression in response to progesterone (Nishida et al., 1997). Thus, AR-mediated control of integrin α6 and the engagement of α6β1 in AR-expressing cells could provide a novel mechanism for prostate cancer cell survival.

Phosphoinositide 3-kinase (PI3-K) signaling is required for survival of most prostate cancers. PTEN, a phosphatidylinositol (3,4,5)-triphosphate phosphatase and negative regulator of PI3-K signaling, is lost in approximately 30% of clinical prostate cancers and in approximately 60% of metastatic cancers, resulting in constitutive activation of PI3-K (Dong, 2001; Schmitz et al., 2007). Akt is a major downstream effector of Class IA PI3-K signaling and regulates survival through inhibition of pro-death proteins, such as Bad, Bax, FOXO, DAP3, and caspase 9, and increased expression of the pro-survival protein survivin and stimulation of NF-κB and mTOR signaling (Duronio, 2008; Reddig and Juliano, 2005).

Nonetheless, PI3-K signaling is not the only survival pathway. The androgen-sensitive prostate cancer cell line LNCaP dies upon PI3-K/Akt inhibition; however, the addition of androgen can rescue this death (Carson et al., 1999; Li et al., 2001). In addition, long-term androgen ablation results in resistance to PI3-K/Akt inhibition (Pfeil et al., 2004) and prostate regeneration studies show that AR and Akt can synergize to promote tumor formation even after androgen ablation (Xin et al., 2006a). This suggests that AR, and in some contexts independent of exogenous androgen, promotes survival independent of PI3-K. In this study, we hypothesized that AR-dependent regulation of integrin α6β1 expression in prostate cancer cells promotes survival independent of PI3-K. We tested the hypothesis by: 1) assessing whether AR-

dependent regulation of α6β1 expression in prostate cancer cells promoted cell survival and if so, we asked whether the suppression of AR within the proper ECM context led to a decrease in cell survival independent of the PI3-K signaling pathway.

## Results

### *AR promotes tumor cell survival through up-regulation of laminin integrin α6β1*

In collaboration with Laura E. Lamb, fluorescence-activated cell sorting (FACS) a specialized method of flow cytometry, was used to detect and compare integrin expression on the cell surface between PC3 cells that were generated by infecting cells with pBabe-puro-hAR (PC3-AR clones) cells and cells that were infected with pBabe-puro vector retroviruses (PC3-Puro clones) as described (Lamb et al., 2011). We discovered that stable AR expression in PC3 cells caused 2-, 3-, and 6-fold reduction in integrin α2, α5, and α3, respectively, but increased integrin α6 levels 6-fold (Fig. 1A). There was a slight 1.5-fold decrease in integrin β1 (Fig. 1A) and a 4-fold decrease in integrin β4. Integrins are expressed as heterodimeric pairs on the cell surface (Miranti and Brugge, 2002), and integrin α6 forms a heterodimer with either β1 or β4. The corresponding decrease in the integrin β1–specific α-subunits, that is, α2, α3, and α5, would generate free β1 integrin, making it available to dimerize with integrin α6. The large decrease in β4 further indicates that α6 is paired with the β1. This predilection for integrin α6β1 expression was striking because during prostate cancer development there is a loss of most integrins and an increase in expression of integrin α6β1 (Bonkhoff et al., 1993; Cress et al., 1995; Davis et al., 2001; Goel et al., 2009; Nagle et al., 1995; Nagle et al., 1994; Pontes-Junior et al., 2009), suggesting that our AR-

expressing PC3 cells recaptures some of what is seen *in vivo*. To verify that AR can regulate integrin α6 expression in other cell lines, AR was knocked-down in LNCaP cells by transfecting AR siRNA. After 48 hours, AR levels were greatly diminished as observed by immunoblotting (Figure 1B). This was accompanied by a ~12% decrease in integrin α6 expression as measured by FACS (Figure 1C). These studies were done in collaboration with Laura E. Lamb as noted in figure 1.

Since integrin-mediated adhesion can promote cell survival and there was an increase in integrin α6 with AR expression, we then sought to test the hypothesis that AR was promoting survival through up-regulation of integrin α6. To test this, integrin α6 expression in the AR expressing clones (PC3-AR clones) was decreased as close as possible to empty vector levels by careful titration of integrin α6 siRNA (Figure 1D). A non-specific siRNA (scram) was used as a control in these and all subsequent experiments. Loss of integrin α6 did not have a significant or consistent effect on AR levels of expression, indicating that integrin α6 is downstream of AR (Figure 1D). In collaboration with Laura E. Lamb, AR expressing clones were treated with integrin α6 or scram siRNA for 72 hours to induce knock-down of integrin α6, and then cells were seeded on LM1 and treated with the PI3-K inhibitor LY294002. After 72 hours, cell viability was assessed by trypan blue staining. A 63-73% reduction in α6 integrin expression in the presence of LY294002 is required and sufficient to induce cell death in AR expressing cells (Figure 1E). These results indicate that AR is promoting survival through integrin α6. To verify that these effects were due to AR expression and not clonal selection, AR expression was knocked in the AR expressing cells using siRNA prior to treatment with LY294002 (Figure 1F, G). Loss of AR in AR expressing clones

leads to decreased integrin α6 levels as measured by immunoblotting (Figure 1F) and increased cell death when the cells were seeded on LM1 and then treated with LY294002 (Figure 1G, H). Overall, these data indicate that AR is a pro-survival factor in PC3 cells that acts independently of PI3-K signaling through increased integrin α6 expression.

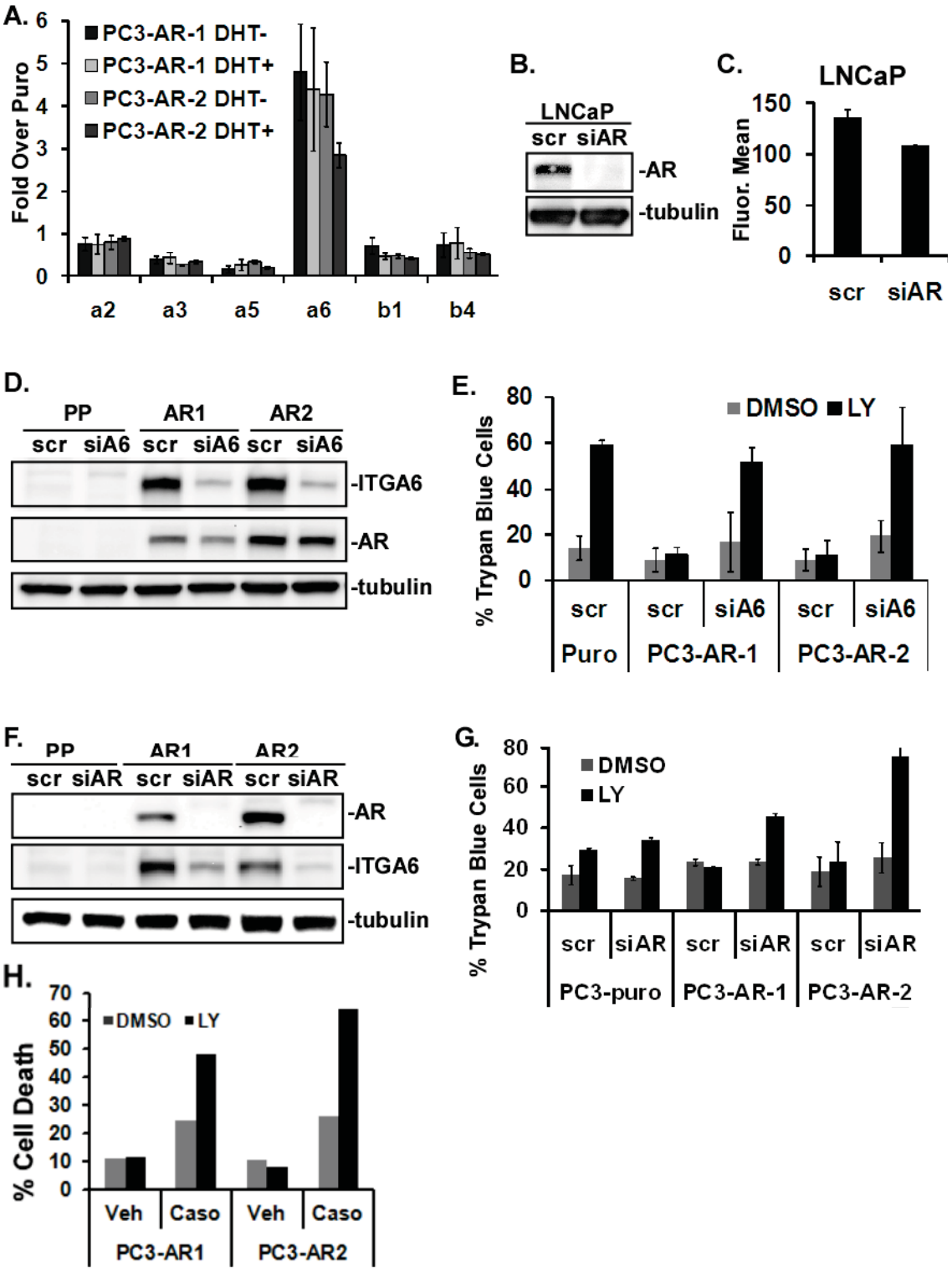


**FIGURE 1. AR promotes survival through up-regulation of integrin α6.**

**FIGURE 1. (CONT'D) A)** PC3-puro, PC3-AR-1, PC3-AR-2 cells were growth factor-starved in charcoal-stripped media and plated on LM1 in the presence of vehicle or 10nM DHT. After 72 hours, cells were treated with fluorescent-conjugated antibodies against the indicated integrins and analyzed by FACS. Mouse and Rat IgG were negative controls. IgG controls were subtracted from mean fluorescent values then values for AR expressing cells were normalized to those of PC3-puro cells. Error bars represent standard error; *n* = 5-8. **B-C)** LNCaP cells were treated with siRNA against AR (siAR) or non-specific sequence (scr) for 72 hours. **B)** LNCaP lysates were immunoblotted to monitor AR expression. Tubulin was used as a loading control. **C)** LNCaP cells were treated with fluorescent-conjugated antibody against integrin α6 and analyzed by FACS. Rat IgG was the negative control. Values given are for mean fluorescent values minus IgG control. Error bars represent standard error; *n* = 2. **D-G)** PC3-puro (PP, Puro), PC3-AR-1 (AR1), and PC3-AR-2 (AR2) cells were treated with siRNA against integrin α6 (siA6), AR (siAR), or non-specific sequence (scr) for 72 hours and plated on LM1. **D, F)** Integrin α6 (ITGA6) and AR expression were monitored by immunoblotting. Tubulin was used as a loading control. **E, G)** Cells were treated with DMSO or 20μM LY294002 (LY) for 72 hours. **H)** Serum starved sub-confluent cells were treated with 10nM Casodex (Caso) and spiked throughout the duration of the experiment. Cells were then seeded on LM1 for 72 hours. Cell viability was determined using trypan blue staining. Error bars represent standard deviation; *n* = 3. *(A, D-F) Laura E. Lamb, (B, G, H) Jelani C. Zarif, (C) Laura E. Lamb and Jelani C. Zarif.*

*AR and integrin α6 regulate Bcl-xL expression*

The pro-survival protein Bcl-xL, which prevents mitochondrial outer membrane permeabilization, has been reported to promote survival independent of PI3-K signaling in prostate cancer cells (Yang et al., 2003). Increased Bcl-xL expression is also associated with prostate cancer progression clinically (Castilla et al., 2006; Krajewska et al., 1996; Sun et al., 2008). Immunoblotting of total cell lysates from AR expressing PC3-AR cells demonstrated that Bcl-xL is up-regulated when compared to the empty vector controls (Figure 2A); suggesting Bcl-xL could be the mechanism by which AR promotes survival independent of PI3-K signaling. To demonstrate that up-regulation of Bcl-xL is due to AR expression, AR-expressing cells were treated with AR siRNA and expression of Bcl-xL was monitored. In collaboration with Laura E. Lamb, we demonstrated that loss of AR in AR expressing clones resulted in down-regulation of both integrin α6 and Bcl-xL (Figure 2B). To determine if Bcl-xL expression is dependent on integrin α6, integrin α6 expression was decreased using integrin α6 siRNA. Decreased integrin α6 resulted in decreased Bcl-xL (Figure 2C). Lastly, we demonstrated that loss of AR in LNCaP cells also resulted in a modest decrease in both integrin α6 and Bcl-xL levels (Figure 2D). Together, these data indicate that AR, acting via integrin α6, drives increased expression of the pro-survival protein Bcl-xL.

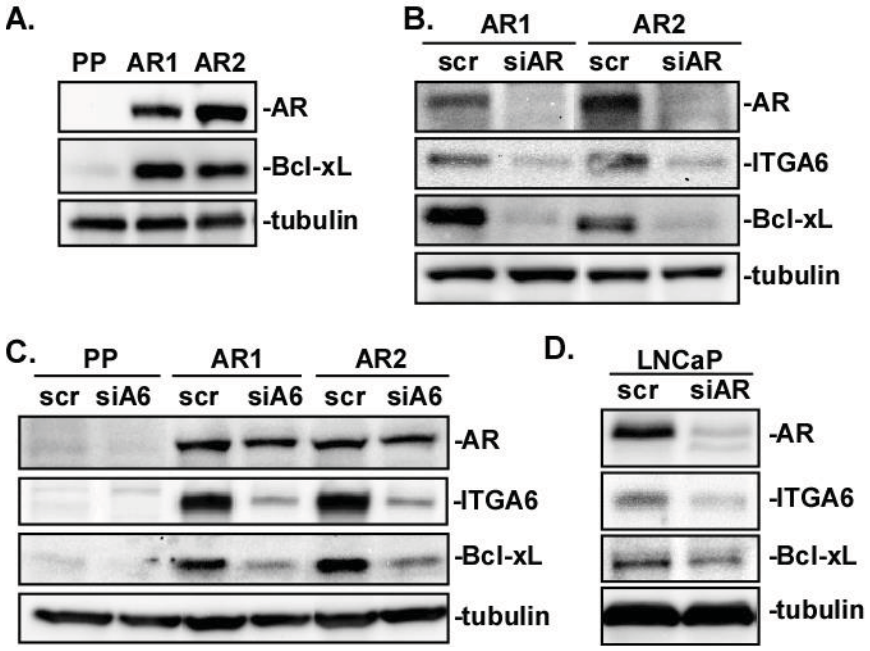


**FIGURE 2. AR and integrin α6 regulate Bcl-xL expression.** PC3-puro (PP), PC3-AR-1 (AR1), PC3-AR-2 (AR2) and LNCaP cells were treated with siRNA against AR (siAR), integrin α6 (siA6), or non-specific sequence (scr) for 72 hours. AR, integrin α6 (ITGA6), and Bcl-xL levels were monitored by immunoblotting of whole cell extracts using AR, integrin α6 (ITGA6), and Bcl-xL specific antibodies. Total levels of protein were monitored by immunoblotting with anti-tubulin. *(A, C) Laura E. Lamb, (B, D) Jelani C. Zarif.*

To further demonstrate that Bcl-xL over-expression is sufficient to promote survival independent of PI3-K signaling, retroviruses were used to infect cells with an empty vector or a vector expressing Bcl-xL and stable clonal cell lines were selected. Bcl-xL over-expression to the levels found in AR expressing clones was confirmed by immunoblotting. As expected, Bcl-xL over expression did not result in any changes of AR expression or changes in integrin expression (data not shown). Moreover, Bcl-xL over-expressing cells did not die when treated with LY294002. These findings further validate that Bcl-xL is regulated by AR and that is a potent pro-survival factor and can promote survival independent of PI3-K signaling (Lamb et al., 2011).

*AR transcriptionally regulates integrin α6 and Bcl-xL mRNA expression*

AR is a steroid receptor and transcription factor whose activity depends on nuclear localization. Work done by Laura E. Lamb demonstrated the importance of AR translocation. Expression of the AR ΔNLS mutant, in which AR is unable to translocate into the nucleus and bind to DNA, was also unable to protect cells from LY294002-induced death (data not shown) (Lamb et al., 2011). To determine whether AR expression was regulating integrin α6 and Bcl-xL transcription, RNA was isolated from PC3-puro and AR expressing clones, reverse transcribed and quantitative RT-PCR (qRT-PCR) was performed. In collaboration with Laura E. Lamb, we observed that there was over a 10-fold increase in integrin α6 and Bcl-xL mRNA levels compared to PC3-puro cells, independent of DHT addition (Figure 3A, B). The AR-dependent increase in Bcl-xL mRNA is in accord with previous studies in LNCaP cells where treatment with androgen or AR-specific siRNA leads to a respective increase or

decrease in Bcl-xL mRNA (Liao et al., 2005b; Sun et al., 2008). To further verify that this was an AR-dependent effect, PC3-AR-1 cells were treated with the partial AR antagonist Mifepristone (RU486) or AR-specific siRNA. RU486 has been reported to recruit co-repressors to AR transcriptional complexes thereby inhibiting AR-mediated transcription (Hodgson et al., 2005). RU486 decreased integrin α6 mRNA expression as measured by RT-PCR, (Figure 3C). RU486 treatment also resulted in a decrease in the protein levels of integrin α6 expression with approximately the same severity as AR-specific siRNA treatment (Figure 3D). There was also a decrease in integrin β1 (Figure 3D). Since integrins must be expressed as heterodimers in order to be stably expressed, loss of the integrin α6 binding partner of integrin β1 may be leading to its degradation.

Since AR must be in the nucleus to act as a transcription factor, we tested the effect of expressing the AR ΔNLS mutant on integrin α6 and Bcl-xL expression. Expression of the ΔNLS AR mutant in PC3 cells did not result in increased integrin α6 expression compared to empty vector PC3-pLKO cells (Figure 3E-F). Lastly, stimulation of LNCaP cells for as little as 24 hours with DHT or the more potent synthetic androgen metribolone (R1881) results in increased integrin α6 and Bcl-xL mRNA expression as determined by qRT-PCR. PSA was used as a positive control. Together, this suggests that AR transcriptionally regulates integrin α6 and Bcl-xL expression. Laura E. Lamb went on to demonstrate that the effect of AR on integrin α6 transcription is direct, in that AR binds directly to the integrin α6 promoter. These studies were done in collaboration with Laura E. Lamb as noted in the figure legend of figure 3.

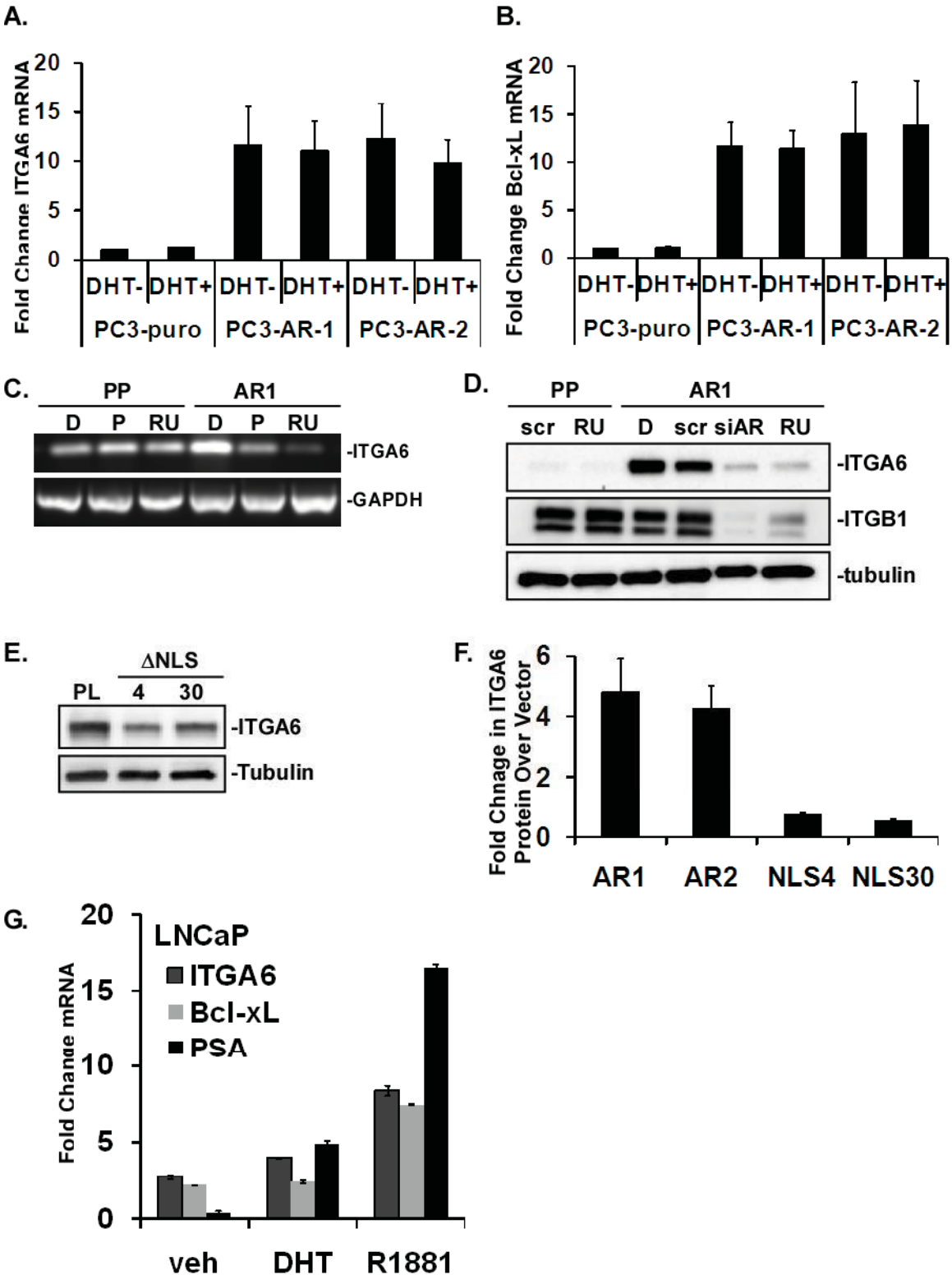


**FIGURE 3. AR regulates integrin α6 and Bcl-xL mRNA expression.**

**FIGURE 3. (CONT'D) A-B)** PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 (AR2) cells were plated on LM1 and treated with vehicle (ethanol) or 10nM DHT for 72 hours. Messenger RNA was then isolated and reverse transcribed. Integrin α6 (ITGA6) **A)** or Bcl-xL **B)** mRNA expression was measured by qRT-PCR. Gene expression was normalized to 18s rRNA then expressed as fold change relative to vehicle-treated PC3-puro cells. Error bars represent standard deviation; *n* = 3. **C)** PC3-puro (PP) and PC3-AR-1 (AR1) were plated on LM1 then treated with DMSO (D), PBS (P), or 10nM RU486 (RU) for 72 hours. Messenger RNA was then isolated and RT-PCR was performed to measure integrin α6 (ITGA6) levels. GAPDH expression was used as a loading control. **D)** PC3-puro (PP) and PC3-AR-1 (AR1) were plated on LM1 then treated with DMSO (D), RU486 (RU), AR siRNA (siAR), or non-targeting siRNA (scr) for 72 hours. Cells were lysed and immunoblotted to monitor integrin α6 (ITGA6) and β1 (ITGB1) expression. Total levels of protein were monitored by immunoblotting with anti-tubulin. **E)** PC3-pLKO (PL) and PC3-ΔNLS-AR (ΔNLS) clone lysates were monitored for integrin α6 (ITGA6) and Bcl-xL expression by immunoblotting. Tubulin expression was used as a loading control. **F)** PC3-puro, PC3-AR-1 (AR1), PC3-AR-2 (AR2)**,** PC3-pLKO, PC3-ΔNLS-AR-4, and PC3-ΔNLS-AR-30 cells were growth factor-starved in charcoal-stripped media and plated on LM1. After 72 hours, cells were treated with fluorescent-conjugated integrin α6 antibody and analyzed by FACS. Rat IgG controls were subtracted from mean fluorescent values then values for AR expressing cells were normalized to those of the corresponding vector cells. Error bars represent standard error; *n* = 2. **G)** LNCaP cells were serum- starved in charcoal-stripped media for 48 hours, then treated with vehicle (veh), 10nM DHT, or 10nM R1881 for 24 hours. Messenger RNA was then isolated and reverse transcribed. Integrin α6 (ITGA6), Bcl-xL, and PSA mRNA expression were measured by qRT-PCR. Gene expression was normalized to 18s rRNA then expressed as fold change relative to untreated cells. Error bars represent standard deviation; *n* = 1. *(A-B, E-G) Laura Lamb, (C-D) Jelani Zarif.*

*Generation of Tet-Inducible shRNA Cell Lines*

One of our objectives was to determine the extent to which AR and integrin α6β1 contribute to prostate cancer survival *in vivo*. We demonstrated that loss of AR or α6β1 causes cell death in cell lines *in vitro*; however, this required simultaneous inhibition of the PI3-K pathway (Lamb et al., 2011). We will determine if this survival pathway is critical *in vivo*, and whether simultaneous inhibition of AR or integrin α6 in combination with PI3-K inhibition could be used as a novel therapeutic strategy to target CRPC in patients. To elucidate the importance of AR and integrin α6 in the survival of prostate tumor cells, we generated stable prostate tumor cell lines expressing a doxycycline-inducible shRNA against AR or integrin α6 (Fig. 4). Each clone was then treated with 200ng/mL of doxycycline and then levels of AR, Bcl-xL, and integrin α6 expression measured by immunoblotting (Fig 4A, B). We will orthotopically inject cells expressing an inducible shRNA against α6 integrin or AR into the prostates of male athymic nude mice. We expect that loss of AR or α6 expression by the inducer doxycycline will not lead to cell death because the PI3-K pathway is still intact (Fig. 5). However, we anticipate that inhibition of PI3-K simultaneously with induction of the shRNA will be required to induce cell death and tumor regression. In a subset of mice, we will test tumor cell lines that are castration-resistant to establish the potential effectiveness of this combination therapy for patients with CRPC.

Discussion

Prostate cancer is a leading cause of cancer death in men in the U.S. In 2013, more than 180,000 men were predicted to be diagnosed and over 28,000 men were

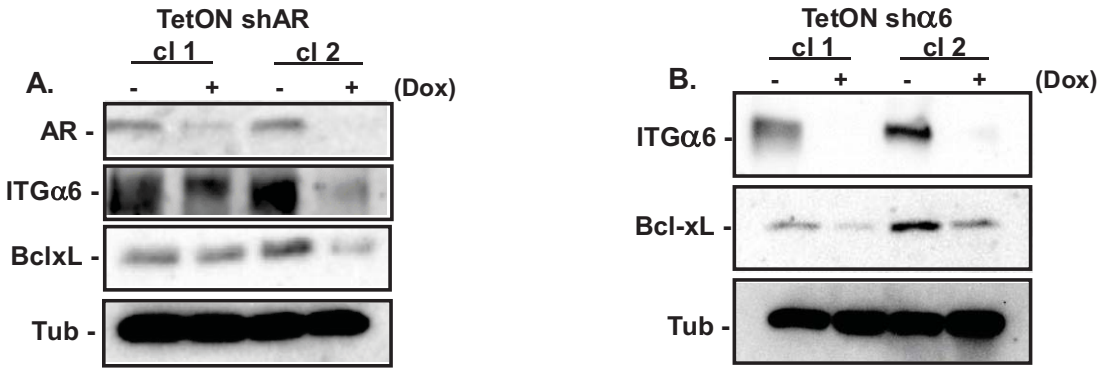


**FIGURE 4. Generation of Tet-Inducible shRNA Cell Lines.** CRPC cell lines were stably infected with tet-inducible shRNA against AR or integrin α6 and stable clones (cl) were isolated after selection (A, B). Treatment of clones with doxycycline (Dox), attenuated (A) AR and (B) integrin α6 (ITGα6) expression which in turn reduced Bcl-xL as measured by immunoblotting. Tubulin (Tub) was used as the loading control.

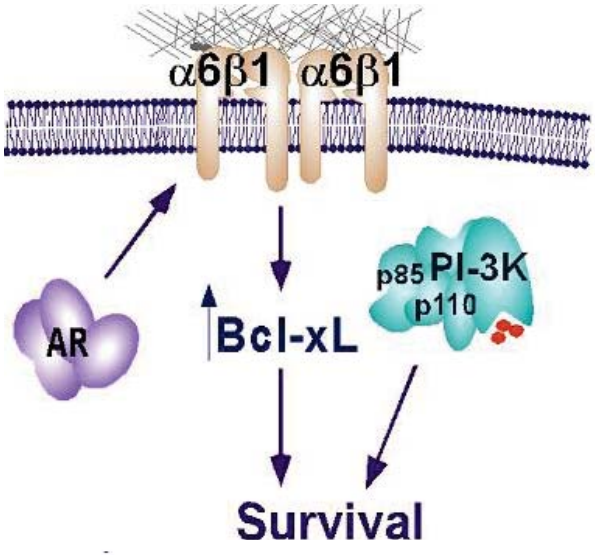


**FIGURE 5. Model for AR and Integrin α6β1 Regulation of Prostate Cancer Survival.** Our model for AR-dependent regulation of α6β1, Bcl-xL, and cell survival in CRPC cell lines. Inhibiting both AR and the PI3-K pathway is required to suppress tumor cell survival *in vitro* and *in vivo*.

predicted to die from this disease (Siegel et al., 2013). It is well established that sex steroids, especially androgens, with the help of their receptor AR, are the driving forces behind prostate cancer development and progression (Feldman and Feldman, 2001). When the disease is at a small focus in the gland, usually its androgen-dependent stage, survival rates are excellent. Treatments for this stage of disease include chemotherapy, removal of tumor(s), implantation of radiation seeds, and prostatectomy. When the cancer has spread androgen deprivation therapy is used and is initially very effective due to the dependence of the tumors on androgens for growth and survival (Feldman and Feldman, 2001; Liao et al., 2005a). However after several years, the disease returns and uses low to no levels of androgens for growth, and when it is in this highly metastatic hormone-refractory stage it is practically inoperable and incurable. Despite the lack of dependence on androgen, recent studies have demonstrated that occluding AR expression suppresses tumor growth and leads to cell death indicating that AR regulates cell survival even in hormone-refractory disease (Cheng et al., 2006; Liao et al., 2005a; Snoek et al., 2009).

The limited number of clinically relevant cell lines available that are metastatic, have the proper integrin profile, and have AR expressed after placement in culture has hampered progress in understanding hormone-refractory metastatic disease. For instance, the prostate cancer cell line PC3, isolated from a bone metastasis are non-responsive to circulating androgens because these cells unfortunately do not express AR, which handicaps researcher who desire to study the metastatic bone disease. In this study, we have developed a new androgen-independent model using PC3 cells to address the role of AR in androgen-independent tumor cell survival. To create a model

that reflects what is seen in patients and to fully study the role of AR in metastatic disease, we have re-expressed AR in PC3 cells, thus creating PC3-AR cells (Fig 1). Immunostaining revealed that all cells in the population express AR and that it is distributed in both the cytoplasm and nucleus (Lamb et al., 2011).

Previous studies have demonstrated that when human AR cDNA was re-expressed in PC3 or DU145 cells, it decreased α6β4 integrin expression, thus suggesting that AR and integrins may have a signaling relationship (Bonaccorsi et al.; Nagakawa et al., 2004). Similarly, our laboratory data has demonstrated that β4 integrin is decreased in stable PC3-AR clones compared to vector control cells (Fig. 1A) (Lamb et al., 2011). However, we also see loss of α3β1 and α5β1, along with a concomitant increase in α6β1 integrin and Bcl-xL (Fig. 1A, 2A). It is important to note that these changes in integrin expression and Bcl-xL in the PC3-AR clones occur in the absence of androgen (Fig 1A, 3A-B). Conversely, loss of AR in PC3-AR clones or in LNCaP cells resulted in decreased α6 integrin (Fig. 1B-C, 1F, 2B). Interestingly, this integrin profile more closely resembles that seen *in vivo*. Furthermore, these data indicate that the expression of AR may contribute to the shift from α6β4 integrin to α6β1 as seen in cancer patients *in vivo* (Cress et al., 1995). The effect of AR on α6 was not due to a clonal artifact, as loss of or antagonism to AR not only decreased Bcl-xL (Fig. 2B) and α6 expression (Fig. 2C), but also it restored the sensitivity to PI3-K inhibition (Fig. 1G-H). These results were successfully validated by using AR siRNA in LNCaP cells (Fig. 2D). Thus, AR promotes survival on LM independently of PI3-K by increasing integrin α6 expression.

Suppression of integrin α6 had no effect on AR levels of expression (Fig. 2C), but did suppress Bcl-xL expression (Fig. 2C) and also sensitized the PC3-AR clones to PI3-K antagonism (Fig. 1D-E). This demonstrates that integrin α6 is upstream of Bcl-xL and it also correlates with the importance of integrin α6 reported by Cress, A.E. *et al*, 1995. We then wanted to determine whether AR transcriptionally regulated integrin α6. LNCaP cells were stimulated with R1881 and RNA was isolated to assess mRNA levels of known AR targets. To our surprise, not only was PSA increased but so was Bcl-xL and integrin α6 (Fig. 3G). Treatment of PC3-AR cells with the transcription AR inhibitor RU486, or AR siRNA, decreased mRNA levels and protein expression of integrin α6 and Bcl-xL (Fig 3C-D). Stable expression of AR mutants that block the ability of AR to translocate to the nucleus (ΔNLS) largely restored the parental PC3 phenotype, including a lower expression of integrin α6 (Fig. 3E-F) and PI3-K dependent survival (data not shown) (Lamb et al., 2011). Lastly, PC3-AR cells were also stably transfected with tetracycline shRNAs against AR or integrin α6 to be further used for orthotopic injections into athymic nude mice (Fig. 4A-B).

Wild-type human AR was stably re-expressed in the prostate cancer cell line PC3. Re-expression of AR into PC3 cells led to an increased transcription and expression of integrin α6, and adhesion to laminin subsequently increased transcription and expression of the pro-survival protein Bcl-xL, and made the cells resistant to cell death induced by PI3-K inhibition. This is also seen clinically where aggressive prostate tumors are resistant to such therapeutic treatments. Thus, we have developed an androgen-independent metastatic prostate cancer model that mimics clinical events seen in patients, i.e. over expressed wild type AR and elevated expression of α6β1

integrin. In this model AR confers androgen independence and enhanced survival. With the use of our tet-inducible shRNA clones (Fig 4A-B), we will test our *in vitro* findings (Fig. 5) *in vivo*. We believe these findings will also reveal the mechanism(s) by which AR, α6 integrin, and Bcl-xL promote prostate cancer progression and survival. Overall, the proposed *in vivo* studies will also yield a better understanding of how intracellular signaling cascades in prostate tumor cells controlled by AR regulates tumor behavior and drug resistance. This information will be valuable for identifying potential therapeutic targets of metastatic prostate cancer.

Materials and Methods

*Cell Culture*

The prostate tumor cell lines, PC3 and LNCaP, were purchased from American Type Culture Collection. PC3 cells were grown in F-12K media supplemented with 10% charcoal-stripped and dextran-treated fetal bovine serum (CSS), 2mM glutamine, 50 U penicillin, and 50 μg/mL streptomycin. LNCaP cells were grown in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U penicillin, 50 μg/mL streptomycin, 0.225% glucose, 10 mM HEPES, and 1 mM sodium pyruvate. For experiments, LNCaP cells were grown in phenol-red free media and 10% CSS for 48 h beforehand and during the experiment duration. Phoenix-ampho cells (Oribigen) and 293-FT cells (Invitrogen) were cultured in DMEM (Gibco) supplemented with 10% HI-FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate. All cells were grown at 37˚C and 5% $CO_2$.

*DNA Plasmids*

The pBabe-puro-hAR and pGL3-basic plasmids were kindly provided by Dr. Beatrice S. Knudsen (Fred Hutchinson Cancer Research Institute). The pCSCG-AR-ΔNLS plasmid, which has three point mutations (K618M, K632M and K633M) that disrupt AR nuclear translocation, was generously provided by Dr. Owen N. Witte (University of California at Los Angeles) (Chen et al., 2004; Xin et al., 2006b). The pLKO.3pg was kindly provided by Dr. Jeff MacKeigan (Van Andel Institute). All AR plasmids were sequence verified to contain wild type sequence or the respective mutations to the sequence. The following primers, designed by Dr. Aaron Putzke (Fred Hutchinson

Cancer Research Institute), were used (sequences listed 5' to 3'):
AAGCTCAAGGATGGAAGTGC, AGCAACCTTCACAGCCGCAG,
AAGCTCAAGGATGGAAGTGC, AGCAACCTTCACAGCCGCAG,
GGGCACTTCGACCATTTCTG, CTACAAGTCCGGAGCACTGG,
GCGGCATGGTGAGCAGAGTG, CTTGTCGTCTTCGGAAATG,
CTTGTCGTCTTCGGAAATG, CTTGTCGTCTTCGGAAATG,
GTGGACGACCAGATGGCTGTC, ACATCCTGCTCAAGACGCTTC,
AATGCTTCACTGGGTGTGG, AACTCTTGAGAGAGGTGCCTC,
AACTCTTGAGAGAGGTGCCTC, AACTCTTGAGAGAGGTGCCTC,
AACTCTTGAGAGAGGTGCCTC, GAGGCTAGAGAGCAAGGCTG,
GCAGCTTCCACATGTGAGAG, GTCCGGAGTAGCTATCCATC,
TTCTCCAGCTTGATGCGAGC, CCAAAAGTGGGGCGTACATG, and
GGCAGCTGAGTCATCCTCGT.

*Establishment of Cell Lines*

PC3-puro and PC3-AR cells were made by infecting cells with pBabe-puro, pBabe-puro-hAR retroviruses respectively. Retroviruses were produced in Phoenix 293 cells by transfecting 5 μg of DNA with 3μl/μg Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) following manufacturer's directions. Cells were maintained at 32˚C and 5% $CO_2$ and the media was changed the next day. Retroviruses in the conditioned medium were collected daily between days five and seven. Viruses were separated from cellular debris by spinning conditioned media for 5 minutes at 1,500 rpm at room temperature. The supernatant was then passed through a 0.45 μm low-binding filter (Millipore) and

frozen at -80˚C until use. For infection of PC3 cells, 2.5 mL of filtered virus were added per 10 cm dish of PC3 cells. After three hour incubation, 5 μg/mL of Polybrene (Sigma) was added to cells. The media was changed the next day and infected PC3 cells were maintained at 37˚C and 5% $CO_2$. Clones were selected for and maintained in 2 mg/mL puromycin (Sigma). Only low passage (i.e. under passage 20) cells were used for experiments.

PC3-ΔNLS cells were made by co-infecting cells with, pCSCG-AR-ΔNLS, lentiviruses along with pLP1, pLP2, and pLP/VSVG lentiviruses. Lentiviruses were made in 293-FT cells that were pre-selected for in 1 μg/mL Diphtheria Toxin (Sigma) and 300 μg/mL of hygromycin (Invitrogen). Four micrograms of each plasmid were transfected into the 293-FT cells with 2.5μl/μg Lipofectamine 2000 in Opti-MEM following manufacturer's directions. Virus was collected from the conditioned medium four and five days after the transfection by spinning at 1,000 rpm for 10 minutes then passing through a 0.22 μm low-binding filter (Millipore). Virus was kept at -80˚C until use. For infection of PC3 cells, 1.5 mL of filtered virus and 5 μg/mL polybrene were added per 10 cm dish of PC3 cells. The media was changed the next day and clones selected thereafter.

*Small Interfering RNA Transfections*

A pool of four small interfering RNAs (siRNA) against androgen receptor (siGENOME SMARTpool; 5 nM for PC3-AR clones and 50 nM for LNCaP cells), integrin α6 (ON-TARGETplus SMARTpool; 20 nM), or a non-targeting sequence were purchased from Dharmacon. Sequences are listed in Table 1. Cells were transfected with siRNA using

siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) media following manufacturer's directions. The media was changed 16 hours after transfection.

Table 1. siRNA Sequences

| Gene | NM# | Product No. | siRNA Target Sequence |
|---|---|---|---|
| AR | NM_000044 | D-003400-01 | GGAACUCGAUCGUAUCAUUU |
| | | D-003400-02 | CAAGGGAGGUUACACCAAAUU |
| | | D-003400-03 | UCAAGGAACUCGAUCGUAUUU |
| | | D-003400-04 | GAAAUGAUUGCACUAAUUGAUU |
| ITGA6 | NM_000210 | J-007214-05 | GGAUCGAGUUUGAUAACGAUU |
| | | J-007214-06 | GGAUAUGCCUCCAGGUUAAUU |
| | | J-007214-07 | GAAAGGGAUUGUUCGUGUAUU |
| | | J-007214-08 | ACAGAUAGAUGAUAACAGAUU |
| scram/ non-targeting | n/a | D-001210-01 | UAGCGACUAAACACAUCAA |
| | | D-001210-02 | UAAGGCUAUGAAGAGAUAC |
| | | D-001210-03 | AUGUAUUGGCCUGUAUUAG |
| | | D-001210-04 | AUGAACGUGAAUUGCUCAA |

*Tet-inducible short hairpin RNA transfections*

PC3-AR tet-ON shRNA clones were generated by using pLKO-Tet-ON vector (Addgene) that contained a single AR shRNA (Open Biosystems) or integrin α6 (Sigma) and cloned upstream of the H1/TO promoter as described (Wee et al., 2008; Wiederschain et al., 2009). Both AR shRNA and integrin α6 shRNA sequences were sequence validated and 4ug of DNA was transformed into Stbl3 competent cells on LB/amp plates. For optimal transformation efficiency, Stbl3 competent cells were pre-chilled on ice, heat shocked for 30-45 s at 42°C, incubated on ice for 5 min. Thereafter, 500 µl of SOC growth media was added and incubated at 37°C while shaking for 30 minutes. Stbl3 colonies that were positive were picked and DNA was isolated using miniprep kit (Qiagen), eluted into EB and 4-5 µl of DNA digested using XhoI (site of shRNA loop) restriction enzyme. Thereafter, isolated from these colonies DNA was

purified by isopycnic centrifugation using cesium chloride (CsCl) maxiprep and was packaged into lentiviruses using pLP1, pLP2, and pLP/VSVG and 293FT cells at a density of $4x10^6$. PC3-AR cells were then infected with either tet-ON ARshRNA or tet-ON α6shRNA lentiviruses. Individual clones were selected using 3 μg/ml of puromycin. Knockdown was validated using immunoblotting after 200 ng/mL of doxycycline (Dox) treatment.

*Immunoblotting*

Whole *C*ell lysates were acquired and prepared for immunoblotting by lysing cells on ice with MAPK lysis buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 50 mM NaF, 100 mM NaCl, 50 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1% Triton-X100, 1 mM $Na_3VO_4$, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM benzamide) or RIPA (10 mM Tris pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton-X100, 1 mMNa3VO4, 1 mM PMSF, 100U mL aprotinin, 10 μg/mL pepstatin, and 10 ug/mL leupeptin) buffers and 40-65 μg of total cell lysates in 2X SDS sample buffer were boiled for 10 minutes. Samples were run on SDS polyacrylamide gels following standard SDS-PAGE protocols and transferred to PVDF membranes. Membranes were blocked in 5% BSA in TBST for two hours at room temperature, and then were probed with primary antibody for two hours at room temperature. Primary antibodies were used at the indicated dilutions in Table 2. Membranes were washed three times, and incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) in 5% BSA in TBST for 1 hour at room temperature. After washing an additional three times, signals were visualized by chemiluminescence reagent with a

CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

Table 2. Immunoblotting Antibodies

| Protein | Clone | WB Dilution | Company |
|---|---|---|---|
| mAb AR | 411 | 1:200 | Santa Cruz |
| rAb Bcl-xL | | 1:1000 | Cell Signaling |
| rAbITGA6 | AA6A | 1:10,000 | Gift of A. Cress |
| mAb ITGB1 | 18/CD29 | 1:1000 | BD Transduction |
| rAb Nkx3.1 | H-50 | 1:500 | Santa Cruz |
| goatAb PSA | C-19 | 1:200 | Santa Cruz |
| rAb Phospho- Src (Y418) | | 1:1000 | Biosource |
| mAb Non-phospho- Src (Y529) | | 1:2000 | Biosource |
| mAb TMPRSS2 | P5H9-A3 | 1:2000 | Gift of P.S. Nelson |
| mAb α-tubulin | DM1A | 1:10,000 | Sigma |

*Cell Surface Integrin Expression Analysis*

Cells were placed in suspension by washing the cells with PBS, detached from plates using trypsin. The action of Trypsin was inhibited using soybean trypsin inhibitor (Invitrogen). Cells were then washed with wash buffer (1% sodium azide/2% FBS/PBS) and then incubated with the appropriate primary antibodies (Table 3) or control IgG molecules for 1 hour at 4˚C. Cells were then washed twice and incubated with fluorescently-labeled secondary antibodies (Molecular Probes, Invitrogen) for 1 hour at 4˚C in the dark. Cells were washed twice more, and fluorescence was detected by a Becton-Dickinson FACSCalibur 4-color flow cytometer with CellQUEST Pro Software v5.2.1 (Becton-Dickinson).

Table 3. Flow Cytometry Antibodies

| Protein | Clone | FACS Dilution | Company |
|---|---|---|---|
| mAb ITGA2 | CBL 477 | 1:250 | Chemicon |
| mAb ITGA3 | MAB 2056 | 1:250 | Chemicon |
| mAb ITGA5 | P1D6 | 1:250 | Santa Cruz |
| mAb ITGB4 | ASC-3 | 1:200 | Chemicon |
| ratAb ITGA6 | GoH3 | 1:200 | BD Pharmingen |
| ratAb ITGB1 | AIIB2 | 1:250 | Iowa State Univ. Hybridoma Bank |

*Cell Death Assays*

Cells were serum starved for 48 hours and plated on 1% BSA blocked 24-well tissue culture plates pre-coated 10 μg/mL natural mouse laminin 1 (Invitrogen) or rat tail collagen 1 (Becton, Dickinson and Company) as described previously (Edick et al., 2007). In some cases, cells had been transfected with siRNA 72 hours prior to assay. After siRNA transfection, DMSO (vehicle; Sigma) or 20 μM of the PI3-K pharmacological inhibitor LY294002 (Calbiochem) was added. Cells were allowed to adhere for 4 hours and then non-adherent cells were removed and DMSO or LY294002 was replaced. Cells were incubated for an additional 72-88 hours. LY294002 was replenished 48 hours after plating. In some cases ethanol (vehicle; Decon Laboratories), 10 nM 5α-dihydrotestosterone (DHT; Sigma or Steraloids) or Bicalutamide (Casodex; Enzo Life Science) was also added to the cells. DHT and/or Casodex were replenished every 24 hours.

To assess cell viability, both attached and floating cells were collected at the end of the experiment. Attached cells were removed using trypsinization, pooled with floating cells, and all cells were washed once. Cells were then spun down for 8 minutes at 800 rpm, resuspended into a 30μl volume. Trypan Blue (Invitrogen) was mixed in an

equal 1:1 ratio with cells and cells were loaded into the hemocytometer via capillary action. Cells that took up Trypan Blue were counted as dead. A minimum of three separate counts per well were performed using a hemocytometer, with two to three wells counted for each condition per experiment. All experiments were replicated a minimum of three times.

*RT-PCR*

Total RNA was isolated from cells using TRIzol (Gibco) and chloroform (Sigma-Aldrich). Contaminating DNA was removed using RNAse-free DNAse kit (Qiagen) following manufacturer's directions. The RNA was further purified with the RNAeasy Total RNA isolation kit (Qiagen) following manufacturer's directions. The purified RNA was eluted in 30 μl water. Concentration and purity were determined by SmartSpec3000 UV spectrophotometer (Bio-Rad).

RT-PCR was performed on 1 μg RNA with the indicated primers (Table 4) using the One-Step RT-PCR kit (Qiagen) following manufacturer's directions. The thermal cycling parameters were 50°C for 30 min, 95˚C for 15 min, followed by 40 cycles of 94°C for 30 s, 50˚C for 30 sec, and 72 °C for 60 s, and a last step of 72˚C for 10 min. RT-PCR products were analyzed on a 2% agarose/TBE gel and DNA was visualized with ethidium bromide and a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

For qRT-PCR, 0.5 μg RNA was reversed transcribed with random primers using a reverse transcription system (Promega). The synthesized cDNA was diluted 1:25 in water and amplified for qRT-PCR analysis using SYBR green master mix (Roche) with the indicated primers (Table 4) and an ABI 7500 RT-PCR system (Applied Biosystems)

following manufacturer's directions. Gene expression was normalized to 18s rRNA by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 4. qRT-PCR Primers

| Gene | Fwd Primer (5'→3') | Rev Primer (5'→3') | Ref. |
|---|---|---|---|
| GAPDH | ACCACAGTCCATGCCATCAC | TCCACCACCCTGTTGCTGTA | Sun et al., 2008 |
| ITGA6 | GCTGGTTATAATCCTTCAATATCAATTGT | TTGGGCTCAGAACCTTGGTTT | Tapia et al., 2008 |
| ITGB1 | GTGGTTGCTGGAATTGTTCTTATT | TTTTCCCTCATACTTCGGATTGAC | Tapia et al., 2008 |
| 18s rRNA | CCGCAGCTAGGAATAATGGA | CGGTCCAAGAATTTCACCTC | Ottosen et al., 2006 |

Acknowledgements

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# CHAPTER THREE:
# AR NON-NUCLEAR REGULATION OF PROSTATE CANCER CELL INVASION THROUGH MATRIPTASE AND SRC SIGNALING

## Introduction

Prostate cancer is one of the leading causes of cancer deaths in U.S. men, and an estimated 240,890 new cases will be diagnosed in 2013 (Siegel et al., 2013). The survival of malignant tumors arising from the prostate gland is dependent on the androgen receptor (AR), a nuclear steroid receptor that binds androgen and activates gene transcription (Agoulnik et al., 2005; Feldman and Feldman, 2001). This dependence on androgen is exploited therapeutically; patients presenting with metastatic disease are treated with anti-androgen therapies that effectively lower androgen levels and cause tumor regression. However, patients typically relapse within one to two years and develop castration-resistant prostate cancer (CRPC) in which the tumors no longer respond to androgen ablation therapy (Seruga and Tannock, 2008). In CRPC, nuclear AR is still critical despite its resistance to anti-androgen therapies. This is further evidenced by the fact that more potent second generation anti-androgen therapies are able to extend patient survival, but they too are not curative.

Studies on the new anti-androgen agent MDV3100 indicate one of the ways it inactivates AR nuclear activity is by preventing nuclear translocation, thus retaining a significant amount of AR the cytoplasm (Chen et al., 2009; Tran et al., 2009). Several steroid receptors have been reported to have non-nuclear (aka non-genomic) signaling functions independent of transcriptional activity (Arnold and Isaacs, 2002; Castoria et al., 2004; McEwen and Alves, 1999; Revelli et al., 1998; Watson and Gametchu, 1999; Wehling, 1997). These non-nuclear signaling mechanisms are associated with rapid responses (within seconds to minutes) of ligand stimulation. However, this mechanism has been poorly characterized for AR. One signaling molecule that is a common target

of steroid nuclear receptors is the non-receptor tyrosine kinase, Src. A study reported that low AR activity correlated with increased Src activation and its sensitivity to the Src/Abl pharmacological inhibitor Dasatinib (Mendiratta et al., 2009). Src activity is reportedly elevated in patients treated with MDV3100 and in patients with castration-resistant disease (Efstathiou E, et al. unpublished observations 2011).

Src is a prototypic member of the non-receptor protein tyrosine kinase family (SFK) and has been shown to be up-regulated or hyper-activated in a high percentage of human cancers (Thomas and Brugge, 1997; Yeatman, 2004). In murine models and *in vitro*, Src has also been long associated with cancer progression in breast, colorectal, and pancreatic cancers. Even more recently, Src activation has been associated with late onset bone metastasis in breast cancer patients (Zhang et al., 2009). Src can exert its effects on cell motility by promoting the ubiquitinylation and endocytosis of E-cadherin and by also its phosphorylation of FAK and of RRAS, p120ctn, STAT3, and cortactin thus promoting cell migration (Mitra and Schlaepfer, 2006; Yeatman, 2004). Src can be activated by chemokines, cytokines, and more interestingly, sex hormone receptors. Studies in breast cancer cell lines demonstrated that Src can become activated downstream of the estradiol and progesterone receptors in a hormone dependent manner to activate MAPK signaling (Migliaccio et al., 1996). Conversely, Boonyaratanakornkit *et al*. demonstrated mechanistically that the progesterone receptor (PR) can directly activate SFKs through interactions with the amino-terminal poly-proline region of PR and the SH3 domain of Src tyrosine kinases (Boonyaratanakornkit et al., 2001).

Src and Lyn were reported to be intimately involved in prostate cancer cell migration and invasion. Inhibition of Src and Lyn decreased prostate cancer invasion, progression, and lymph node metastasis (Park et al., 2008). While decreasing Src in prostate cancer cell lines has a dramatic effect on tumor cell invasion, AR is critical for the disease at all stages and may be a positive regulator of Src activation. In this regard, Src was shown to form a complex with AR upon androgen stimulation in via the SH3 domain of Src (Migliaccio et al., 2000). Additionally, AR forms a tertiary complex with a modulator of non-genomic activity of estrogen receptor, MNAR, and Src which leads to the activation of Src in this complex (AR/MNAR/Src) and subsequent activation of the downstream effector, MEK. The activation of this pathway stimulates tumor cell proliferation and survival of prostate tumor cells (Unni et al., 2004). However, the role that AR non-nuclear signaling may have on other tumor cell behavior such as migration and invasion has not been explored.

We previously demonstrated that AR activation increases integrin α6β1 transcription and expression, which through adhesion to laminin increases Bcl-xL expression (Lamb et al., 2011), conferring resistance to PI3K inhibitors (Edick et al., 2007). We also observed that AR activation induced morphological changes that led us to hypothesize that AR may contribute to metastasis. In this report, we explore non-nuclear signaling of AR and demonstrate its involvement in prostate cancer migration and invasion.

Results

*AR stimulation alters cell shape, migration, and invasion via laminin integrins*

Previously we generated PC3 cells stably expressing wild type AR (Lamb et al., 2011). We demonstrated that AR was constitutively nuclear localized and activated in the PC3-AR cells as measured by immunofluorescent staining and expression of PSA. During those studies we observed marked changes in cell morphology in the AR-expressing cells compared to the parental vector cells (PC3-Puro). To quantify these differences, PC3-Puro and PC3-AR cells were plated on laminin and immunostained to visualize actin structures. Laura E. Lamb found that there was a marked increase in cell spreading on laminin by the AR-expressing cells compared to the vector cells (Fig. 6A), which was accompanied by an 8-fold increase in filopodial structures (Fig. 6B). The observed increase in filopodia formation specifically in PC3-AR cells correlated with increased migration in Boyden chambers (Fig. 6C) as well as increased activity of matrix metalloproteinase 2 and 9 (MMP2/9) (Fig. 6D) compared to PC3-Puro vector cells. Furthermore, inhibiting AR activity with AR antagonists Casodex or RU486 attenuated Src activation (not shown) and the AR-specific increase in Matrigel invasion (Fig. 6D). Complementary experiments were conducted using LNCaP and C4-2 cells which are both androgen responsive and express endogenous AR. In LNCaP and C42 cells, stimulation of AR using R1881 increased matrigel invasion (Fig. 6E) and suppression of AR using siRNA decreased matrigel invasion stimulated by R1881 (Fig. 6G). We previously demonstrated that the transcriptional activity of AR increases integrin α6β1 expression (Lamb et al., 2011). However, blocking integrin α6 expression

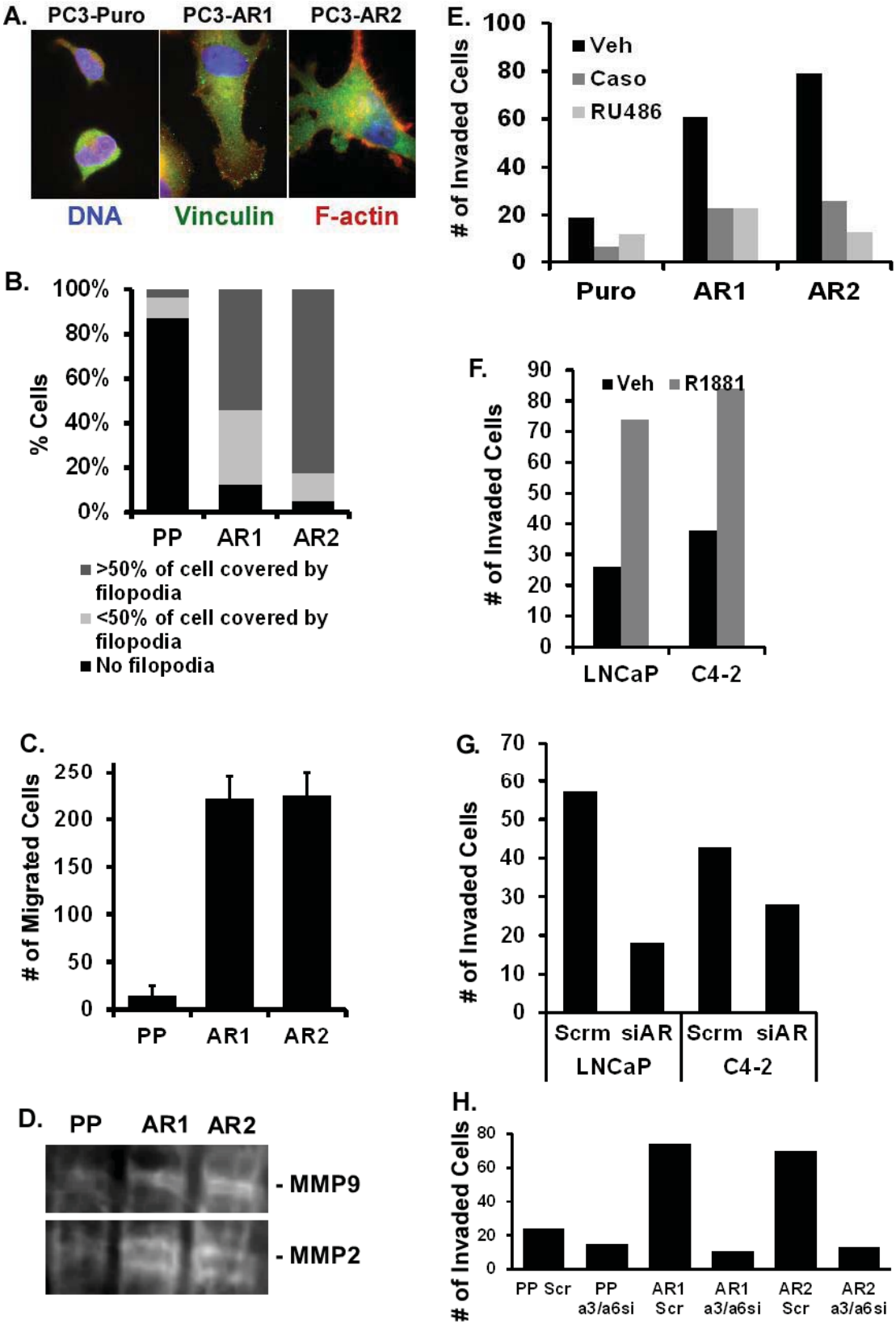


**FIGURE 6. AR stimulation alters cell shape, migration, and invasion via laminin integrins.**

**FIGURE 6. (CONT'D) A, B)** PC3 (Puro) and 2 PC3-AR clones (AR1 and AR2) were plated on laminin for 1 hour then **A)** immunostained for vinculin (green) or stained for F-actin with phalloidin (red) and counterstained with Hoecsht (blue). **B)** Filopodia production was quantified. **C)** Migration on laminin-coated Boyden chamber inserts was quantified in PC3-Puro (PP) versus PC3-AR clones. **D)** Zymography was performed on media from PC3 (PP) and 2 PC3-AR clones (AR1 and AR2) to assess MMP 2/9 activity. **F)** Extent of invasion through Matrigel-coated Boyden chambers was quantified following treatment of PC3 (Puro) or PC3-AR clones with ethanol (veh), 10nM Casodex (Caso) or 10nM RU486. **E, F)** Matrigel invasion was measured in LNCaP or C4-2 cells treated with **E)** ethanol (veh) or 10nM R1881 for 24 hours to stimulate AR activity, or **F)** with AR-specific siRNA (siAR) or scrambled siRNA (Scrm) to block AR. **G)** Level of Matrigel invasion by PC3-Puro (PP) or PC-AR clones treated with scrambled (Scr) or combined integrin α3 and α6 siRNA (a3/a6) was compared. *(A-C) Laura E. Lamb, Jelani C. Zarif, (D-H)*

did not significantly block invasion (not shown). Depletion of both α6 and α3 integrins was required to suppress cell invasion (Fig. 6H). Thus AR, independently of its actions on integrin α6 expression, promotes the migration and invasion of prostate tumor cells on laminin.

*AR stimulates Src activation*

Src is a major effector of cell spreading, migration, and invasion (Thomas and Brugge, 1997; Yeatman, 2004). Therefore, we investigated Src activation and expression in the PC3-Puro and PC3-AR cells. We found both elevated Src expression as well as increased Src activity in PC3-AR cells as measured by anti-phospho-[$Y^{416}$]-Src antibody (Fig. 7A). Suppression of AR expression in PC3-AR clones with siRNA resulted in decreased Src activation, but not total Src expression (Fig. 7B) demonstrating that AR regulates Src activation but not its expression. Androgen stimulation of LNCaP or C4-2 cells with R1881 for twenty minutes was sufficient to increase Src-$Y^{416}$ activation, but caused no change in Src expression (Fig. 7C). Conversely, inhibiting AR expression with siRNA in LNCaP and C42 cells suppressed Src-$Y^{416}$ activation in response to R1881 (Fig. 7D). Thus, AR stimulates Src activity in several different prostate tumor cell lines.

Inhibiting Src expression with siRNA did not alter AR expression (Fig. 7E) indicating Src does not control AR expression. The increase in Src-$Y^{416}$ activation or Src expression in PC3-AR cells was not dependent on the ability of AR to induce integrin α6 expression (Lamb et al., 2011) since siRNA against integrin α6 did not change Src activity (not shown). Src is also not involved in the AR and integrin α6

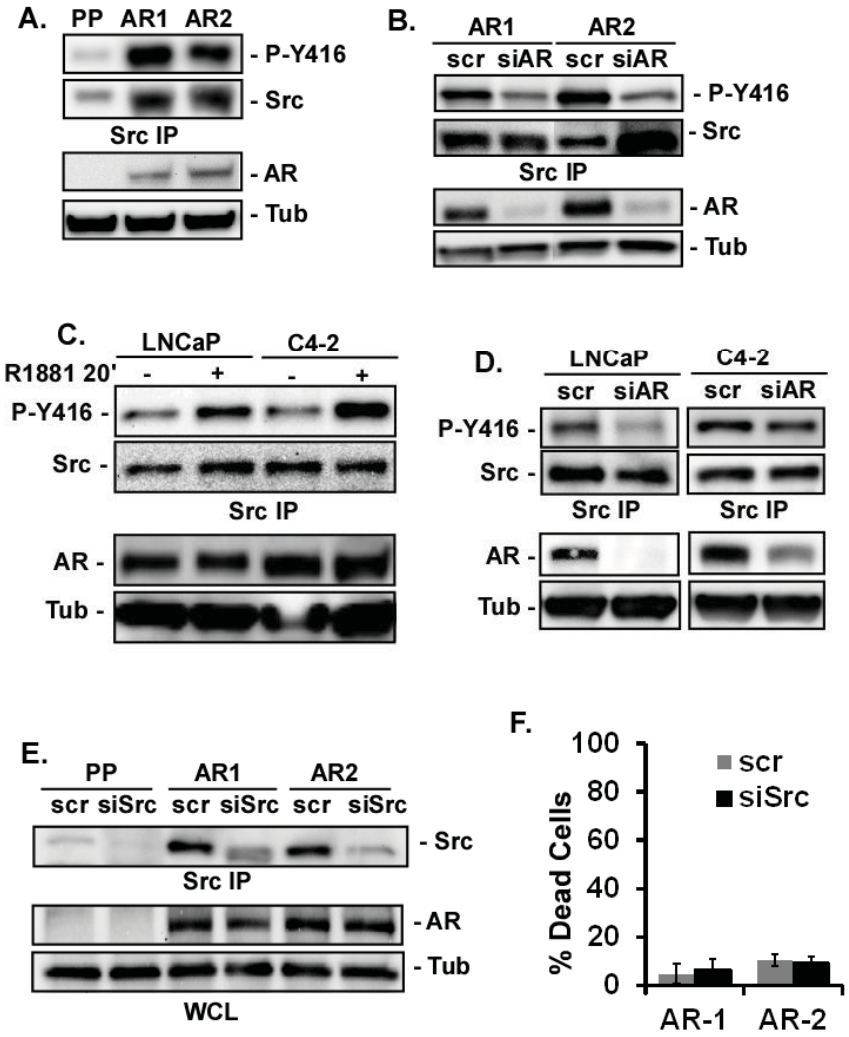


**FIGURE 7. AR stimulates Src activation.** **A,B)** Levels of Src activation and expression in PC3-Puro (PP) or PC3-AR clones (AR1/AR2) were measured by immunoblotting of Src immunoprecipitates with anti-Y416 phospho-specific antibody or total anti-Src antibody respectively in **A)** untreated cells or **B)** cells treated with scrambled siRNA (scr) or AR-specific siRNA (siAR). Total levels of AR expression were monitored and tubulin (Tub) served as loading controls. **C,D)** Src activation was measured in LNCaP or C4-2 cells stimulated with ethanol (-) or 10nM R1881 for 20 minutes in **C)** untreated cells or **D)** cells treated with scrambled siRNA (scr) or AR-specific siRNA (siAR). **E, F)** PC3-Puro (PP) or PC3-AR clones (AR1/AR2) were treated with scrambled siRNA (scr) or Src-specific siRNA (siSrc). **E)** Levels of Src and AR expression were measured by immunoblotting. **F)** PC3-Puro (PP) or PC3-AR clones (AR1/AR2) were treated with scrambled siRNA (scr) or integrin α6-specific siRNA (siα6). Levels of Src activation and integrin α6 expression were measured by immunoblotting. **G)** Percentage of cell death as measured by trypan blue dye exclusion following adhesion to laminin. *(A) Laura E. Lamb and Jelani C. Zarif, (B-C) Jelani C. Zarif (E-F) Laura E. Lamb.*

tumor survival pathway previously reported (Lamb et al., 2011) since suppressing Src expression with siRNA in PC3-AR clones caused no increase in cell death (Fig. 7G). Thus AR, independently of its actions on integrin α6 expression and cell survival, stimulates Src activity.

*AR and Src are required for invasion*

To determine if AR-dependent stimulation of Src is responsible for the increase in invasiveness, cells were transfected with scram siRNA or siRNAs against AR or Src. Inhibition of AR or Src expression attenuated PC3-AR invasion (Fig. 8A). To assess whether AR stimulates Src activity and invasion via a non-nuclear mechanism, Src-$Y^{416}$ activation was measured in PC3 cells expressing AR with a mutated nuclear localization sequence (ARΔNLS), or AR with a mutated ligand binding domain (ARΔLBD) (Fig. 8B). Src activity was elevated in all AR-expressing mutants, indicating that neither nuclear localization nor ligand binding activity is required for AR stimulation of Src activity. Cells expressing the ARΔNLS mutant also displayed increased Matrigel invasiveness, which was suppressed when Src was inhibited with siRNA (Fig. 8C). However, in spite of elevated levels of Src activity in the ARΔLBD, mutants, their invasiveness was unaffected by Src inhibition (Fig. 8D), indicating ligand binding activity may be crucial for Src-dependent invasion. Inhibiting Src expression in LNCaP, C4-2, or VCaP cells with siRNA similarly decreased R1881-stimulated Matrigel invasion (Fig. 8E). These data demonstrate that AR stimulation of Src activity via a non-nuclear, but LBD-dependent mechanism is required for prostate tumor cell invasion.

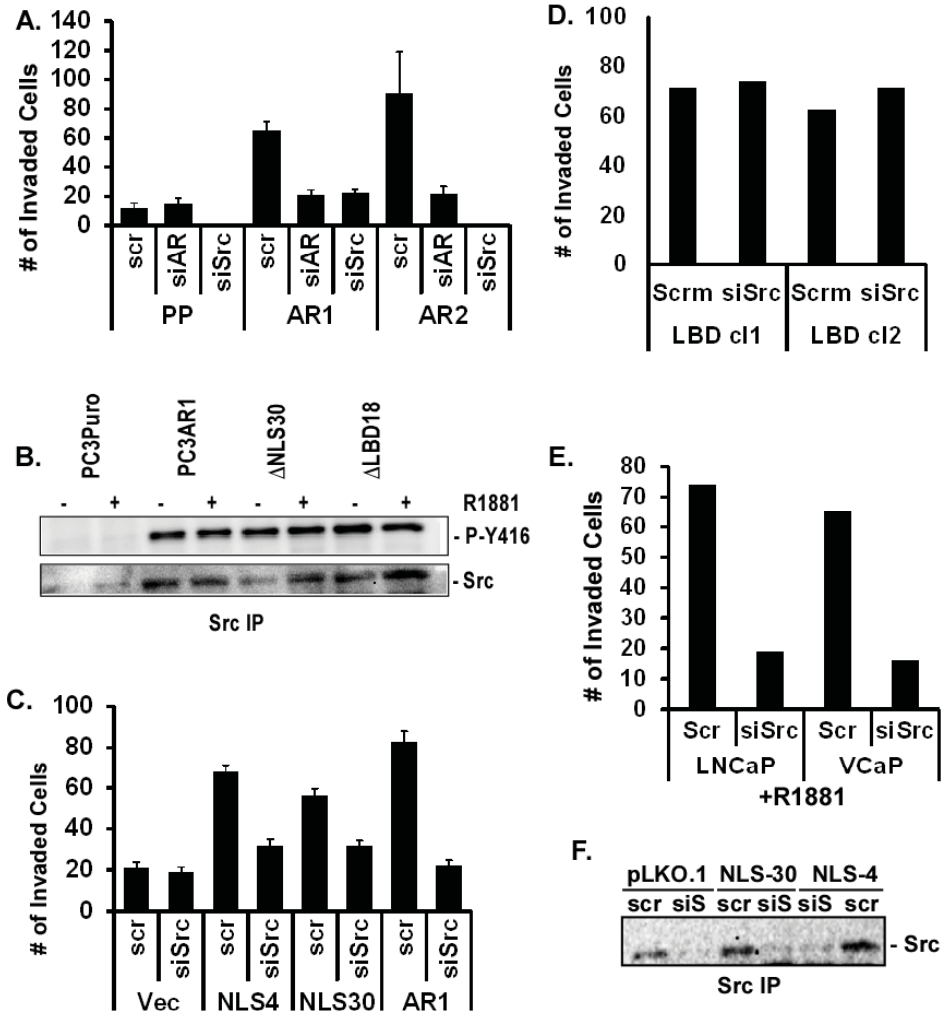


**FIGURE 8. AR and Src are required for invasion.** A) Invasion through Matrigel was measured in PC3-Puro (PP) or PC3-AR clones (AR1/AR2) transfected with scrambled siRNA (scr) or AR- or Src-specific siRNA (siAR, siSrc). B) Levels of Src activation and expression were measured in PC3 clonal cell lines expressing wild-type (AR1/AR2), nuclear localization deficient (NLS), or ligand binding deficient (LBD) AR mutants by immunoblotting of Src immunoprecipitates with anti-Y416 phospho-specific antibody or total anti-Src antibody respectively. C, F) Invasion through Matrigel was measured in PC3 (Vec), PC3-AR1, AR-NLS, or AR-LBD clones treated with scrambled siRNA (scr) or Src-specific siRNA (siSrc). E) Invasion through Matrigel was measured in R1881-stimulated LNCaP, C4-2, or VCaP cells following treatment with scrambled siRNA (scr) or Src-specific siRNA (siSrc).

*AR stimulates Matriptase activation and extracellular shedding in a non-nuclear fashion*

Matrigel invasion requires proteolytic degradation of laminin substrates; therefore, we measured the activity of several secreted proteases and found that Matriptase (Uhland, 2006) was specifically activated in PC3-AR cells compared to the parental control PC3-Puro cells (Fig. 9A). PC3-AR cells had constitutively elevated levels of activated intracellular and secreted Matriptase compared to PC3-Puro cells, which was not further stimulated by androgen. Suppression of AR using a stably expressed tetracycline-inducible shRNA decreased active Matriptase expression (Fig. 9E). To assess whether the activation of Matriptase by AR occurs in a non-nuclear fashion, we stimulated PC3 cells expressing the AR∆NLS and AR∆LBD mutants with R1881 and measured activate Matriptase levels. Only the AR∆NLS mutant displayed elevated levels of intracellular and secreted levels of active Matriptase (Fig. 9B). Thus, the AR-dependent increase in Matriptase activity requires ligand binding, but not nuclear activity. Complementary experiments using LNCaP and C42 lines indicate that AR induces active Matriptase extracellular shedding 24 hours after androgen stimulation (Fig. 9C). Active intracellular Matriptase can be detected within twenty minutes of ligand stimulation (Fig. 9E). The appearance of active Matriptase within 20 minutes of androgen stimulation was resistant to mRNA synthesis inhibition by Actinomycin D (Fig. 9F) indicating androgen stimulates Matriptase activation independent of transcription, further supporting the non-nuclear action of AR. Suppression of Src with siRNA decreased expression of Matriptase in PC3-AR cells (not shown) or in androgen-stimulated LNCaP or C4-2 cells. Together these data indicate that AR activates Matriptase through Src using a ligand-dependent, but non-nuclear mechanism.

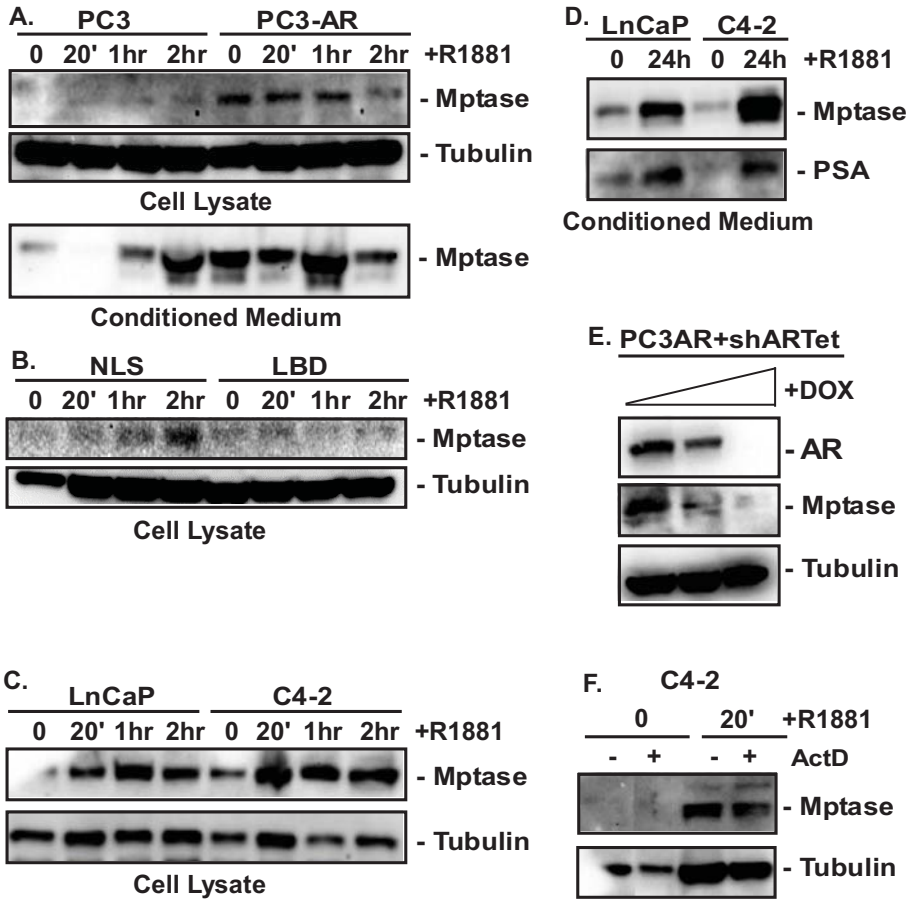


**FIGURE 9. AR stimulates Matriptase activation and extracellular shedding in a non-nuclear fashion.** A) PC3-Puro or PC3-AR clones were stimulated with 10nM R1881 for 0, 20, 60, 120 minutes (0, 20', 1hr, and 2hr). Levels of active Matriptase (Mptase) in cell lysates and secreted into the conditioned medium were measured by immunoblotting. B) PC3 cells expressing ARΔNLS or ARΔLBD were stimulated with 10nM R1881 for 0, 20, 60, 120 minutes (0, 20', 1hr, and 2hr). Levels of active Matriptase (Mptase) in cell lysates and secreted into was measured by immunoblotting. LNCaP and C4-2 cells were stimulated with 10nM R1881 for 0, 20, 60, 120 minutes (0, 20', 1hr, 2hr) C) or D) 24 hours. Levels of active Matriptase in cell lysates C) and secreted into the conditioned medium D) were measured by immunoblotting. PC3-AR cells stabling expressing a Tet-inducible AR shRNA (shARTet) were stimulated with 200 and 400ng/ml doxycycline for 16 hours and the levels of AR and active Matriptase in the cell lysate measured by immunoblotting E). C4-2 cells were stimulated with ethanol (0) or 10nM R1881 for 20 minutes (20') after 2 hours of pretreatment with vehicle DMSO (-) or 5μg/ml Actinomycin D (+). Levels of active Matriptase in the cell lysate were measured by immunoblotting F).

*CDCP1 activity is regulated by AR and Src*

Cub Domain Containing Protein 1 (CDCP1/p140/Trask) is a transmembrane glycoprotein that facilitates integrin-dependent migration and invasion and is associated with metastasis in several cancers (Wortmann et al., 2009). CDCP1 can be cleaved extracellularly by several serine proteases, including Matriptase (He et al., 2010). Full-length CDCP1 and the cleaved form are also Src substrates via the Y734 residue of CDCP1, and phosphorylation by Src intracellularly is required for promoting cellular de-adhesion from matrix and invasion (Casar et al., 2012). However, recently it has been reported that CDCP1 does not have to be cleaved in order to be phosphorylated (Spassov et al., 2011). AR expression in PC3 cells increased the appearance of tyrosine-phosphorylated and cleaved (70kD) CDCP1, which was blocked by AR or Src siRNA or by treatment with the Src inhibitor dasatinib (Fig. 10A). Treatment of C4-2 cells with R1881 resulted in increased CDCP1 cleavage and phosphorylation within 5 to 20 minutes of stimulation (Fig. 10C). Inhibition of Src with siRNA or dasatinib blocked androgen stimulation of CDCP1 cleavage and phosphorylation (data not shown). CDCP1 cleavage was also observed in PC3 cells expressing AR$\Delta$NLS and AR$\Delta$LBD (Fig. 10B) and elevated Src activity was observed in these cell lines (see Fig. 8B). Furthermore, knock-down of Src in these cells using Src specific siRNAs attenuated CDCP1 levels of expression (Fig. 10C). These data indicate that non-nuclear and ligand-independent AR stimulation of Src activity results in the phosphorylation of one of its substrates, CDCP1, involved in migration and invasion. Altogether our data demonstrate that AR, acting via a non-nuclear mechanism, is required for Src-dependent activation of CDCP1 and activation of Matriptase.

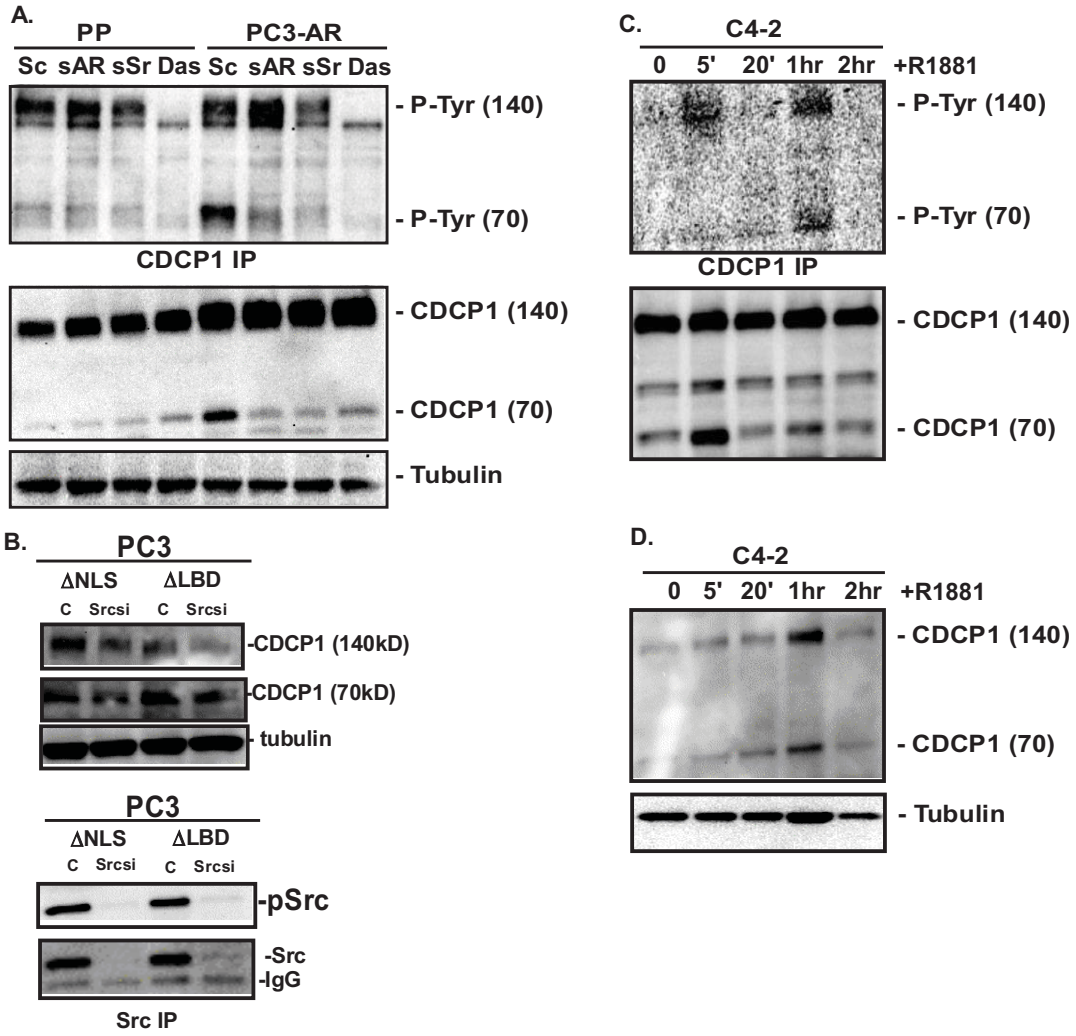


**FIGURE 10. CDCP1 activity is regulated by AR and Src. A)** PC3-Puro (PP) or PC3-AR cells were transfected with scrambled siRNA (Sc), AR- or Src-specific siRNA (sAR, sSr), or 10nM dasatinib for 16 hours. Levels of tyrosine phosphorylation on full length (140kD) or cleaved (70kD) CDCP1 from CDCP1 immunoprecipitates were measured by immunoblotting with anti-phosphotyrosine antibody. Total levels of CDPC1 were also measured by immunoblotting. **B)** PC3 cells expressing ARΔNLS or ARΔLBD were transfected with scram siRNA (c) or siRNAs against Src (Srcsi). Levels of CDCP1 were measured by immunoblotting. Src knockdown was validated by immunoprecipitation using anti-Src antibody. **C, D)** C4-2 cells were stimulated with 10nM R1881 for 0, 5, 20, 60 or 120 minutes (0, 5', 20', 1hr, and 2hr). **C)** Levels of tyrosine phosphorylation on full length (140kD) or cleaved (70kD) CDCP1 from CDCP1 immunoprecipitates were measured by immunoblotting with anti-phosphotyrosine antibody. **D)** Levels of total CDCP1 cleavage were measured by immunoblotting.

*Matriptase is sufficient for Matrigel Invasion*

To assess the importance of CDCP1 or Matriptase in promoting AR-dependent invasion, CDCP1 or Matriptase expression was suppressed by siRNA in PC3-AR1 cells and the effect on invasion assessed. The loss of Matriptase by matriptase specific-siRNA (Fig 11A), but not CDCP1 (Fig 11B), dramatically inhibited Matrigel invasion of PC3-AR cells. To test the sufficiency of extracellular Matriptase in promoting invasion, conditioned medium from PC3-AR cells was then tested by isolation of conditioned media from PC3-AR cells. Matriptase secreted from PC3-AR cells occurred independent of androgen was pulled down by immunoprecipitation using an anti-matriptase antibody in conditioned medium. Rabbit IgG was used as a positive control. Supernatant from the control and the matriptase antibody pull down was added to the Matrigel invasion chambers containing C4-2 cells. The invasive ability of C42 was decreased in chambers containing conditioned medium in which matriptase had been reduced when compared to the control (Fig 11C). The ability of Src to stimulate the AR-dependent activation and cleavage of Matriptase was determined by inhibiting Src activation in PC3-AR cells with the pharmacological inhibitor, Dasatinib. Inhibition of Src activity suppressed levels of active matriptase but did not prevent its shedding outside of the cell entirely (Fig 11D). These findings suggest that matriptase is sufficient for invasion of C42 cells and that blocking the kinase activity Src does not completely inhibit the shedding of matriptase.

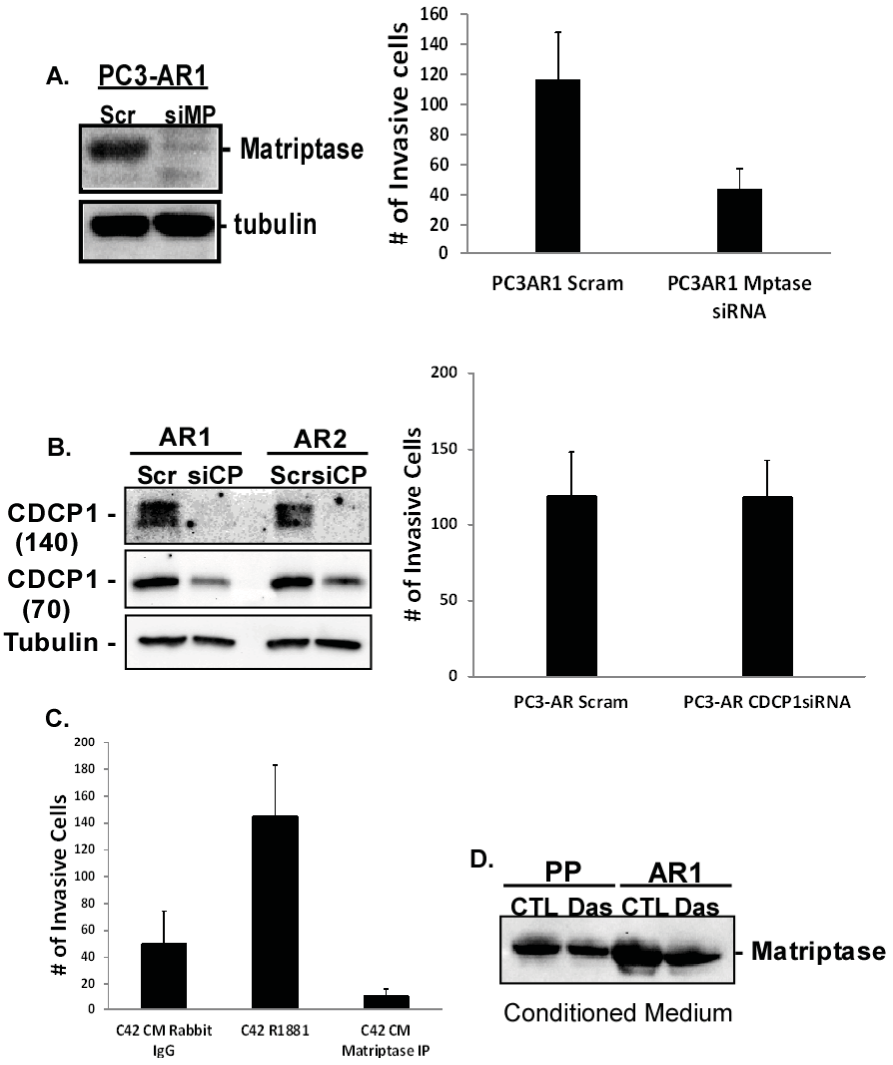


**FIGURE 11: Matriptase is sufficient for Matrigel Invasion. A)** PC3-AR cells were transfected with scrambled siRNA (Scr), or Matriptase-specific siRNA (siMP). Levels of active matriptase (Mptase) were measured by immunoblotting with anti-matriptase antibody. Invasion through Matrigel was measured in PC3-AR1 cells transfected with scrambled siRNA (scram) or Matriptase-specific siRNA (Mptase siRNA). **B)** PC3-AR clones (AR1 and AR2) cells were transfected with scram siRNA (Scr) or siRNAs against CDCP1 (siCP). Levels of full length and cleaved CDCP1 (140kD and 70kD) were measured by immunoblotting to assess knockdown. Invasive potential of PC3-AR1 cells that were transfected with scrambled siRNA (Scram) and CDCP1-specific siRNA (CDCP1siRNA) was measured using Matrigel invasion chambers. **C)** Supernatant of Conditioned Media (CM) from PC3-AR cells was added to the invasion chamber of C42 cells. Significance was determined using the two-tailed student T-test for unequal variance **D)** PC3-Puro (PP) and PC3-AR1 (AR1) cells were treated with EtOH (CTL) and 10nM Dasatinib (Das) for 24 hours. The levels of active Matriptase expression in conditioned medium were measured by immunoblotting.

Discussion

The limited number of cell lines available in which AR remains expressed after placement in culture has hampered progress in understanding hormone-refractory metastatic disease. For instance, the prostate cancer cell line PC3, isolated from a bone metastasis is non-responsive to circulating androgens because these cells unfortunately do not express detectable levels AR, which hinders the ability to study AR in metastatic bone disease. Our laboratory has developed a new androgen-independent model using PC3 cells to address the role of AR in castration resistant prostate cancer. To create a model that reflects what is seen in patients and to fully study the role of AR in metastatic disease, we have re-expressed AR in PC3 cells, thus creating PC3-AR cells. In the PC3-AR model, AR is both nuclear and cytosolic, and as we demonstrated constitutively active (Lamb et al., 2011), reflecting the characteristics of castration-resistant tumors. Initial studies in these PC3-AR cells demonstrated that re-expression of AR promoted laminin-dependent survival, independent of PI3-K. However, in these same studies we observed an AR-dependent increase in cell migration, Matrigel invasion, and MMP2/9 activity and increased Src activation. The increase in Src activity, migration, and matrigel invasion were independent the AR/α6 integrin mediated survival pathway; having no effect on tumor cell viability. Altogether, these results suggested that AR enhances invasiveness through up regulation of Src *in vitro*. The next step in the course of this study was to determine the mechanism by which AR increases Src activity and invasive capability. First, to assess if AR could regulate Src in a non-nuclear fashion, we made use of the PC3AR∆NLS cells which exhibit AR non-nuclear signaling. Our data from PC3AR∆NLS cells indicate that the

non-nuclear signaling properties of AR were sufficient to increase Src activation but not overall expression levels relative to the PC3 parental cell line. Androgen stimulation had no effect on Src activity in these cells. In prostate cancer cell lines that respond to androgen, our approach to assess non-nuclear regulation of Src was a little different. We relied heavily on the speed in which R1881 was able to bind to AR. In this case, R1881 time-course experiments were carried out beginning with AR stimulation of serum deprived sub-confluent cells seeded on laminin as early as 10s to two hours. We were able to observe an increase in Src activation as early as 20 minutes with no change in AR levels of expression or PSA expression levels.

Increased invasive and migratory properties were observed in both PC3-AR cell lines relative to PC3-Puro cells, and when AR was antagonized, Matrigel invasion was attenuated. Also, when the PC3-AR cells were transfected with Src siRNA then seeded into Matrigel invasion chambers, the ability of the cells to migrate and invade was decreased. We then assessed if cells that exhibited AR non-nuclear signaling, i.e. the PC3AR$\Delta$NLS cells, were invasive or, as invasive as the PC3-AR cells given that these cells also had increased Src activation. When we seeded these cells into Matrigel invasion chambers, these cells were as invasive as PC3-AR and suppression of Src by siRNA in both cell types, suppressed their ability to invade Matrigel. Thus the non-nuclear regulation of Src by AR is crucial for invasion.

The role of AR in promoting invasion through Src activation led us to investigate potential downstream Src effector proteins and proteases. Firstly, CDCP1 (also known as gp140/Trask) was one of these effectors that had been previously reported to promote cellular de-adhesion and tumor escape (Casar et al., 2012; Deryugina et al.,

2009; Perry et al., 2007). In our studies, we report that the expression, cleavage and activation of CDCP1 are substantially higher in the PC3-AR cells than the PC3-Puro. In androgen sensitive cells C42 and LNCaP, CDCP1 expression and cleavage was increased within 20 minutes of androgen stimulation (Fig. 10C-D), similar to what we observed with the activation of Src (Fig. 7C), suggesting a non-genomic mechanism for CDCP1 activation like is observed for Src. Furthermore, blocking Src activation blocked CDCP1 phosphorylation and cleavage. However, despite CDCP1 activation and cleavage by AR and Src, loss of CDCP1 by siRNA had no impact on AR-dependent invasion, indicating CDCP1 is not required for invasion. While elevated CDCP1 expression has been correlated with local prostate cancer invasion, its expression is actually lost in metastatic prostate cancer lesions suggestive that its loss further potentiates metastasis (Dr. Beatrice Knudsen personal communication). This has been reported in other human cancers previously cancers (Spassov et al., 2012). Thus, other targets of Src must be involved in controlling metastatic tumor cell invasiveness.

Another effector we discovered that was up-regulated was the serine protease Matriptase. Matriptase had been recently reported to cleave laminin, which is lost during prostate cancer progression and is expressed in aggressive prostate cancer tissues (Saleem et al., 2006; Tripathi et al., 2011). We observed that in PC3-AR cells, which were more invasive, Matriptase expression was higher and it was shed into the media in the absence of androgen. Conversely, LNCaP and C42 cells exhibited higher intracellular levels of Matriptase expression only upon androgen stimulation, which was also subsequently shed into the medium (Fig. 9D). These cells were also more invasive when stimulated with androgen in the matrigel invasion assay. Unlike CDCP1, loss of

Matriptase dramatically inhibited PC3-AR invasion. Furthermore, conditioned medium containing shed Matriptase was sufficient to enhance Matrigel invasion of C4-2 cells in the absence of androgen. Finally, the ability of AR to induce Matriptase activation and shedding was dependent on Src. Thus, AR-induced Matriptase activation, via Src, is required and sufficient for inducing prostate cancer cell invasion of Matrigel.

Prostate cancer is a very serious disease that is initially curable using androgen ablation therapy. However, patients ultimately develop resistance to androgen ablation and develop hormone-refractory disease, a malady that is untreatable and practically unstoppable-thus, claiming the lives of over 28,000 men per year in the US. This has piqued the interests of many scientists to find new methods that are inexpensive, lack adverse side-effects and increase the survival rate of patients with the metastatic disease. Conversely, the lack of available prostate cancer models that are representative of the metastatic disease has been an encumbrance to researchers in the past and present. Our report describes a newly developed prostate cancer model that possesses hormone-refractory enhanced migratory and invasive behavior.

These cellular behaviors are dependent on both androgen receptor (AR) and the regulation of Src activation and its downstream effector Matriptase. These tumor cell behaviors also correlate very closely to what is observed in patients, thus providing a model that is clinically relevant. To date, very few groups have outlined the mechanisms by which AR can act in a non-nuclear signaling fashion to promote hormone-refractory enhanced invasive behaviors that is seen in metastatic prostate cancer. In this report, we show a closer and unique analysis of androgen/AR and its impacts in a non-nuclear fashion on prostate tumor cell behavior. In cells that are

androgen sensitive, treatment with androgen increases Src activation, invasion, matriptase shedding and activation, and CDCP1 cleavage and activation while having no effects on AR levels of expression. Importantly, our report highlights an alternative signaling cascade that allows AR to elude antagonism from pharmacological inhibitors such as MDV3100. This elusion can lead to the spread of castration resistant prostate cancer which leads metastasis and subsequent death. Altogether, the essence of these studies will also provide hopeful drug targets and approaches for treating prostate cancer patients.

## Materials and Methods

### *Cell Culture*

The prostate tumor cell lines, PC3, LNCaP, and VCaP were purchased from American Type Culture Collection. C4-2 cells were obtained from Dr. Robert Sikes, University of Delaware (cite paper). PC3 cells were grown in F-12K media supplemented with 10% charcoal-stripped and dextran-treated fetal bovine serum (CSS), 2mM glutamine, 50 U penicillin, and 50 µg/ml streptomycin. LNCaP, VCaP and C4-2 cells were grown in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum, 2mM glutamine, 50 U penicillin, 50 µg/ml streptomycin, 0.225% glucose, 10 mM HEPES, and 1mM sodium pyruvate. For experiments, LNCaP, VCaP, and C4-2 cells were seeded on laminin (Millipore) and grown in phenol-red free media and 0.1% CSS for 24 hrs beforehand and throughout the experiment. All cells were grown at 37˚C in 5% $CO_2$.

*DNA Constructs*

PC3-Puro, PC3-AR cells were generated by infecting cells with pBabe-puro, pBabe-puro-hAR retroviruses respectively. Clones were selected and maintained in 2μg/mL puromycin. The PC3-pLKO.1, PC3-ΔNLS, and PC3-ΔLBD cell lines were generated by infecting cells with pLKO.1, pCSCG-AR- ΔNLS, or pCSCG-AR-N705S lentiviruses as described previously (Lamb et al., 2011). PC3-AR tet-ON shRNA clones were generated by using pLKO-Tet-ON vector (Addgene) that contained a single AR shRNA cloned upstream of the H1/TO promoter as described (Wee et al., 2008; Wiederschain et al., 2009). AR shRNA sequences were sequence validated and transformed into Stbl3 competent cells. Stbl3 colonies that were positive were isolated and DNA from these colonies was packaged into lentiviruses using 293FT cells at a density of $4X10^6$. PC3-AR cells were then infected with tet-ON ARshRNA lentiviruses and individual clones were selected using 3 μg/ml of puromycin. Knockdown was validated using immunoblotting after doxycycline treatment.

*siRNA Transfections*

A pool of four small interfering RNAs (siRNA) against androgen receptor (siGENOME SMARTpool; 20 nM), integrin α6 (ON-TARGETplus SMARTpool; 20 nM); integrin α3 (ON-TATRGETplus SMARTpool; 5nM)); Src (ON-TARGETplus SMARTpool; 20 nM); CDCP1 (ON-TATRGETplus SMARTpool; 5nM), Matriptase (Santa Cruz Biotechnology, Inc.; 3.3 nM) or a non-targeting sequence were purchased from Dharmacon. Serum-deprived sub-confluent cells were transfected with siRNA using siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) media following manufacturer's directions. The

media was changed 16 hours after siRNA transfection. All pools were titrated to determine the lowest optimal concentration for inhibition of protein expression by immunoblotting 72 hours after transfection.

*Drug Treatments*

Mifepristone (RU-486) was purchased from Tocris Bioscience (Ellisville, MO). Dasatinib was a gift from the lab of Dr. Matthew Steensma. Bicalutamide (Casodex) was purchased from Enzo Life Science (Farmingdale, NY). Each drug was diluted into ethanol and used at a final concentration of 10 nM. Metribolone (R1881) was purchased from PerkinElmer (Boston, MA). R1881 was diluted into ethanol and then into phenol red free media and used at a final concentration of 10nM in all experiments. Actinomycin D was purchased from Calbiochem and reconstituted in DMSO at a concentration of 10 µg/mL.

*Immunoblotting*

Total whole cell lysates were prepared for immunoblotting as previously described (Edick et al., 2007; Miranti, 2002). Briefly, cells were lysed on ice with MAPK lysis buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 50 mM NaF, 100 mM NaCl, 50 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1% Triton-X100, 1 mM $Na_3VO_4$, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 10 µg/ml aprotinin, 1 mM benzamide) or RIPA (10 mM Tris pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton-X100, 1 mM $Na_3VO_4$, 1 mM PMSF, 100U ml aprotinin, 10 µg/ml pepstatin and 10 µg/ml leupeptin) buffers and 40-65 µg of total cell lysates in 2X SDS sample buffer were

boiled for 5 minutes. Samples were run on SDS polyacrylamide gels following standard SDS-PAGE protocols and transferred to PVDF membrane. Membranes were blocked in 5% BSA in TBST for two hours at room temperature, and then were probed with primary antibody for two hours at room temperature. Membranes were washed three times, and incubated with horseradish peroxide-conjugated secondary antibodies (Bio-Rad) in 5% BSA in TBST for 1 hour at room temperature. After washing an additional three times, signals were visualized by chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad). Primary antibodies and dilutions are as follows: anti-AR monoclonal antibody (Santa Cruz Biotechnology, Inc.; diluted 1:200); anti-Bcl-xL polyclonal antibody (Cell Signaling Technologies, Inc.; diluted 1:1,000); anti-matriptase monoclonal antibody (Bethyl Laboratories, Inc.; diluted 1:2,000); anti-phospho-[$Y^{416}$]-Src monoclonal antibody (Invitrogen; diluted 1:1,000); anti-Src antibody (diluted 1:1,000); anti-CDCP1 (Cell Signaling Technologies, Inc.; diluted 1:1,000); anti-tubulin monoclonal (Sigma-Aldrich; diluted 1:10,000)

*Immunoprecipitation*

For detection of phosphorylated CDCP1 and phospho-[$Y^{416}$]-Src, cell lysates were prepared as described above. Protein samples (500 µg), in a total volume of 500 µL of 1X MAPK buffer, were incubated with 1µg of mouse monoclonal anti-Src antibody (Lipsich et al., 1983) or anti-CDCP1 (Cell Signaling Technologies, Inc.) overnight at 4°C. Protein-antibody complex was precipitated with protein G or protein A agarose beads (Thermo Scientific) respectively and subsequently followed by immunoblotting with

rabbit anti-phospho-[$Y^{416}$]-Src antibody (diluted 1:1000; Invitrogen) or mouse 4G10 anti-phoshotyrosine antibody (Millipore; diluted 1:2000) as described above.

*Immunofluorescence*

Cells were fixed with 4% paraformaldehyde in PBS at 4˚C for twenty minutes and permeabilized for ten minutes with TBS (10 mM Tris, pH 8.0, 150 mM NaCl) + 0.5% TritonX-100 at room temperature. Cells were then blocked with 2% BSA in TBS + 0.1% TritonX-100 for twenty minutes at room temperature before incubation with vinculin antibody (Sigma) and Alexafluor 546-Phalloidin (Molecular Probes, Invitrogen) for one hour. Cells were incubated with goat anti-mouse Alexa Flour-488 secondary antibody for one hour at room temperature. DNA was visualized by staining with Hoechst 33258 for 10 minutes at room temperature. Cells were washed four times with TBS + 0.1% TritonX-100 over ten minutes between all steps. Epifluorescent images were acquired on a Nikon Eclipse TE300 fluorescence microscope using OpenLab v5.5.0 image analysis software (Improvision).

*Migration Assays*

Cell migration was measured using the modified Boyden chamber assay. Cells ($5 \times 10^4$) were suspended in the upper well of the 8.0 μm pore size polyethylene terephthalate membrane culture inserts for 12 well plates (BD Biosciences) in 400μL phenol-red free DMEM containing no additives. The lower chamber was filled with 400μL phenol-red free DMEM and used laminin 1 (LM1) gradient as a chemoattractant. After six hours of incubation, the culture inserts were removed and washed with 1X PBS. Cells that had

migrated to the lower membrane surface were stained with chemicon crystal violet stain (Millipore) and counted under a microscope at 100x magnification in five random fields per insert in triplicate.

*Matrigel Invasion Assays*

Serum-starved sub-confluent cells ($6.25 \times 10^4$) were suspended in the upper well of the 8.0 µm pore size matrigel membrane culture inserts (BD Biosciences) coated with 1 µM laminin. The upper chamber was filled with 400 µL phenol-red free DMEM containing no additives and the lower chamber was filled with 400 µL phenol-red free DMEM no additives. After 72 hour incubation, the culture inserts were removed and washed once with 1X PBS. Cells that invaded through Matrigel to the lower membrane surface were stained with chemicon crystal violet stain (Millipore) and counted under a microscope at 100x magnification in five random fields per insert in triplicate. Significance was determined using the two-tailed student T-test for unequal variance where noted.

*Isolation of Conditioned Medium*

To examine matriptase within the media, cells were seeded and grown on plates in the presence of laminin matrix or in the case of PC3-Puro and PC3-AR cells, the absence of laminin until 75-80% confluence. Cells were then were then subject to serum starvation using DMEM for 24 hrs. Between ten to fifteen mL of media was collected on ice from the plates and loaded into Amicon Ultra-15 centrifugal filter units (Millipore Corp. Billerica, MA). The Amicon filter tubes were centrifuged at 4000xg for 30 minutes at 4°C. Concentrated media was placed on ice, collected from the Amicon filter

tubes vials and 15-25 µL of concentrated media was combined with 30uL of loading buffer (Bio-Rad) and heated for ten minutes. Lysates were then loaded onto SDS PAGE and subject to electrophoresis as described above. Gel was run for about 2 hours at constant 125 volts. Ponceau S stain (Sigma-Aldrich) was made up in 0.1 NaOH and immunoblots were stained after transfer was complete to assess loading.

*Zymography*

Between ten to fifteen mL of media was concentrated using Amicon Ultra-15 (Millipore Corp.) as described above. Conditioned media was then collected and 15µL of conditioned was mixed with 15µL of zymogram sample buffer (Bio Rad Laboratories, Inc.). Samples were then electrophoresed on gelatin based gels (Invitrogen) at 125 volts for 2 hours. Gel was then washed for 1.5 hours using 1X Renaturation buffer (Triton X-100, 25% (v/v) in water) at room temperature while changing buffer every half hour. Gel was then washed in 50mL 1X Development buffer (50mM Tris base, 0.2M NaCl, 5mM $CaCl_2$ and 0.02% Brij 35) for 2 hours while changing buffer every half hour. Gel was then stained with 40mL of Coomassie blue stain for half hour and destained using destaining buffer (10% Methanol, 10% glacial acetic acid) for 12-16 hours. Gel was then photographed using CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

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# CHAPTER FOUR:
# CONCLUSIONS AND FUTURE PERSPECTIVES

Summary

In conclusion, we have generated a model for understanding the nuclear role of AR/integrin α6β1 in regulating cell survival independent of PI3-K signaling when cells are adherent to LM1 and an AR non-nuclear role in the regulation of Src in prostate cancer cell invasion. In this model, wild-type or mutated AR was stably re-expressed in the metastatic prostate tumor cell line, PC3. Similar to what is observed clinically in prostate cancer, there was an increase in integrin α6β1 with a concomitant decrease in in the other integrins such as β4 integrin. We also observed an increase in secretion of both MMP2 and MMP9 which have been implicated clinically in prostate cancer invasion and angiogenesis (Stearns et al., 1997; Stearns et al., 1999). Since previous reports that have re-expressed AR in prostate tumor cell lines reported reduced proliferation or cell survival due to activated AR (Bonaccorsi et al., 2000; Evangelou et al., 2002; Heisler et al., 1997), mainly four extra precautions were taken to keep AR minimally active in our cells. The first precaution that was taken was to sequence verify that the AR cDNA was human and wild-type, and not mutated or an activated splice variant. Second, AR was not highly over-expressed but maintained at the expression levels similar to LNCaP cells. Third, the only cells that were used were of low passage. This was important as cell phenotypes can drift in culture and during passaging. Fourth, all cells were selected using puromycin, isolated, validated, and constantly maintained in CSS and phenol red–reduced media to prevent AR hyper-activation. Immunostaining demonstrated that even under these conditions a large portion of AR is both nuclear and cytoplasmic in the absence of androgen (Lamb et al., 2011). Thus, AR is constitutively active, as further evidenced by constitutive expression of PSA. It is

plausible that the constitutively active nature of AR in our cells is a reflection of the known intratumoral androgen synthesis activity that is present in PC3 cells and is clinically important in CRPC (Dillard et al., 2008; Locke et al., 2008; Stanbrough et al., 2006; Yamaoka et al., 2010). This could potentially also explain why addition of exogenous androgen to PC3-AR cells does not enhance AR functionality to contribute to its nuclear regulation of integrin α6 or its non-nuclear signaling to increase Src activation, active Matriptase, or to change CDCP1 levels of expression or activation. Conversely, LNCaP and C42 cell lines, which respond to exogenous androgen, have an increased level of integrin α6, Src activation, CDCP1 activation/expression, and active matriptase levels upon androgen addition. Furthermore, continual addition of exogenous androgens in this system, such as propagation of cells in non-stripped serum, could over-activate AR in such a way that it acts in a suppressive manner giving cells a growth disadvantage which is why it might lead to suppressed growth, invasion, and reduced survival as seen in other studies (Tararova et al., 2007).

Regarding the nuclear signaling of AR in our model, we have successfully identified a new AR-dependent pathway acting through α6β1 that stimulates survival of LM-adherent prostate cancer cells independently of PI3-K signaling. This nuclear signaling pathway (AR/α6β1) stimulates the activity of Bcl-xL, whose up-regulation is highly associated with advanced CRPC. Application of this new knowledge may lead to the development of better prostate cancer therapies and supports the importance of targeting more than one pathway to effectively treat the lethal stage of this malady.

With regard to the non-nuclear function of AR, we have identified that constitutively active AR or mutated AR (ΔNLS) and (ΔLBD) in PC3 cells leads to an

increase in Src activation independent of androgen. In cells that are sensitive to androgen, stimulation of AR with R1881 in these cells increases Src activation in a matter of minutes and increases their likelihood of being more invasive through Matrigel. These findings were very surprising to us. Suppression of AR or Src using specific siRNAs or pharmacological inhibitors attenuated Matrigel invasion in cell lines expressing wild-type AR or the AR∆NLS mutant. Because neither AR nor Src have been reported to possess the ability to proteolytically cleave any matrix ligands, we investigated several proteases that may be AR regulated. We investigated Matriptase due to previous reports on its ability to cleave LM, specifically the β3 chain of laminin 332 which is found in the prostate epithelia and its loss in expression in human prostate corresponding with tumor grade (Saleem et al., 2006; Tripathi et al., 2011). Matriptase is similar to Hepsin, another serine protease that cleaves its substrates after arginine/lysine residues (Hooper et al., 2001; Lin et al., 1999), and is implicated in the invasive and metastatic potential of PC3 cells (Cheng et al., 2013; Tsui et al., 2008), is shed extracellularly (Lin et al., 2008), and has been shown to be regulated by AR (Kiyomiya et al., 2006). We found that AR regulated the shedding of Matriptase in a matter of minutes which correlated with the time in which AR regulated Src activation. Unlike the report from Kiyomiya *et al.*, we found that blocking transcription and then stimulating androgen sensitive cells with androgen still led to an increase in active Matriptase. We also found that AR stimulation led to the extracellular shedding of Matriptase in a matter of minutes and was independent of transcription. To further explore the non-nuclear regulation of Matriptase, we demonstrated that our PC3 cells that express AR with a mutated NLS (∆NLS), but not the ∆LBD mutant had an increase

in active Matriptase levels of expression upon androgen stimulation. Further investigation from several studies highlighted that Matriptase is important in cancer progression and can cleave the extracellular CUB domains a known glycoprotein, gp140/Trask/CDCP1 that has been implicated in tumor escape and metastasis (Bugge et al., 2007; Deryugina et al., 2009; Fukuchi et al., 2010). We observe that the stable expression of full length wild-type human AR or AR that has been mutated at its NLS (ΔNLS) or LBD (ΔLBD) exhibit elevated expression of CDCP1. Knocking down AR using AR specific siRNAs decreased CDCP1 levels of expression and inhibition of Src activity with Dasatinib led to a decrease in CDCP1 activation and cleavage. Stimulation of androgen-sensitive cells with androgen increased CDCP1 cleavage and activation. However, suppression of CDCP1 had no effect on AR expression, nor did it decrease Matrigel invasion. On the other hand, inhibition of Matriptase blocked the ability of AR to stimulate invasion.

Future Perspectives

In addition to investigating AR and integrin survival pathways and AR non-nuclear signaling in additional cell lines, I established cell lines using lentiviral constructs that stably express AR tet-inducible shRNAs specifically directed against either AR or integrin α6. I have tested and validated knock down after doxycycline treatment of either AR or integrin α6 treatment *in vitro*. I have observed that suppression of either AR or integrin α6 decreases Bcl-xL levels of expression as we observed previously (Lamb et al., 2011). We can now use these tet-inducible cell lines *in vivo* to validate whether or not if these pathways exist and are important for tumor survival and metastasis *in vivo*. We know that PC3 cells form metastatic lesions in the lung and

lymph nodes of mice when orthotopically implanted. To determine if AR and PI3-K signaling cooperate to promote survival *in vivo*, PC3-AR cells infected with the shRNA constructs will be orthotopically injected into nude mice. After primary tumor formation, the mice will be treated with a specific class I PI3-K inhibitor, PX-866 (Howes et al., 2007), tetracycline to drive AR or α6 specific shRNA expression, or in combination and we will monitor tumor size. In addition, the mice can be castrated to determine androgen dependence, or PC3 cells expressing AR mutants can also be tested. The tumors can also be analyzed for expression of AR, integrin α6, and Bcl-xL as well as Matriptase, Src, and CDCP1 by either immunohistochemistry or immunoblotting. Complementary experiments to validate the importance of non-nuclear AR signaling can be undertaken by using PC3 cells that stably express AR mutated at its NLS (ΔNLS). Each of these studies will help elucidate the importance of both the nuclear and non-nuclear signaling mechanisms of AR and will aid in the development of new drug targets that may be useful for not only tumor cell survival but also potentially for *in situ* invasion and subsequent metastasis of the disease.

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