ANALYSIS OF ENDOTHELIN DURING ANDROGEN DEPRIVATION: IMPLICATIONS FOR PROSTATE CANCER PROGRESSION

by

Jason M. D'Antonio

BA, University of Colorado at Boulder, 1997

Submitted to the Graduate Faculty of

The School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2007

UNIVERSITY OF PITTSBURGH

THE SCHOOL OF MEDICINE

This dissertation was presented

by

Jason M. D'Antonio

It was defended on

August 21, 2007

and approved by

Billy Day, Ph.D.
Dissertation Committee Chair
Professor, Department of Pharmaceutical Sciences

Luyuan Li, Ph.D.
Associate Professor, Department of Pathology

Alan Wells, M.D., D.M.S.
Professor, Departmental Pathology

Michael J. Becich, M.D., Ph.D.
Professor, Department of Pathology

Beth R. Pflug, Ph.D.
Dissertation Advisor
Assistant Professor, Department of Pathology

ANALYSIS OF ENDOTHELIN DURING ANDROGEN DEPRIVATION:

IMPLICATIONS FOR PROSTATE CANCER PROGRESSION

Jason M. D'Antonio, PhD

University of Pittsburgh, 2007

Background. Androgen deprivation has been in use for the treatment of advanced prostate cancer since 1941; however, most patients develop resistance to treatment leading to incurable, androgen-independent disease. Previous reports have correlated endothelin A receptor (ET_A) expression with increasing prostate cancer grade and stage, and have shown that endothelin-1 (ET-1) treatment of ET_A-expressing prostate cancer cells inhibits apoptosis. ET_A blockade has emerged as a potential strategy in the treatment of advanced prostate cancer. Here, the potential role of endothelin signaling in promoting prostate cancer cell survival during androgen ablation therapy is evaluated in efforts to establish the potential value of ET_A blockade in improving hormone therapy.

Methodology and Principle Findings. Androgen-dependent human prostate cancer cells were androgen deprived and evaluated for expression changes in ET-1, ET_A, ET_B, and AR. Ligand binding, real time quantitative PCR, and immunohistochemical studies show that androgen deprivation increased ET-1, ET_A, ET_B, and AR expression in prostate cancer cell lines, and ET_A expression in human prostate tissue. Using the specific AR inhibitor bicalutamide, acute androgen receptor blockade increased prostate cancer cell ET-1 secretion. Following androgen deprivation, LNCaP cells acquired androgen independence (LNCaP-AI), but retained sensitivity

to androgens. ET-1 treatment of ET_A over-expressing prostate cancer cells induced a more rapid and sustained activation of Akt, and ET_A blockade significantly reduced Akt activation. *In vivo* ET_A blockade, in combination with castration, significantly reduced LNCaP xenograft cell growth, compared to either treatment alone. Affymetrix GeneChip HG-U133 Plus 2 expression array analysis of androgen deprived prostate cancer cells discovered dramatic changes in gene expression patterns throughout the transition to androgen independence. Lastly, the role of ET_B signaling in prostate cancer cell apoptosis was examined but remains to be further elucidated.

Conclusions and Significance. During androgen deprivation, prostate cancer cells up-regulate ET-1 and ET_A expression. Upon engagement of ET-1, ET_A invokes activation of the survival factor Akt. *In vivo*, ET_A blockade plus castration inhibits prostate cancer growth. Collectively, these results implicate endothelin survival signaling in promoting progression to androgen-independent disease, and lend support to the targeted disruption of endothelin survival signaling in treating advanced, metastatic prostate cancer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

In recognition of their scientific and intellectual contributions to the development and synthesis of this research, special thanks go out to: Dr. Talal El-Hefnawy for his expertise in real time quantitative PCR; Dr. Drazen M. Jukic for his expertise in analyzing the histopathology, grading of the human prostate immunohistochemistry and providing the digital images of the ET_A and ET_B stained human prostate tissue sections; Dr. Anil Parwani for his expertise in grading the LNCaP xenograft immunohistochemistry; Marie Acquafondata for her expert technical assistance in performing much of the immunohistochemistry; Dr. Moira Hitchens for critical reading of manuscripts; Dr. Robert R. Bies for his expertise in mathematical modeling of the *in vivo* mouse model data; Mike Meyers for his assistance in performing Flow cytometry; and Dr. Yun Wang for statistical analysis of the xenograft immunohistochemical scoring.

Sincere thanks to my dissertation advisor, Dr. Beth R. Pflug, for years of scientific guidance and thoughtful patience in sculpting my scientific development, as well as to my thesis committee for their assistance in directing my scientific endeavors.

I dedicate this work to my parents, Jim and Joane, for their lifelong support, encouragement, and the opportunities they have afforded me – they have opened my eyes to the world. Also, I want to thank my loving wife Katherine Elizabeth for being my most passionate supporter, for believing in me, and for guiding me throughout this entire experience.

A quote that has been with me for 10 years; let it ring in my ears for all my years to come:

Who knows only his own generation remains always a child

- Dr. George Norlin (1871 – 1942)

1.0   INTRODUCTION

1.1   PROSTATE DEVELOPMENT

Normal prostatic development is regulated by the interactions of various hormonal, cellular and molecular mechanisms. Early in development, the prostate arises from the ambisexual endodermal urogenital sinus (UGS), which is surrounded by the androgen receptor-positive (AR+) urogenital sinus mesenchyme (UGM). In the presence of fetal testicular androgens, prostatic epithelial buds grow into the UGM, forming the AR-negative (AR-) urogenital sinus epithelium (UGE) (1). Pivotal tissue recombination studies generating chimeric prostates of UGM and UGE from wild-type and AR- testicular feminized mice established that early prostatic development is indeed controlled by mesenchymal-epithelial interactions (2). Given that the UGE lacks AR expression early on, androgenic stimulation induces the UGM to dictate prostate epithelia determination, epithelial bud formation and branching, and to promote cell differentiation into secretory epithelium (3,4). Additional recombination studies revealed that UGE AR expression is eventually required for the production of AR-dependent secretory proteins (5,6). Following AR expression in the prostatic epithelia, androgenic stimulation of the UGE influences the UGM to undergo smooth muscle differentiation (7). In the prostate there exists a fine balance between luminal epithelial cell proliferation and apoptosis, which is controlled by mesenchymal-epithelial paracrine signaling (8); a loss in the coordination of these

hormonal, cellular and molecular interactions tips the scale in favor of proliferation, resulting in the development of prostate adenocarcinoma.

1.2   PROSTATE CANCER INCIDENCE AND RISK FACTORS

By the end of 2007, there will be an estimated 218,890 new cases of prostate cancer, making it the most frequently diagnosed cancer in American men. With an anticipated 27,050 annual disease related deaths, prostate cancer remains the second leading cause of cancer related deaths in American men. Although the age-adjusted death rate for prostate cancer has been decreasing since the early 1990's, incidence rates dramatically decreased from 1992-1995 but have moderately increased since 1995[1]. This varying trend is, in large part, due to the advent of improved prostate cancer screening via detection for prostate specific antigen (PSA) in the blood.

Unlike other types of cancers, the risk factors for prostate cancer, which include age, ethnicity, family history, environment, and diet, are not as well defined. Because prostate cancer develops later in life and typically exhibits a more slowly progressing phenotype, age is the primary risk factor for developing the disease with the other factors contributing to penetrance and other aspects of disease development and progression. Recently, an increasing amount of

---

[1]   American Cancer Society 2007 facts and figures, Inc., Surveillance Research. http://www.cancer.org/downloads/STT/CAFF2007PWSecured.pdf

research has focused on the potential role of environmental and dietary factors in prostate carcinogenesis. Additionally, extensive research is dedicated to understanding the genetic and epigenetic mechanisms associated with prostate cancer development. Specific genetic alterations, such as single-nucleotide polymorphisms at 8q24 and loss of PTEN at 10q23, have been linked with elevated risk for developing prostate cancer (9), and AR mutations at Xq11 may play a role in disease progression (10). Epigenetic modifications, such as DNA methylation and alterations in histone architecture via acetylation or methylation, play a critical role in regulating gene expression during development (11-13). Due to factors such as environmental exposures, diet, and mutations that affect protein function, changes in the maintenance or generation of these modifications lead to deregulated gene expression, which can promote disease initiation or progression (14-23).

1.3   PROSTATE CANCER TREATMENT

Currently there are a limited number of treatment options for prostate cancer patients, and treatment will vary depending on patient age and stage of the disease. For older patients or those with less aggressive disease, watchful waiting is often more appropriate than initiating treatment. Patients with localized disease that has not penetrated the prostatic capsule typically receive surgery (radical retropubic prostatectomy), external beam radiation or brachytherapy (radioactive seed implants), and are often supplemented with hormone therapy (24). However, patients diagnosed with metastatic disease or those who relapse following first line intervention can receive a variety of treatments including hormone therapy, chemotherapy or a combination of

both, and are often supplemented with antiandrogens in efforts to delay disease progression (25). Lastly, many clinical trials now focus on the applicability of immune-based therapies as alternatives for treating prostate cancer (26,27).

Since 1941, we have known two things regarding advanced prostate cancer: that almost every patient undergoes and initially responds to androgen ablation therapy (AAT), and in time they will progress to develop androgen-independent prostate cancer (28). According to the American Cancer Society, the 5-year survival rate for individuals with metastatic prostate cancer is only 33.3%[1]. In addition to lymph nodes, lungs, and liver, prostate cancer metastasizes to bone producing extremely painful and lethal osteoblastic bone lesions (29). Based on the critical role of the AR in prostatic development and regulation of prostate physiology, hormone therapy remains the cornerstone in treating advanced prostate cancer. Hormone therapy is effective because it can target cancer that has spread beyond the prostate gland and thus beyond the reach of surgery or local radiation. Nonetheless, there is growing controversy over the timing of when androgen deprivation should be administered (30,31).

At the onset, "chemical castration" induces significant androgen-dependent apoptosis causing a substantial reduction in tumor size; however, through a combination of mechanisms that remain unclear, the majority of cases eventually evolve into androgen-independent recurrence. Changes in AR levels, mutations that enhance AR function, and alterations in expression and function of AR co-regulators play a role in prostate cancer progression; however, additional survival mechanisms likely exist, contributing to the development of androgen-independent prostate cancer.

1.4   ENDOTHELIN

1.4.1   Endothelin-1

Endothelins are a peptide family consisting of three isoforms: ET-1, ET-2, and ET-3, which are 21 amino acids in length and show great homology to sarafotoxins (32). All three ET peptides possess two intrachain disulfide bonds creating a hairpin loop, a highly conserved hydrophobic C-terminal end, and a variable N-terminal domain; these distinct N- and C-terminal regions, in large part, regulate ligand binding to ET receptors (33). ET-1, the most powerful vasoconstrictor known, is secreted primarily by endothelial cells in addition to various epithelial cells, Sertoli cells, macrophages, neurons, cardiac myocytes, hepatocytes and vascular smooth muscle cells (34-37). In fact, levels of immunoreactive ET-1 in human seminal fluid are among the highest in the body (38). Experimentally, ET-1 has been shown to promote mitogenesis in a variety of cell types (34,36,39-41), and anti-apoptotic responses in human smooth muscle cells (42), cancer cells (43) as well as prostate cancer cell lines (44). ET-1 was discovered in 1988 in the culture supernatant of porcine endothelial cells and found to have close homology to neurotoxins that act on voltage-dependent Na^+ channels.   The ET-1 precursor, preproendothelin (PPET), is proteolytically cleaved in a unique manner suggesting that regulation of ET-1 biosynthesis occurs post-translationally in response to specific stimuli (45). The human ET-1 gene (*EDN1*), which maps to 6p23-24 (46), encodes a 2026-nucleotide mRNA for PPET, consisting of 5 exons (47). In humans, a furin-like convertase cleaves the 203 amino acid PPET to a 38 amino acid prohormone, big ET. Big ET is further cleaved between tryptophan-21 and valine-22, by the membrane-bound metalloprotease endothelin-converting enzyme (ECE-1), resulting in active

5

ET-1 (48); an essential post-translational conversion necessary for optimizing the biological activity of ET-1 (49).

1.4.2   Endothelin-1 regulation

PPET sequence analysis located AP-1 and NF-1 binding elements in the 5'UTR in addition to an acute-phase reactant regulatory element suggesting an mRNA induction regulatory mechanism of ET-1 secretion, particularly in response to cell stress (47). Insulin, thrombin, transforming growth factor-β (TGF-β), angiotensin II, vasopressin, insulin-like growth factor-1 (IL-1), adrenaline, and cell stress are known to stimulate PPET mRNA production whereas factors such as nitric oxide (NO), cyclic adenosine monophosphate (cAMP), prostacyclin, atrial natriuretic hormone, vasoactive intestinal peptide and prostaglandin E2 repress expression (34,37,47,50,51). In the 3'UTR, three AUUUA motifs may mediate selective translation-dependent destabilization of PPET mRNA, possibly accounting for the short half-life of PPET mRNA (47). *In vitro* analysis demonstrated that endothelins, once secreted, are degraded by neutral endopeptidase (NEP) 24.11, a metallopeptidase enzyme produced by endothelial and epithelial cells (52). A 1998 study demonstrated that *in vitro*, NEP expression and activity is lost in androgen-independent prostate cancer cell lines, yet maintained in androgen-dependent cells. *In vivo*, they showed that NEP expression is decreased in metastatic prostate cancer samples from patients with androgen-independent prostate cancer. These studies concluded that expression of NEP, a zinc-dependent metallopeptidase, is transcriptionally regulated by androgens and that loss of NEP contributes to progression to androgen independence (53).

1.4.3   Endothelin receptor signaling

Endothelins operate through binding two endothelin receptor subtypes ET_A and ET_B, which were isolated and cloned in 1990 (54,55). Encoded by 4q31.22 and 13q22.3 respectively (56,57), ET_A and ET_B are 63% identical (58), and both have been identified in mammalian tissue. Both receptors belong to the seven-transmembrane G-protein coupled receptor (GPCR) superfamily (54,55). ET_A binds ET-1 and ET-2 with greater affinity than ET-3, whereas ET_B binds all three ET peptides equally (58). Upon binding ET-1, both ET_A and ET_B are endocytosed; ET_A is recycled to the membrane surface whereas ET_B is degraded (59,60).

ET-1 binding ET_A receptor activates a variety of intracellular signaling pathways. ET-1 induces phospholipase C (PLC) activation producing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which in turn affect intracellular calcium mobilization and protein kinase C (PKC) activation, leading to proliferation (40), inhibition of FasL-induced apoptosis and inhibition of caspase-induced apoptosis (50). ET-1 binding ET_A also results in elevation of cAMP as well as extended elevation of calcium levels via extracellular means (61). Via GPCR (ET_A) crosstalk with non-receptor protein-tyrosine kinases ($pp60^{c\text{-}src}$), ET-1 induces Shc phosphorylation and association with Grb2 and Sos1, Ras recruitment with subsequent activation of mitogen activated protein kinase (MAPK) (62). In 1996, Sugawara *et al*. demonstrated that ET-1 induces a strong mitogenic response in Chinese Hamster Ovary (CHO) cells, transfected to express ET_A, by G-protein induction of phosphatidylinositol 3 kinase (PI3K), resulting in activation of MAPK independent of PLC (41). Additionally, ET-1 signaling through the ET_A receptor has been shown to enhance the mitogenic properties of IL-1, IL-2, platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and epidermal growth factor

(EGF) (34). In prostate cancer epithelial cells, ET_A signaling recruits PI3K to the inner membrane surface where PI3K converts inositol 4,5-bisphosphate (IP2) to IP3. IP3 phosphorylates and activates protein dependent kinase (PDK) which in turn phosphorylates and activates protein kinase B/Akt. Akt activation results in phosphorylation and deactivation of pro-apoptotic factors such as Bad, Bax and Bak, leading to cell survival. Anti-apoptotic proteins such as Bcl-2 and Bcl-X_L were found to be unaltered by ET_A signaling (63).

The ET_B receptor functions in ET-1 clearance (64) and autoregulation of ET-1 secretion (65), effecting signal transduction counter regulatory to that of ET_A. ET-1 stimulates ET_B-G-protein-mediated IP3 formation and intracellular Ca^{2+} mobilization, activating Ca^{2+}/calmodulin-dependent nitric oxide synthase production of NO (66). NO has been associated with decreased ET-1 secretion, yet this remains to be shown in the prostate. However, Nelson *et al*. discovered decreased ET_B receptor expression, compared to benign prostatic epithelial cells, in many cases of advanced prostate cancer due to extensive hypermethylation of the 5' CpG island of the ET_B receptor gene, *EDNRB* (67). This suggests that decreased ET_B expression may be due to gene silencing, which would result in increased availability of ET-1. ET_B expression is lost in 70% of men with prostate cancer while ET_A expression is maintained, if not up-regulated (68). Complete nucleotide sequence analysis of ET_B located, in the 3'UTR, three AUUUA motifs that may act to mediate selective translation-dependent destabilization of ET_B mRNA (55). ET_B signaling potentially contributes to apoptosis in prostate cancer cells; however, further investigation is needed to identify and characterize the ET_B-downstream signaling molecules in prostate cancer epithelial cells.

1.4.4   Endothelin and the hormonal milieu

As a major premise to this research, several studies have examined endothelin expression in the context of the dynamic hormonal milieu. In the human menstrual cycle, ET_A and ET_B mRNA levels fluctuate: during the proliferative stage, ET_A is expressed solely by the endometrium, with increases in ET_B expression levels detected in the secretory and menstrual phases (69). ET-1 mRNA is present throughout (69), with concentrations greater in the proliferative and menstrual phases than in the secretory and ovulatory phases (70). Studies that measured plasma ET-1 concentrations after cross-gender hormone treatment in female-to-male transsexuals, treated with testosterone, observed increases in ET-1; however, in male-to-female transsexuals, using antiandrogens and estradiol, decreases in ET-1 were noted (71). Lastly, a 1998 study looking at human osteoblasts showed that glucocorticoids increased both *in vitro* and *in vivo* levels of ET-1 and ET_A specifically, with a more than 2-fold increase in total ET-1 binding capacity per osteoblastic cell (72).

1.4.5   Endothelin and androgens

Androgen withdrawal represents a form of cell stress that triggers an array of emergency response mechanisms, possibly including that of endothelin signaling. Androgens have been shown to down-regulate ET-1 production in androgen-sensitive LNCaP cells but not in androgen-insensitive PC3 and DU145 cells. However, PC3 cells transfected with full-length AR demonstrate reduced ET-1 production when treated with androgens while AR blockade with flutamide or bicalutamide restored ET-1 secretion levels (73).

In terms of androgen withdrawal, an immunohistochemical analysis of human prostate cancer tissue showed a decrease in immunodetectable ET-1 in areas of regression, with ET-1 retention in areas unaffected by androgen withdrawal (74). Additionally, recent studies that employed animal castration models revealed a 2.31-fold increase (canine) and a 2.1-fold increase (rat dorsolateral) in prostate endothelin receptor levels, both noting ET_A to be the predominant receptor subtype (75,76). However, it is unclear how androgens regulate endothelin expression at the molecular level: the AR may operate directly by occupying promoter regions with certain co-regulators, or indirectly by regulating the expression of ET-1 processing molecules, ECE-1 and NEP 24.11.

One study proposed that NEP is transcriptionally activated by androgens and showed that NEP decreases with androgen withdrawal (53). In 2000, a homologous androgen receptor response element (ARE) and androgen response region (ARR) were identified in the 24th exon and promoter region of NEP, respectively, and characterized in prostate cancer cell lines. Each *cis*-element was manipulated to induce NEP expression, but combining the two more than doubled the transcriptional activity as measured in PC-3/AR transfected cells (77). Lastly, Usmani *et al.* confirmed that, in prostate cancer cell lines, NEP expression is lost and ECE expression increases with increasing malignancy, supporting the hypothesis that ET-1 secretion increases in advanced disease (78).

Computer-based analysis of ET-1, ET_A, ET_B, and ECE upstream regions (-2000bp) and full gene sequences failed to locate potential ARE sites; however, the previously published ARE in the last exon and the ARR in the promoter region of NEP were confirmed. This sequence analysis looked at entire gene sequences in order to account for any distant enhancer sites in

addition to the promoter region. If previous studies are correct, fluctuations in ET-1 levels may be due to indirect androgen regulation of NEP.

1.4.6 Endothelin signaling in cell migration and invasion

Endothelin has been linked to cell migration and invasion in several cell types. The ET_B receptor has been shown to be essential for neural crest cell migration (79), and it may have a potential role in modulating invasion of Ewing's sarcoma and neuroblastoma cell lines (80). A group in Italy observed that ET-1 induced Kaposi's sarcoma cells, KS IMM, to secrete and activate matrix-metalloproteinase (MMP) MMP-2,-3,-7,-9, and -13, inducing extracellular matrix remodeling. Blocking ET_A and ET_B was required to completely inhibit KS IMM MMP-dependent migration and invasion (81). An additional study looking at vascular endothelial cells, a major source of ET-1, showed that ET-1 induces a 3-4 fold increase in VEGF expression in cultured human vascular smooth muscle cells. This secreted VEGF, in turn, is believed to stimulate the proliferation and invasive potential of the near-by endothelial cells (82). Just recently, Dawson *et al.* investigated prostate stromal-epithelial cell interactions, and demonstrated that stromal cells, which express ECE-1, stimulate invasion of malignant prostate cancer cells (PC-3 and DU-145) which lack NEP. Prostate cancer cell invasion was specifically inhibited by blocking ECE-1 activity or by adding recombinant NEP, and this inhibition was reversed by exogenous ET-1 (83).

1.4.7 Clinical significance of endothelin in prostate cancer

ET_A receptor blockade has emerged as a new strategy in the treatment of advanced prostate cancer. In a phase-I clinical trial using the selective ET_A antagonist, atrasentan (ABT-627), 68% of men experienced decreases in PSA and 70% had reduction in pain (29). Two phase-II clinical trials, addressing the efficacy and safety of atrasentan, found that atrasentan extended the median time to disease and PSA progression, decreased pain and narcotic use, and decreased markers of bone deposition and resorption. Atrasentan was tolerated very well and maintained quality of life parameters (84,85). Of note, Pecher *et al*. showed that atrasentan does not alter PSA secretion in prostate cancer cell lines LNCaP and LAPC4 (86), indicating that changes seen in PSA levels after treatment with atrasentan resulted from a decrease in tumor growth rather than an inhibition of the cell's ability to produce PSA. Additionally, our lab has previously shown that ET_A blockade effectively enhances the anti-tumor activity of paclitaxel and docetaxel in reducing tumor growth of malignant MLL and ET_A-overexpressing PPC-1 cell xenografts, respectively, in athymic male mice (87,88). Lastly, a recent study by Banerjee *et al*. examined both the *in vitro* and *in vivo* efficacy of ET_A blockade (ABT-627) in combination with Taxotere (docetaxel), an agent that shows great promise in treating advanced prostate cancer. *In vitro*, they found that ABT-627 significantly enhanced LNCaP and C4-2b prostate cancer cell sensitivity to docetaxel-induced apoptosis. They also demonstrated a 90% reduction in C4-2b xenograft tumor growth in an *in vivo* prostate cancer mouse model when combining ABT-627 with docetaxel relative to untreated mice (89).

1.5   PURPOSE

The rationale behind this research is 1) ET-1 is secreted by human prostate epithelial cells, and that ET_A and ET_B subtypes are expressed in human prostate, 2) *in vitro*, ET-1 is a mitogen/survival factor known to induce growth regulation and modulate apoptosis through ET_A and ET_B receptor subtypes, and 3) ET-1 secretion and ET_A receptor expression are detected in men with advanced prostate cancer and have been shown to contribute to the pathobiology of metastatic prostate cancer, particularly of the bone.

It is hypothesized that the endothelin may be a key factor in the evolution of advanced, androgen-independent prostate cancer, providing prostate epithelia an alternate survival route when faced with androgen deprivation. By identifying the timing of the endothelin response to androgen deprivation, inhibition of ET-1 signaling through the ET_A receptor may provide additional benefit to androgen ablated patients by optimizing current therapy.

1.6   MATERTIALS AND METHODS

1.6.1   Cell culture and androgen ablation

LAPC4 (provided by Dr. Rob Reiter, UCLA), and LNCaP and PPC-1 (obtained from ATCC) cells were cultured in growth media (IMDM plus 10% FBS; RPMI plus 10% FBS, respectively). LA98 and LN96 cells are long-term androgen clones of LAPC4 and LNCaP, respectively, grown in androgen-stripped media containing charcoal/dextran filtered FBS (10% csFBS, Hyclone) for

at least 4 years. Control cells were passaged approximately once per 10 days. Androgen deprived cells were grown in csFBS (single lot) supplemented media and passaged twice within one month, zero times through 10 months, and then once per 7 days thereafter. PPC-1-pCMV and PPC-1-ET$_B$ cells were maintained in RPMI-1640 + 10%FBS + 100μg/ml G-418 (Neomycin). In 100mm tissue culture dishes, PPC-1-pCMV and PPC-1-ET$_B$ cells were treated with 0.01-100nM ET-1 in serum-free RPMI or 0-10% FBS in RPMI. Cells photographed in cell culture on Olympus microscope using a Canon digital rebel SLR camera and compiled in photoshop.

1.6.2 Ligand binding studies

Cells were homogenized in cold buffer (50 mM Tris-HCl, pepstatin1 μg/ml, leupeptin 1μg/ml, pH 7.7 at 4°C) and, centrifuged at 60,000g for 30 minutes. The subsequent and pellets were resuspended in warm buffer (50mM Tris-HCl, pepstatin1 μg/ml, Leupeptin 1μg/ml, pH 7.7 at 37°C), kept at 37°C for 40 minutes, and then centrifuged at 60,000 g for 30 minutes at 30°C. To define Kd and Bmax, duplicate aliquots (2 x 125μl) of membrane suspensions were incubated with increasing concentrations (0.98 to 500 nM) of non-radioactive ET-1 and corresponding [^{125}I]ET-1 (Amersham Pharmacia Biotech, 2000 Ci/mmol), added at increasing concentrations (3.9-2000pM) as well.   The membrane suspensions were vacuum-suctioned to Whatman filters (Kent, United Kingdom), placed in 5 ml tubes, and counted on Wallac 1470 gamma counter (PerkinElmer Life Sciences, Turku, Finland). Non-ablated LAPC4 and LNCaP were used as controls.

1.6.3   Real Time Quantitative Polymerase Chain Reaction (PCR)

RNA isolation and RT first strand synthesis. Total RNA was extracted using RNA-Bee (Tel Test Inc, Friendswood, TX), as per manufacturer's instructions, and quantified by spectrophotometric measurements at 260/280nm. After removal of contaminating DNA (Ambion DNase kit, Austin, TX), Hex-Reverse Transcription was performed in a 100µl final volume, containing 1 µg of total RNA, 10 U/µl of MMLV RT enzyme (Epicentre MMLV RT enzyme, Madison, WI) 40 U/µl of RNase Inhibitor (Promega, Madison, WI), 1.25 mM hexamer primers, 25 mM of each dNTP's, 10 µl of 10x PCR buffer and 75 mM $MgCl_2$.

Quantitative PCR. Primer Express (Applied Biosystems, Foster City, CA) was used to design the following primers and probes. RT-PCR was used to confirm q-PCR primer specificity prior to use in real time qPCR.

Gene	forward primer (5'-3')	reverse primer (5'-3')	probe (5'-3')
ET_A	CGCTCTTAGTGTTGACAGGTACAGA	AGACAATTTCAATGGCAGTTACCA	TGGAGTCGTGTTCAGGGAATTGGGA
ET_B	TATCAATGTCTACAAGCTGCTGGC	TGATTCCCACGGAGGCTTT	AGGACTGGCCATTTGGAGCTGAGATG
ET-1	TCAACACTCCCGAGCACGTT	TGGCATCTATTTTCACGGTCTGT	ACTTGGAAGCCCTAGGTCCAAGAGAG
AR	TACTTCGCCCCTGATCTGGTT	TCATTCGGACACACTGGCTGTA	TCAATGAGTACCGCATGC
NEP	GTTAACTCCATTAAAACAGATGTGCAC	AAGGCTTCTGAAAACTCTGCAGA	CCAGGCAATTTCAGGATTATTGGGACTTTG
GUS	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA	TGAACAGTCACCGACGAGAGTGCTGG

Amplicons: ET_A = 92bp; ET_B = 95bp; ET-1= 109bp; AR = 75bp; NEP = 89bp. FAM fluorescent dye and 3' TAMRA quencher dye. Each 50µl amplification reaction mix contained 1x Taqman

universal PCR mastermix (containing AmpliTaq Gold™ DNA polymerase, 25mM dNTP's, passive reference 1 (ROX) and optimized buffer components including 3.5mM MgCl2) with, 5μl of cDNA and, 200nM primers and 100nM probes (final concentration). Amplification was performed at 95ºC for 12min followed by, 40 cycles of 15sec at 95ºC, and 1min at 60ºC, with using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) at the University of Pittsburgh Taqman facility. Each PCR was carried out at two dilutions (1μg and 250ng of cDNA each in 50μl) to verify potential real-time quantitative-PCR inhibition, which contained 1μg and 250ng of cDNA in 50 μl, respectively. All reactions were carried out alongside a non-template control containing sterile water and a positive control containing 1μg/μl cDNA. Expression of genes of interest was normalized for any unknown sample by solving for the RNA loading represented in the housekeeping gene Glucuronidase β (GUS) expression. For quantification, analysis was done with the Δ cycle threshold (Ct) value (Ct gene of interest - Ct housekeeping gene) to generate relative expression. Fold change in gene expression was obtained by the ΔΔ Ct method (Δ Ct sample-Δ Ct value calibrator) using the baseline week as a calibrator for comparison of every unknown sample gene's expression levels. The conversion between ΔΔ Ct and relative gene expression is fold induction = 2-ΔΔ Ct. ET-1 over-expressing LNCaP cells, ET_A over-expressing PPC-1 cells, ET_B over-expressing PPC-1 cells, DHT treated LAPC4 cells, and Ambion (Austin, TX) human kidney RNA were used as positive controls (ET-1, ET_A, ET_B, AR, NEP calibrators, respectively).

1.6.4 ELISA

ET-1 secretion: LNCaP and LAPC4 cells were plated in 24-well plates (1×10^{6}/well) and left for 24 hours to attach. Serum free media containing increasing amounts of DHT (0.01-100nM) with or without 10μM bicalutamide (Casodex) was added for 48 hours. Supernatants were collected, centrifuged at 10,000g for 5 minutes, and 100μl used for the ET-1 determination by ELISA (Amersham Biosciences, Piscataway, NJ). ET-1 concentration was determined by computer software interpolation (BioRad, Hercules, CA) from the standard curve and normalized for cell numbers. Bicalutamide obtained from AstraZeneca, (Wilmington, DE).

PSA secretion: LNCaP-AI cells were plated 20,000/well in a 24-well plate and allowed to attach 36 hours in phenol-red free RPMI containing 10% csFBS growth media. Cells were then incubated in serum free, phenol-red free media containing increasing amounts of DHT (0.001-100nM) or vehicle for 4 days. Fresh media was added on day 2. On day 4, culture media was collected, spun at 400g for 10 minutes, and 25μl assayed using the Active PSA ELISA according to manufacturer's instructions (Diagnostic Systems Laboratories, Webster, TX).

Apoptosis: PPC-1-pCMV and PPC-1-ET$_B$ cells were plated 20,000 cells/well in duplicate 24 well tissue culture plates in growth media (RPMI-1640 + 10%FBS) and allowed to adhere overnight. For the serum starvation time course, cells were treated in 1ml serum-free media for 0 to 24 hours at the indicated time intervals. For serum dose response, cells were treated in 1ml serum-free media with increasing amounts of serum (0 – 10% FBS) for 12 hours. For apoptosis induction, cells were treated in 1ml media containing 1% serum or serum-free media with

increasing concentrations of ET-1 (0.01 – 100nM) or vehicle and incubated for 12 hours. Additional assays were performed incorporating both ET_A and ET_B antagonists ABT-627 and ABT-621, respectively. Media was removed and centrifuged to pellet cells. Pelleted cells were added back to appropriate wells and cells lysed according to manufacturer's instructions (Roche, Indianapolis). 100μl of lysate added to the wells of the 96-well apoptosis plate, in triplicate, and processed according to manufacturer's instructions. Plate measured at 405nm. Duplicate plate used for cell count assessment, which was used for lysate normalization.

1.6.5   Immunoblotting

Cells were washed in ice-cold PBS, harvested by cell scraping, lysed in 20mM HCl, 135mM NaCl, 10% Glycerol, 1% Triton-X 100, pH 8, 1% HALT protease inhibitor (Pierce, Rockford, IL) and placed on ice for 20 minutes. Lysates were centrifuged at 14,000rpm at 4°C for 10min. Equal amounts of protein (20μg), as determined by BioRad Protein assay (BioRad, Hercules, CA), were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto Immobilon-P PVDF (Millipore, Bedford, MA). Membranes were blocked in 4% milk in PBST and incubated with Akt and pAkt polyclonal antibody (Cell Signaling, Danvers, MA) and AR polyclonal antibody (Santa Cruz, CA) overnight or β-actin monoclonal antibody (Sigma, St. Louis, MO) for 1hr. After washing with PBST, blots were incubated for 1hr with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnologies, Santa Cruz, CA) for Akt, pAkt, and AR or β-actin, respectively, washed again and subjected to ECL (GE Healthcare, Buckinghamshire, UK) procedures. Immunoblots were subjected to densitometric analysis,

18

normalized for β-actin, and protein expression quantified using Quantity One analysis software (BioRad). ABT-627 obtained from Abbott Laboratories, Chicago, Il.

1.6.6   Growth Assays

LNCaP-AI + DHT: LNCaP-AI cells were plated and cultured as described in PSA ELISA section. On day 4, after media was removed, adherent cells were dislodged in 250μl 0.05% trypsin, neutralized in 750μl media and counted using hemacytometer and trypan blue exclusion to collect live versus dead cells. For hemacytomoter count analysis, 50,000 LNCaP-AI cells were plated per well in a 6-well plate, in triplicate, and treated with 10nM DHT or vehicle for 1 week.

Cell doubling time: LNCaP and LNCaP-AI cells were plated in growth media at 50,000 and 25,000 cells per well, respectively, in 6-well plates, and allowed to attach overnight. Cells were counted via Z1 Coulter Counter (Beckman Coulter, Fullerton, CA) each day for six days. Cell growth was determined by calculating cell doubling time (hr) = inv (((log (final count) - log (initial count)) * 3.32) / time).

1.6.7   Tissue specimens

Formalin fixed and paraffin embedded prostate tissue sections were prepared from radical prostatectomy (RP) and TURP specimens from patients that have undergone three month (short-term, ST) and at least 6 months (long-term, LT) androgen ablation, respectively. Patient age

ranged from 50 to 94 years and Gleason sums ranged from 3 to 9. Numbers of tissue samples obtained for analysis: ST: androgen ablated (11), non-ablated (13). LT: androgen ablated (17), non-ablated (24). For normal prostate controls samples, paraffin embedded donor prostate tissue was obtained from 17 organ donors, aged 45 to 74 years. Donor peripheral zones were matched for ST and donor central zones were matched for LT androgen ablated tissues. All tissues were obtained from the University of Pittsburgh Tissue Bank.

1.6.8 Immunohistochemistry

Immunohistochemical staining was performed by a UPMC Department of Pathology histologist on 5μm paraffin-embedded human prostate tissue samples described above. Slides were deparaffinized in xylene and step re-hydrated in alcohol. Slides were incubated with primary polyclonal rabbit-anti-ET_A and anti-ET_B antibodies at a dilution of 1:250 (Abbott Laboratories), followed by horseradish peroxidase-linked goat anti-rabbit antibody, and then counterstained in Mayer's Hematoxylin solution (Sigma-Aldrich, St. Louis, MO). Sections were step dehydrated and cover-slipped. Non-immunized rabbit IgG was applied as a negative control. Semi-quantitative assessment of the slides was performed by a pathologist (D.M.J.) in a blinded fashion to evaluate the staining intensity in areas containing tumor and normal adjacent to tumor (NAT), with a score ranging from 0 to 3 (0 representing no stain and 3 representing high staining in comparison to background levels).

1.6.9 MTT cell viability

5,000 or 10,000 cells were plated in 96-well tissue culture plates (Becton Dickinson, San Jose, CA) and allowed to adhere overnight. In triplicate wells, cells were treated as follows: 1) serum free media + vehicle; 2) 100nM ET-1; 3) 10nM docetaxel; 4) ET-1 + docetaxel; 5) 1μM ABT-627 + ET-1; 6) ABT-627 + ET-1 + docetaxel. MTT cleavage by live cells measured according to manufacturer's instructions (Chemicon MTT assay, Millipore, MA). Briefly, following 24, 48 or 72 hour docetaxel treatment, 10μl of AB solution (MTT) was added to each well and the plate incubated at 37ºC for 4 hours. 100μl solution C (isopropanol with HCl) added to each well, mixed thoroughly and absorbance measured within 1 hour at a wavelength of 570nm.

1.6.10 Flow cytometry

$1x10^6$ cells were plated in 100-mm tissue culture dishes (Becton Dickinson, San Jose, CA) and allowed to adhere overnight (PPC-1) or for 48 hours (LNCaP). Cells were washed with RPMI, and six groups were formed according to administered treatment: 1) serum free media + vehicle; 2) ET-1 (100nM); 3) docetaxel (10nM); 4) ET-1 for 1 hour followed by docetaxel; 5) ABT-627 (1μM) for 1 hour, followed by ET-1; 6) ABT-627 for 1 hour, followed by ET-1 for 1 hour, and then followed by docetaxel. Each group receiving docetaxel was treated for a total of 4 hours (LNCaP) or 24 hours (PPC-1). Cells were evaluated for apoptosis by Annexin V-FITC staining.

For Flow cytometry using the Annexin V assay, cells were collected and double-stained with fluorescein isothiocyanate–conjugated Annexin V and propidium iodide (PI) (BD PharMingen, San Diego, CA). Cells were counted and 10^5 cells for each condition (in 100μl of

Annexin V binding buffer) were placed in 5ml round-bottom tubes (Becton Dickinson). Annexin V and PI were added according to the manufacturer's recommendations. Annexin V−positive cells were considered apoptotic and their percentage of the total number of cells was calculated. Cells taking up vital dye PI were considered dead. Samples of 10,000 cells were analyzed by FACScan Flow cytometer with LYSYS II software package (Becton Dickinson).

1.6.11 *In vivo* combination therapy model

For xenograft studies, 3 groups of 40 athymic male mice (Charles River Laboratories, Wilmington, MA) received subcutaneous flank injections of $1x10^6$ LNCaP, LNCaP-ET$_A$, or LNCaP-ET$_B$ cells in 100μl Matrigel (Becton Dickinson, San Jose, CA). When the average tumor size had reached 0.05 cm^3, mice in each group were randomly divided into 4 subgroups (10mice/arm) as follows: 1) intact + vehicle, 2) intact + antagonist, 3) castrate + vehicle, and 4) castrate + antagonist. Abbott laboratories provided the ET$_A$ antagonist (ABT-627) and the ET$_B$ antagonist (ABT-621). Antagonist treatment was started the day of castration at 20mg/kg based on a 25gm mouse drinking 4ml water/day. LNCaP and LNCaP-ET$_A$ mice received ABT-627 and LNCaP-ET$_B$ mice received ABT-621. Vehicle was unsupplemented drinking water. Tumors were measured every week and the volumes were calculated (length x height x width). When tumor volumes reached approximately 2cm^3, the mice were sacrificed and the tumors were removed, measured, weighed, fixed in 4% paraformaldehyde, and embedded in paraffin for immunohistochemical analysis of ET$_A$, ET$_B$, and CD31 expression as described above. Serum was also collected at the time of castration, every 2 weeks by saphenous vein puncture, and at time of sacrifice by cardiac puncture for the quantification of prostate specific antigen (PSA) by

ELISA, in accordance with the manufacturer's instructions (Diagnostics Systems Laboratories, Webster, TX). PSA velocity is an estimation of the rate of change in PSA, determined by the ((final ng/ml – initial ng/ml) / total number of weeks) = ng/ml/week.

1.6.12 cRNA preparation and gene expression profiling

Total RNA was further cleaned up with Qiagen RNeasy Mini Kit (Qiagen, San Diego, CA) following the manufacturer's instruction. Quality of RNA was reassessed by ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) at OD 260/280nm and by capillary electrophoresis with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression profiling experiments were performed at the Clinical Genomics Facility of the University of Pittsburgh Cancer Institute. cRNAs were prepared and hybridized to Affymetrix Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions as previously described (90). Briefly, recommended amount of total RNA was reverse transcribed to double-stranded cDNA with T7-Oligo(dT). After purification, cDNA was *in vitro* transcribed to cRNA with T7-RNA polymerase. Proper amount of cRNA was then purified, fragmented and hybridized on HG-U133 2.0 arrays. Each array was washed, and stained in a GeneChip Fluidics Station 450 (Affymetrix) and scanned by a GeneChip Scanner 3000 (Affymetrix) as recommended by the manufacturer. All reagents were from Affymetrix unless otherwise specified.

1.6.13 Affymetrix gene expression data analysis

The scanned raw image files were converted to probe cell intensity files (.CEL) by GCOS software (Affymetrix). Gene expression data was further derived from probe intensity files using MAS5.0 algorithm. Data normalization was also performed with MAS5 by scaling the mean of intensity value of an array to a target intensity of 500. Quality control (QC) parameters were derived from the MAS 5.0 algorithm of the GCOS software (version 1.1; Affymetrix). Avadis 3.3 Pride Software (Strand Life Sciences, Bangalore, India) was used for data analysis and presentation. Gene expression data has been submitted to NCBI's Gene Expression Omnibus (GEO) under accession number GSE8702

1.6.14 Statistical analysis

Results for the quantitative RT-PCR, Affymetrix expression data, and ET_B apoptosis were analyzed for statistical significance by one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison post-test using GraphPad Prism (GraphPad Software, San Diego, CA). Student's T-test was used for initial qPCR, ET-1 ELISA and growth assays. Statistical analysis for IHC studies was performed by a University of Pittsburgh Cancer Institute biostatistician. One-way ANOVA with Dunnett's multiple comparison post-test and two-way ANOVA with Bonferroni post-test was used for MTT assays. ANOVA method was used to establish the influence of treatment on tumor growth in LNCaP-ET_B mice. All analyses were done with an α level for significance set at 0.05.

1.6.15 Mathematical Modeling

Tumor growth patterns in LNCaP xenograft mice were assessed using an exponential growth model ($Y = \text{baseline} \times e^{\alpha \times \text{time}}$), with the determination of doubling time for each treatment (Doubling Time = $0.693 / \alpha$). This growth pattern served as a template for determining growth parameters by nonlinear mixed-effects modeling (NONMEM). Each treatment group was assessed while preserving within-individual and between-individual variances present in measurements of tumor growth over time. In addition, the mixed-effects modeling approach used every data point to determine growth parameters, both as a mean and as a variance across individuals. Therefore, balance in the groups was not as critical (weighting across individual contributions to this model is based on data contributed, as well as on the informativeness of data to a particular parameter). In mixed-effects modeling, population average and variance were calculated using a maximum likelihood estimation. In the case of an exponential model, population average and variance were based on the population average baseline tumor size (S0) and a population average growth rate parameter (α). Each treatment group was tested as a covariate in the growth model to examine whether that group exhibited unique growth characteristics. The threshold for determining significance was based on the objective function returned by the NONMEM software program, which is equal to -2 times the log likelihood. The difference between nested models of this objective function approximates a chi-square distribution and thus provides a means of statistically comparing growth descriptions across models. The α level for significance was set at .05, corresponding to an objective function change of 3.84 points for 1 df.

25

2.0 ET$_A$ RECEPTOR SIGNALING AS A KEY MECHANISM IN THE EMERGENCE OF ANDROGEN-INDEPENDENT PROSTATE CANCER

(Adapted from manuscript Jason M. D'Antonio, Geeta Godara, Drazen M. Jukic, Robert R. Bies, and Beth R. Pflug, Departments of Urology, Pathology, and Pharmaceutical Sciences, University of Pittsburgh School of Medicine, *submitted for publication*)

2.1 INTRODUCTION

Following AAT it remains unclear as to whether the development of androgen-independent prostate cancer results from adaptive mechanisms, clonal outgrowth, or a combination of both. ET-1, a 21 amino acid peptide originally characterized as a potent vasoconstrictor (91), plays an important role in development, as well as cell proliferation, survival, apoptosis, and migration (34,63,80,81). Secreted predominantly by endothelial cells, ET-1 is also produced by a variety of epithelial cells, Sertoli cells, macrophages and vascular smooth muscle cells (34-37). ET$_A$ and ET$_B$ are seven-transmembrane G-protein coupled receptors (58), which affect a variety of signaling molecules, including MAPK, Akt and PKC (41,62,92).

In normal prostate tissue, ET$_A$ is predominantly expressed in the stromal compartment whereas ET$_B$ is predominantly expressed in the luminal epithelial cells. In cancer, Gohji *et al.* observed greater ET$_A$ receptor expression in prostate tissue from patients with extraprostatic

disease, Gleason scores of 5 to 10, and lymph node or bone metastases as compared with tissue from patients with organ-confined disease, Gleason scores of 2 to 4, and non-metastatic disease (93). Additionally, Godara *et al.* previously found that high ET_A expression in human prostate cancer tissue is associated with shorter time to disease progression (94). In the prostate, ET_B function has been linked to ET-1 clearance and inhibition of ET-1 secretion (67). However, ET_B expression is much lower in prostate cancer cells compared to benign prostatic epithelial cells due, in part, to hypermethylation of the promoter region of the ET_B gene, *EDNRB* (68), resulting in increased ET-1 availability. In support of this, ET-1 levels were found to be elevated in some patients with androgen-independent, metastatic prostate cancer compared to patients with localized disease or healthy donors (36).

Published data from our laboratory supports a model that ET-1 treatment of ET_A-expressing prostate cancer cells generates a survival mechanism. We have previously shown that, *in vitro*, ET_A blockade significantly increased sensitivity of high ET_A-expressing Dunning Rat prostate cancer cells (MLL) to paclitaxel-induced apoptosis. *In vivo* studies employing MLL xenograft tumors revealed that ET_A blockade combined with paclitaxel significantly reduced tumor size compared with either treatment alone (87). Additionally, Nelson *et al.* demonstrated that ET-1 treatment of ET_A-expressing prostate cancer cells promotes the activation of the survival factor Akt through PI3K induction, leading to apoptosis inhibition (63). In addition, phase II and phase III clinical trials have demonstrated that ET_A blockade, using the selective ET_A antagonist atrasentan (ABT-627), in a subset of patients with androgen-independent prostate cancer, resulted in increased time to disease progression, pain relief, and a decrease in markers of bone remodeling compared to placebo groups (84,85).

27

Selective ET_A receptor blockade represents a rational, targeted approach in controlling the pathophysiologic effects of endothelin in cancer. In this study, the effects of androgen deprivation on ET-1 and ET_A expression in prostate cancer were investigated. Believing that ET-1 acts as a survival factor through ET_A signaling, the goal was to further examine the specificity of ET-1 induction of Akt and test the efficacy of ET_A-blockade in combination with castration on prostate cancer cell growth in a mouse model.

2.2   RESULTS

2.2.1   Chronic androgen deprivation increases ET_A receptor expression in prostate cancer cells.

To date, the effect of androgen ablation on the expression of endothelin receptors in human prostate cancer has not been reported. To determine so, ligand binding studies using androgen-dependent LAPC4 cells, which express a wild-type AR and secrete PSA, and chronically androgen deprived LAPC4 clone, LA98 were performed by our post-doc Dr. Geeta Godara. [^{125}I] ET-1 binding showed 4-fold greater total endothelin receptor binding in LA98 cells compared to parental LAPC4 cells (Fig. 1). Although not identifying which ET receptor sub-type, ET_A or ET_B, is increased following chronic androgen deprivation, these results show that expression and thus signaling of endothelin is likely affected during androgen withdrawal, establishing the working hypothesis for further investigation.
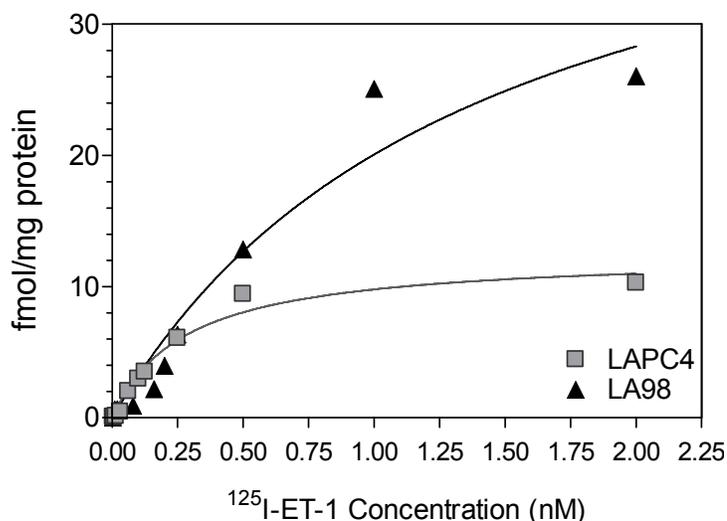
Figure 1. [^{125}I] ET-1 binding in LAPC4 and LA98 prostate cancer cells.
Duplicate aliquots of membrane suspensions were incubated with twelve different concentrations, ranging from 3.9-2000 pM of [125I] ET-1. (Bmax were 12.5 and 48 fmol/mg, Kd were 0.278 and 1.393nM, for LAPC4 and LA98 respectively).

Antibodies specific for ET_A and ET_B immunoblot analysis are currently not available; therefore, real-time quantitative PCR (qPCR) was used to delineate changes in expression of ET receptor sub-types following chronic androgen deprivation. Performed by Dr. Geeta Godara, qPCR analysis showed 4.5-fold higher ET_A and 2.4-fold lower ET_B mRNA levels in LA98 compared to LAPC4 cells (Fig. 2A). Additionally, chronically androgen deprived LNCaP clone, LN96, exhibited a 3.4-fold increase in ET_A and a 1.9-fold decrease in ET_B levels compared to parental LNCaP cells (Fig. 2B). These results show that up-regulation in the ET_A receptor, and not ET_B, accounts for the increase in endothelin receptor levels following androgen deprivation; these binding and qPCR findings served as the foundation for my dissertation research in further investigating the role of endothelin in the progression to androgen-independent prostate cancer.
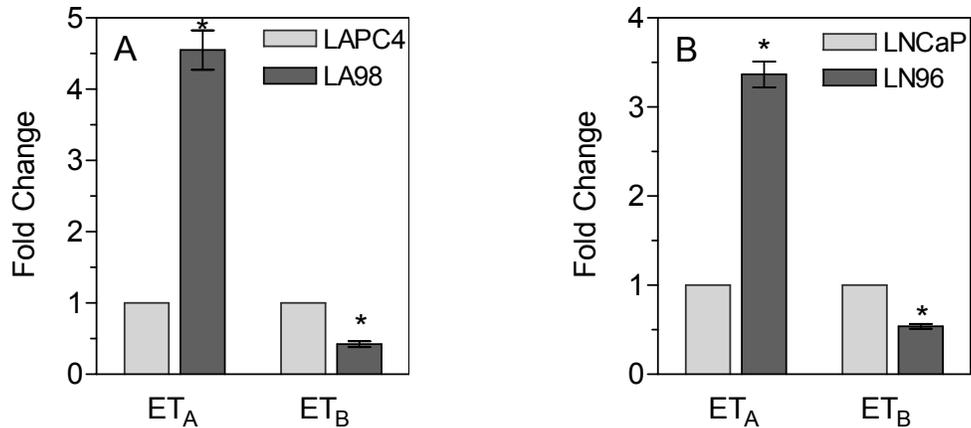
Figure 2. Real time quantitative PCR analysis of ET_A and ET_B expression in parental and chronically androgen deprived prostate cancer cells.

Androgen-dependent *A*. LACP4 and *B*. LNCaP prostate cancer cells compared to LA98 and LN96 clones, respectively. For all qPCR analyses, expression of genes of interest were normalized to the housekeeping gene Glucuronidase β (Gus) and expressed as fold change. Data are representative of three separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$

2.2.2 Changes in ET_A, ET-1, ET_B, AR, and NEP expression following long-term time course androgen deprivation, *in vitro*.

To examine changes in gene expression and most importantly ascertain the timing of when ET_A expression increases in response to androgen withdrawal, LAPC4 and LNCaP prostate cancer cells were subjected to long-term androgen deprivation: at each time point total RNA was extracted from control and androgen deprived cells and subjected to qPCR analysis. Following one month and two weeks of androgen withdrawal, respectively, ET_A and ET-1 expression increased significantly compared to untreated zero time point cells (Fig. 3).
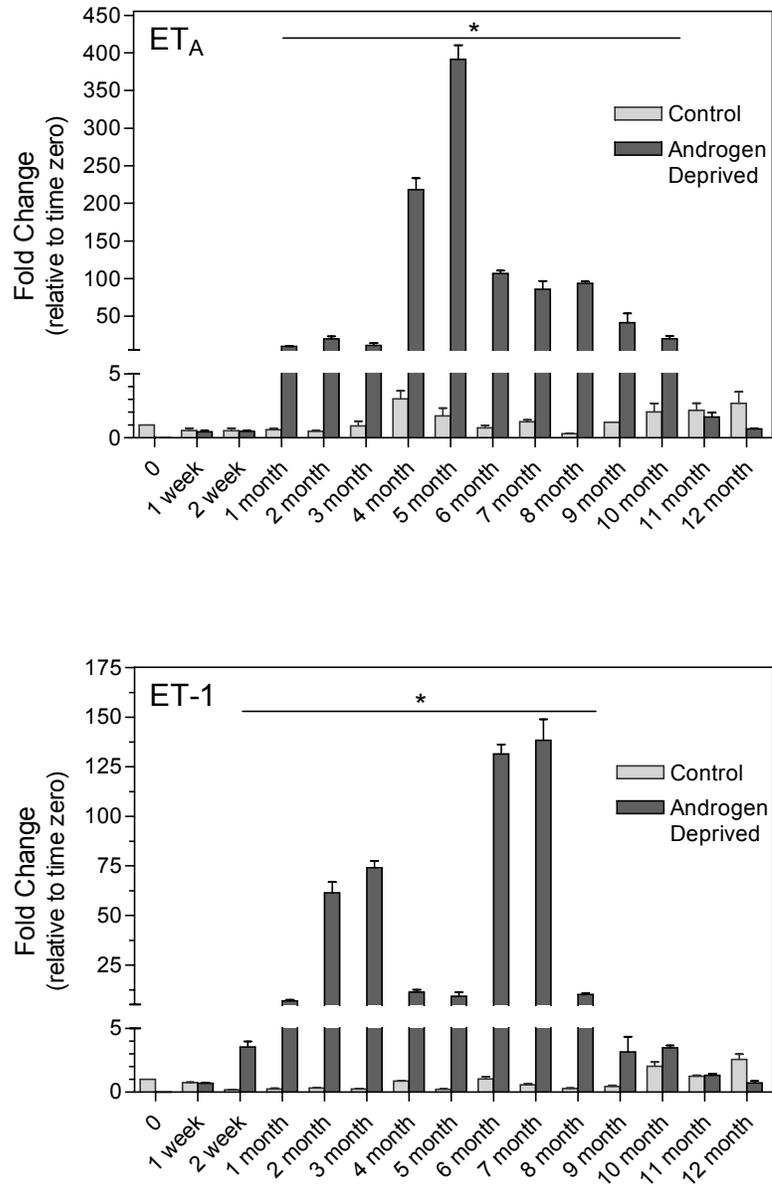
Figure 3. Changes in ET$_A$ and ET-1 expression following long-term androgen deprivation.
Data representative of two separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$.

Based on the analysis of ET$_B$ expression in LA98 and LN96 cells and previous studies demonstrating decreased NEP expression following androgen withdrawal (53), it was surprising to see that expression of both ET$_B$ and NEP also increased significantly in androgen deprived cells (Fig. 4). Of note, ET$_B$ expression reached maximum induction of roughly 21,000-fold over

control cells and remained significantly elevated at 12 months. Increases in NEP expression, which is transcriptionally regulated by AR, suggests that AR transcriptional activity my indeed be maintained or even enhanced during androgen withdrawal.
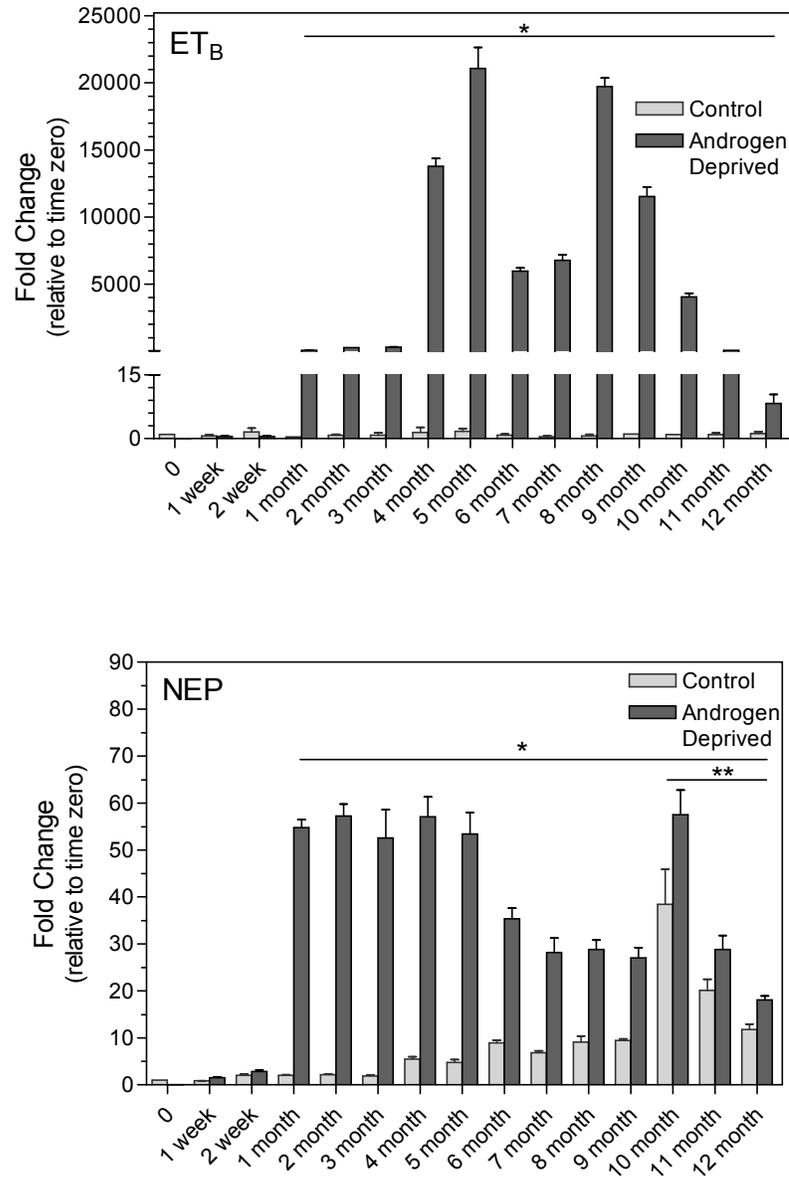
Figure 4. Alterations in ET_B and NEP expression in long-term androgen deprived LNCaP cells.
Data are representative of two separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$. **, $P < 0.05$ (control samples compared to zero control).

To validate our model of androgen deprivation by culturing cells in csFBS supplemented, phenol-red free media, AR expression was examined and found to be significantly increased within two months (Fig. 5). This short-term increase in AR expression may help explain why NEP expression is increased early on, but after four months AR expression returns to levels detected in control cells, leaving the observed sustained increase in NEP expression up to various interpretations. Stabilization of the AR/DNA interaction, promiscuous activation of AR, or alterations in AR co-factor function could promote AR activity therefore explaining the sustained increase in NEP expression. It is also possible that other nuclear hormone receptors, such as the glucocorticoid receptor or the retinoic acid receptor could possibly aid in driving NEP expression given castrate levels of androgens.
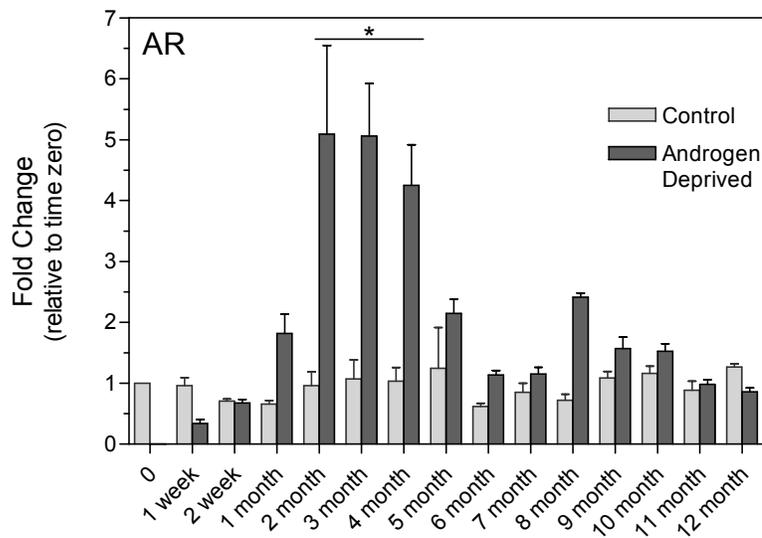
Figure 5. AR expression following long-term androgen deprivation of LNCaP cells.
Data representative of two separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$.

To confirm that alterations in gene expression were not an artifact of time in culture, gene expression for ET_A, ET_B, ET-1 and AR was evaluated and found to remain unchanged in control cells maintained for 12 months (Figs. 3-5). This suggests that the effects of androgen withdrawal, and not time in culture or serial passaging, account for altered ET_A, ET_B, ET-1 and AR expression. However, NEP expression increased significantly in 10 to 12 month control cells, suggesting that cells maintained for long periods of time in culture undergo certain genetic changes that are reflected only in specific genes.

Changes in mRNA stability, promoter methylation, and alterations in transcription factor activity as regulated by upstream signaling events represent several of the mechanisms that regulate gene expression. ET_A is upstream of several key signaling molecules, including AR, PKC, PI3K, and MAPK. To address whether ET_A up-regulation may result from the acute activation of these downstream molecules, LNCaP prostate cancer cells were treated with 5-α-dihydrotestosterone (DHT), a PKC activator [phorbol-12-myristate-13-acetate (PMA)] or epidermal growth factor for 12-72 hours. Quantitative PCR analysis demonstrated no change in ET_A mRNA levels due to any of these treatments compared to vehicle control, confirming that ET_A expression is not regulated by acute activation of AR, PKC, PI3K, or MAPK. Additionally, ET_A expression in LNCaP cells was unaffected by treatment with a demethylating agent (Zebularine, 0.1 mM + decytobine 10 μM) for four days, excluding the possibility that DNA promoter methylation modulates ET_A transcription. Therefore, modification in the ET_A transcriptional machinery, resulting in de novo gene transcription, is the most likely scenario for ET_A up-regulation during androgen deprivation.

LAPC4 cells demonstrated significant changes in ET-1 and ET_A expression patterns with androgen deprivation (Fig. 6) during one month time course experiments. ET-1 expression

34

increased within one week but ET_A expression decreased within the first two weeks compared to untreated zero control cells. These results suggest that, in conjunction with LAPC4 cells expressing a wild-type AR, down-regulation in the ET_A receptor may contribute to diminished cell survival in comparison to LNCaP cells; however, the LAPC4 cells that go on to survive long-term androgen deprivation have higher levels of ET_A (Fig. 2A, B). In conjunction with the changes in ET-1 and ET_A expression, ET_B mRNA levels also decreased in the androgen deprived LAPC4 cells compared to untreated control cells (Fig. 6).
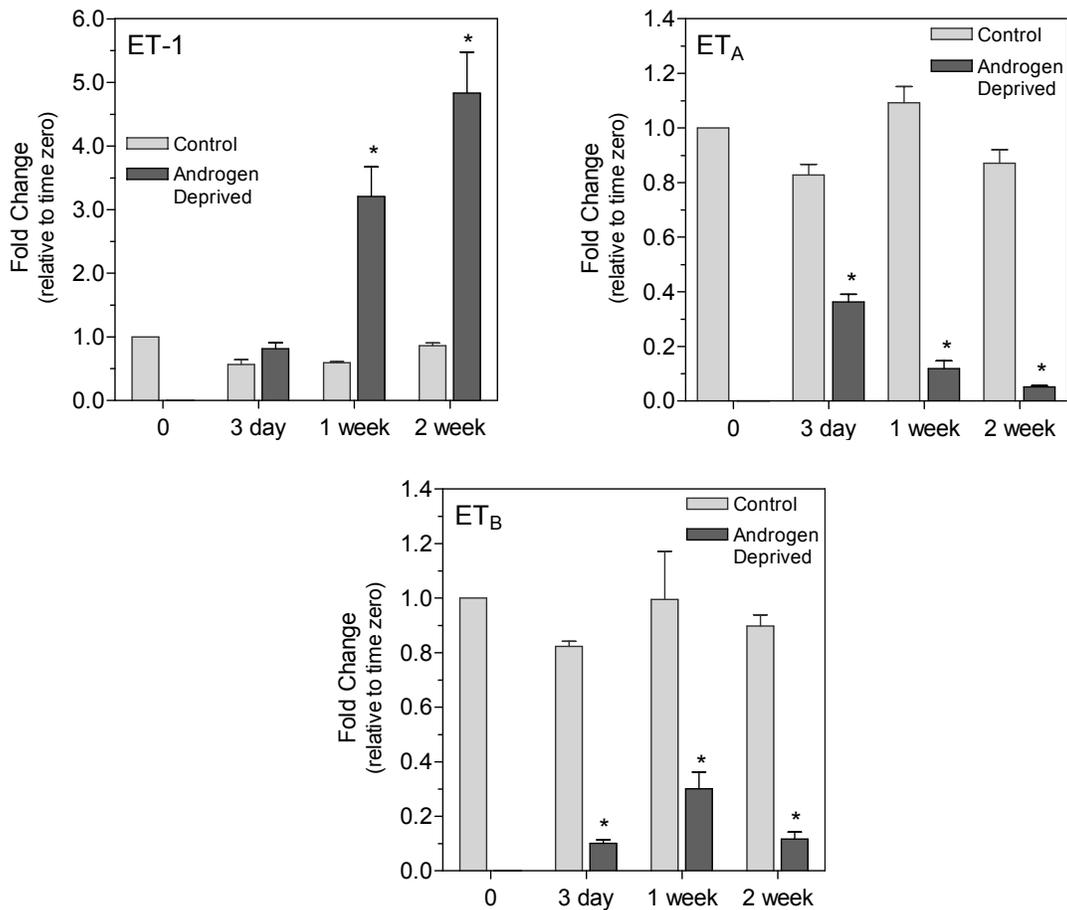
Figure 6. ET-1, ET_A, and ET_B expression changes in short-term androgen deprived LAPC4 cells.
Data representative of two separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$.

Between one and ten months, androgen deprived LNCaP cells entered a quiescent state not requiring a single passage in culture. By 12 months surviving cells had re-entered a proliferative state and expression for ET_A, ET-1 and AR returned to levels similar to control cells. Importantly, increased ET_A expression was not mimicked by agents that activate AR (DHT), PKC (PMA), or PI3K and MAPK (EGF). Due to ET_B promoter methylation in prostate cancer, methylation was suspected as a potential source of ET_A expression regulation, but found that ET_A expression was not affected by the demethylating agent Zebularine (5-aza) plus decytobine. These observations are important because they show that ET_A up-regulation is likely a direct cellular response to the stress of androgen deprivation and not due to indirect activation of alternate intracellular signaling mechanisms or epigenetic regulation. Collectively, these results demonstrate that ET-1/ET_A survival signaling is likely exploited over the majority of time it takes for cells to survive androgen deprivation and develop androgen independence.

2.2.3   Characterization of androgen-independent LNCaP-AI cells.

At 12 months the remaining LNCaP cells that survived the androgen deprivation, characterized as androgen-independent (LNCaP-AI), had not only re-entered exponential growth, fully adapted to androgen-depleted media, but exhibited a significantly accelerated growth rate. In comparison to parental LNCaP cells, which exhibit a doubling rate of approximately 41 hours, LNCaP-AI cells double nearly every 22 hours (Fig. 7).

36
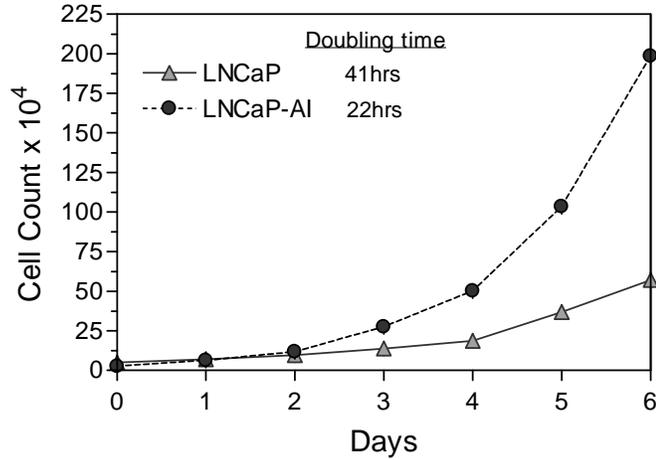
Figure 7. Growth comparison of parental LNCaP versus LNCaP-AI cells.

LNCaP and LNCaP-AI cells were plated at 50,000 and 25,000 cells per well, respectively, and cultured in regular growth media for 6 days. Performed in triplicate. Error bars (smaller than symbol size) indicate ± SEM.

To examine the nature of androgen independence in LNCaP-AI cells, AR protein expression was first evaluated via immunoblot in un-stimulated, exponentially growing LNCaP and LNCaP-AI cells. Typical AR protein expression levels were 15-fold lower in LNCaP-AI compared to LNCaP cells (Fig. 8).
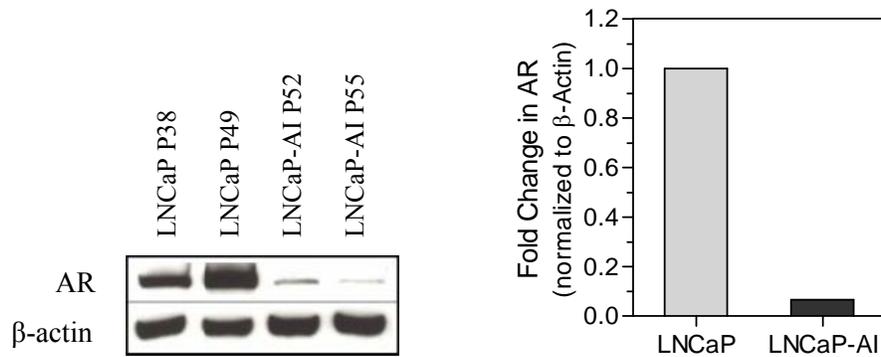
Figure 8. AR expression in unstimulated LNCaP and LNCaP-AI cells.

Average AR (110kD) expression normalized to β-actin (43kD) and quantified by densitometric analysis. Data representative of two separate experiments.

In contrast to androgen-dependent, PSA-secreting LNCaP cells (95-97), LNCaP-AI cells exhibited no change in cell growth when treated with increasing doses of DHT (0.001-100nM) for 96 hours (Fig. 9). There appears to be a trend towards decreased cell growth at higher concentrations on DHT, but the results were not significant. Additionally, PSA was undetectable in the culture media of LNCaP-AI cells treated in serum-free or increasing doses of DHT, suggesting that traditional AR transcription activity, of at least PSA, in LNCaP-AI cells is significantly reduced.
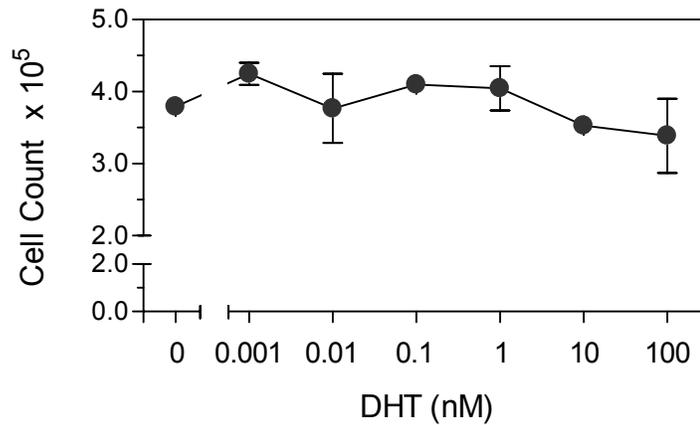
Figure 9. LNCaP-AI cell growth response to increasing doses of DHT (0.001-100nM) for 96 hours.
20,000 cells plated per well and treated in triplicate. Error bars indicate ± SEM.

Contrary to these findings, previous studies have demonstrated an inhibitory effect on androgen-independent cell growth when stimulated with androgens. Therefore, LNCaP-AI cells were treated with 10nM DHT or vehicle for up to one week and total cell counts and cell viability were assessed via hemacytometer and trypan blue exclusion . Although total cell counts were nearly identical in control versus DHT treated cells, LNCaP-AI cells treated with 10nM DHT exhibited a 198% increase in the number of dead cells (Fig 10).
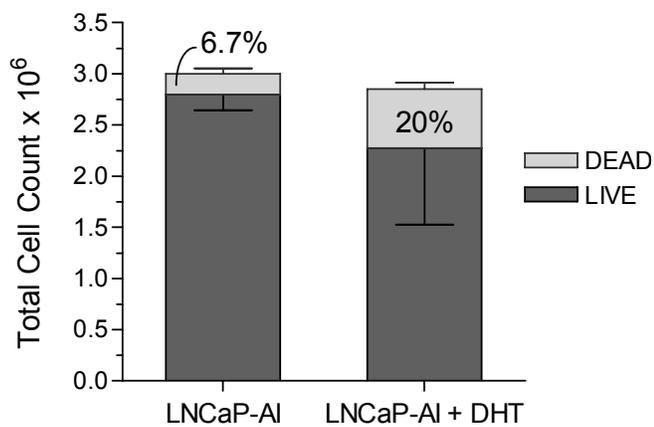
Figure 10. LNCaP-AI cell growth response when treated with 10nM DHT for 1 week.
50,000 cells plated per well and treated in triplicate. Cell counts determined by hemacytometer and trypan blue exclusion. Error bars indicate ± SEM.

Additionally, over that same increasing range of DHT stimulation used in figure 9, AR expression in LNCaP-AI cells increased, reaching a 2.3-fold maximum induction at a concentration of 1nM DHT (Fig. 11).
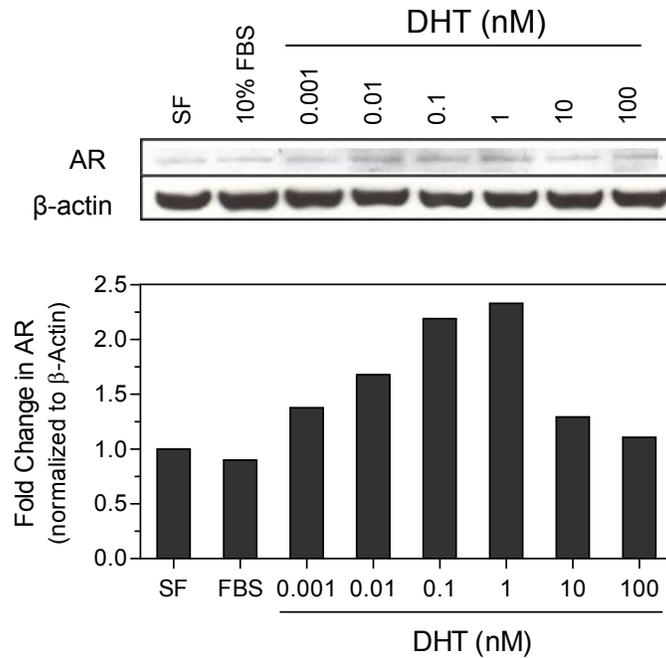
Figure 11. AR expression in LNCaP-AI cells following dose DHT stimulation for 96 hours.
AR (110kD) expression normalized to β-actin and quantified by densitometric analysis. Data representative of two separate experiments.

These results illustrate two important characteristics of malignant, androgen-independent prostate cancer cells as exhibited by LNCaP-AI cells: accelerated rates of proliferation and growth independent of androgenic signaling. In support of these findings, Pflug *et al.* previously reported that chronically androgen deprived LNCaP clones are significantly more tumorigenic than parental LNCaP cells when injected into both castrate and intact male nude mice. More importantly, when injected with androgen deprived LNCaP clones, 87% of castrate mice formed tumors compared to 47% of intact mice (98). In conclusion, it appears that LNCaP-AI cells exhibit growth independent of androgens but with enough time, demonstrate sensitivity to androgens that results in growth inhibition.

2.2.4   Reintroduction of DHT to LNCaP-AI cells shows retained sensitivity to androgens.

It has been well documented that 10nM DHT induces growth arrest in LNCaP cells (95-97), and recent evidence sheds light on this phenomenon by demonstrating that AR is stabilized during mitosis thus inhibiting the re-licensing of the DNA replication machinery (99). To test this hypothesis, cells were treated with 10nM DHT and qPCR was used to examine ET_A, ET-1 and AR expression in LNCaP-AI cells compared to parental LNCaP cells. Quantitative PCR analysis showed that by two weeks the stress of 10nM DHT induced significant increases in ET_A and ET-1 expression in both cell types (Fig. 12).
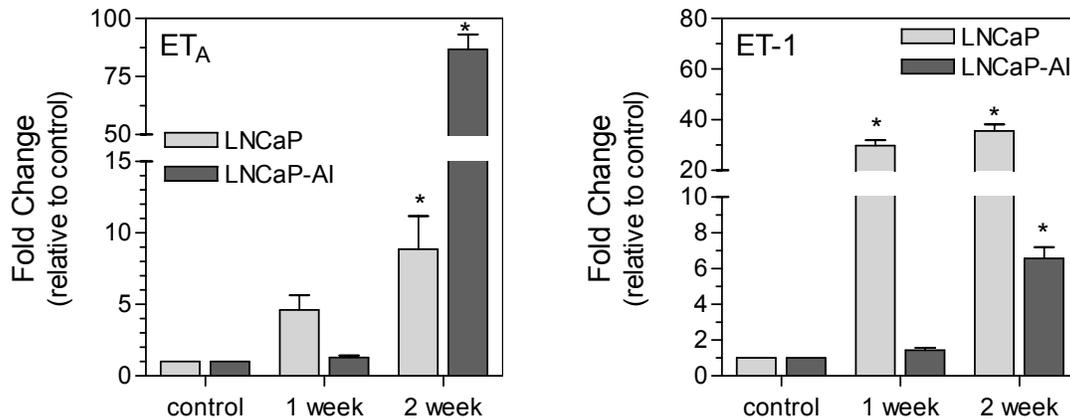
Figure 12. Induction of ET_A and ET-1 gene expression following 2 week treatment with 10nM DHT.
Data representative of three separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$.

If 10nM DHT induces AR stabilization, it would seem reasonable that, through a negative feedback mechanism, parental LNCaP cells down-regulate de novo AR synthesis in response to decreased AR turnover. Also, increased ET_A and ET-1 expression in LNCaP-AI cells suggests that LNCaP-AI cells are stressed by this level of androgenic stimulation, observations that directly support the findings in figure 10 where 10nM DHT resulted in a

significant increase in LNCaP-AI cell death. In support of the previous studies (95-97,99), AR

mRNA levels decreased 11-fold in parental LNCaP cells; however, in agreement with the

immunoblot analysis shown in figure 11, AR expression increased in LNCaP-AI cells (Fig. 13)

on a similar time scale.
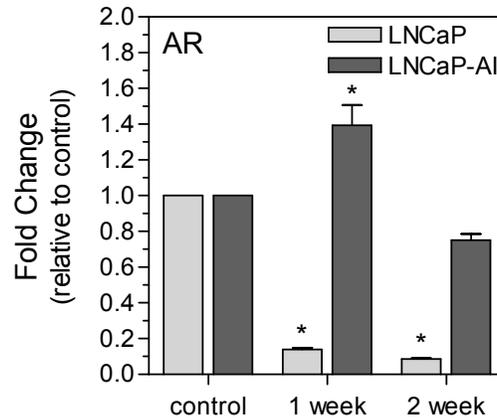
Figure 13. Changes in AR expression following 2 week treatment with 10nM DHT.
Data representative of three separate experiments each analyzed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$.

There exists conflicting data regarding the effect of androgens on the growth of

androgen-independent prostate cancer cells: some studies report a stimulatory effect while others

report growth inhibition (100,101). Evidence of altered ET_A and ET-1 mRNA, and AR mRNA

and proteins levels following 10nM DHT stimulation illustrates that LNCaP-AI cells, which

appear to grow independent of androgenic signaling, remain responsive to the stimulation of

androgens.

2.2.5 AR blockade increases ET-1 secretion in androgen-dependent prostate cancer cells.

Having shown that androgen deprivation increases ET-1 and ET_A expression in prostate cancer cells, an ELISA based assay was used to examine the effect of acute AR blockade on ET-1 protein secretion in androgen-dependent LAPC4 cells. ET-1 secretion was affected over an increasing range of DHT (0.01-100nM) within 48 hours (Fig. 14). The pattern of ET-1 secretion inversely mimics the bi-phasic response that androgens have on prostate cancer cell growth: at 0.1 to 1nM DHT, cell growth is maximal whereas concentrations outside this range are stressful to cells and retard cell growth (95-97,102). Knowing this, LAPC4 cells were then cultured in the same increasing concentrations of DHT plus 10μM bicalutamide (Casodex) for 48 hours. Compared to DHT-only treated cells, ET-1 secretion increased with AR blockade over the entire range of androgenic stimulation, most significantly between 0.1 to 10nM DHT (Fig. 14), signifying that prostate cancer cells secrete ET-1 as an immediate response to the stress of AR blockade.
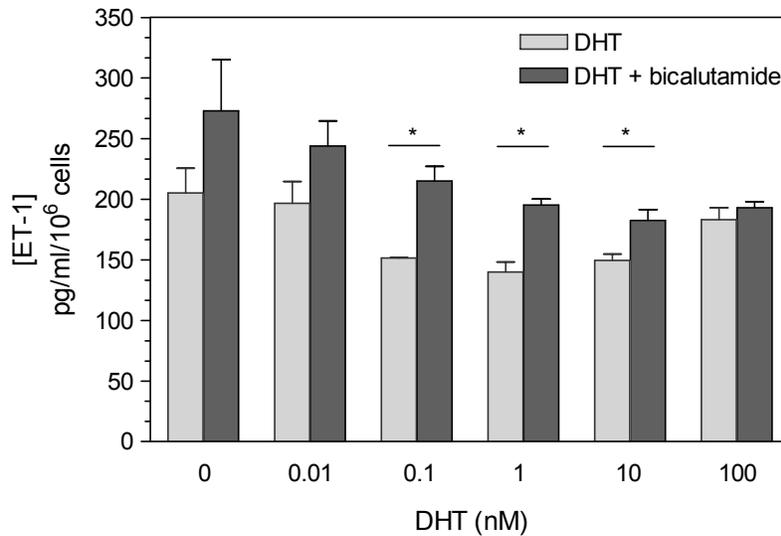
Figure 14. Changes in ET-1 secretion following acute AR blockade in LAPC4 prostate cancer cells.
ET-1 secretion in LAPC4 cells treated in serum-free media containing increasing DHT (0.01 – 100nM) or vehicle, with or without 10μM anti-androgen bicalutamide, for 48 hours, was measured by ET-1 ELISA at pg/ml/million cells. Results shown are the mean ± SEM of two separate experiments each performed in duplicate. *, $P < 0.05$.

It should be noted that these results are supported by the findings from a 2001 study by Granchi *et al*. (73), which demonstrated that acute (9hr) androgen treatment significantly decreases ET-1 mRNA production and protein secretion in PC-3 cells transfected to express AR. When treated with acute (24hr) anti-androgens flutamide or bicalutamide, ET-1 mRNA production and protein secretion increases compared to DHT-only treated cells but not significantly above control treated cells.

The effect of AR blockade on ET-1 secretion in LNCaP cells was also investigated. In addition to treating cells with increasing doses of DHT with or without 10μM bicalutamide, LNCaP cells were also cultured in 10nM DHT with or without increasing doses of bicalutamide (0.01 - 100μM) to examine the effect of high versus low levels of AR blockade. Because LNCaP cells secrete much lower levels of endogenous ET-1 than LAPC4 cells, attempts to generate a discernable effect following acute AR blockade, given either treatment regime, were

44

unsuccessful. In fact, the pattern of ET-1 secretion was typically inconsistent, and at many times ET-1 secretion was immeasurable for various treatment conditions, on several different occasions. These observations, consistent with previous findings (73), demonstrate that the mechanisms and timing of ET-1 induction during a cell stress, such as AR blockade, likely vary for different cell types.

2.2.6 ET-1 induces phosphorylation of Akt through the ET_A receptor.

The next goal was to draw a correlation between ET-1 activation, such as during AR blockade, and Akt induction to substantiate the importance of ET-1 survival signaling as an ET_A-specific mechanism during AAT. In a time-dependent manner 100nM ET-1 treatment induced the phosphorylation of Akt (pAkt) as early as five minutes after treatment in both PPC-1 and ET_A-overexpressing PPC-1 cells (PPC-1-ET_A). In ET-1 treated PPC-1-ET_A cells pAkt levels reached maximum intensity sooner (30 minutes) than in PPC-1 cells (60 minutes), and were sustained longer compared to PPC-1 cells (Fig. 15).
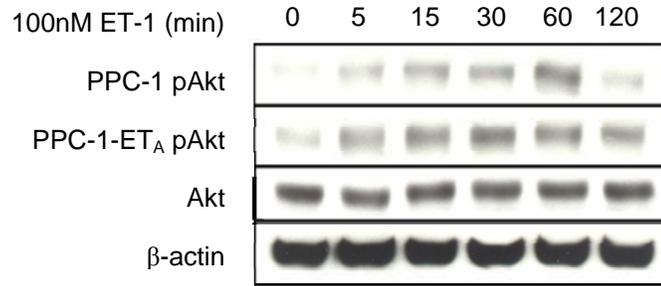
Figure 15. Time course induction of Akt phosphorylation by 100nM ET-1 in PPC-1 and PPC-1-ET$_A$ prostate cancer cells.

Immunoblots were re-probed with β-actin (43kD) (to confirm equal loading) and Akt antibody (to ensure ET-1 treatment did not affect total Akt levels). pAkt (60kD) expression was normalized for β-actin and densitometric analysis performed to determine time of maximum pAkt induction. pAkt was also compared with total Akt (60kD). Data are representative of three separate experiments.

By pre-treating cells with the selective ET$_A$ inhibitor, ABT-627 (atrasentan), there was a 34% reduction in pAkt in PPC-1-vector-only cells and a 77% reduction in pAkt in PPC-1-ET$_A$ cells (Fig. 16), demonstrating that ET-1 induction of pAkt occurs directly via the ET$_A$ receptor. Induction and knock-down of pAkt was also more significant in PPC-1-ET$_A$ cells compared to parental PPC-1 cells.
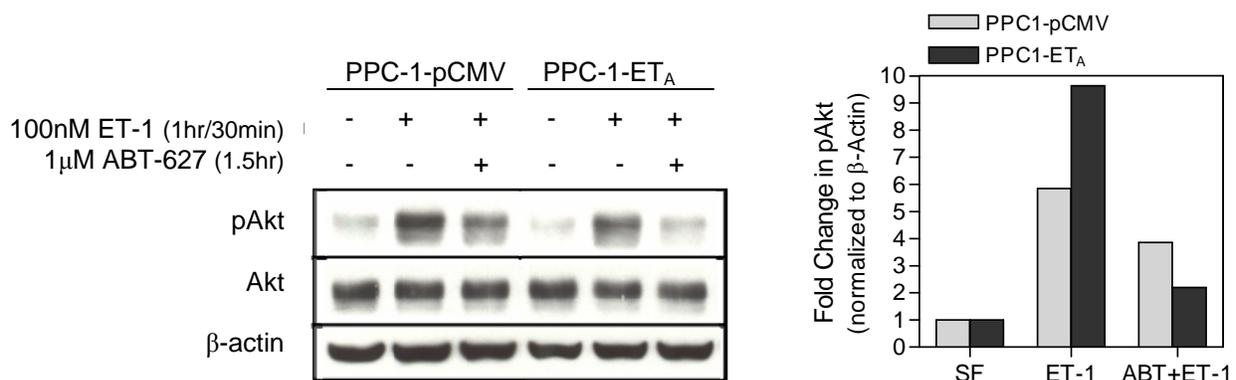
Figure 16. Specificity of ET-1 induction of pAkt through the ET$_A$ receptor.

pAkt (60kD) expression normalized for β-actin and quantified via densitometric analysis relative to serum-free (SF) treated cells, and was also compared with total Akt. Data are representative of three separate experiments.

Additionally, when plated at similar densities and treated with 100nM ET-1 over the same time course, LNCaP-AI cells exhibited increased pAkt within five minutes and reached maximum induction by 30 minutes (Fig. 17).
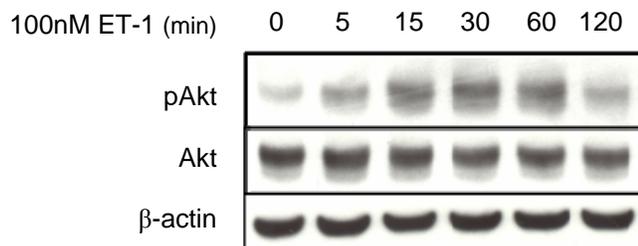
Figure 17. ET-1 time course induction of pAkt in LNCaP-AI cells.

pAkt (60kD) expression was normalized for β-actin and densitometric analysis performed to determine time of maximum pAkt induction. pAkt was also compared with total Akt. Data are representative of three separate experiments.

Pre-treating LNCaP-AI cells with ABT-627, there was a 53% reduction in Akt activation (Fig. 18), demonstrating that LNCaP-AI cells may retain elevated levels of functional ET_A, observations supported by the qPCR analysis of the chronically androgen deprived LA98 and LN96 cells (Fig. 2A, B).
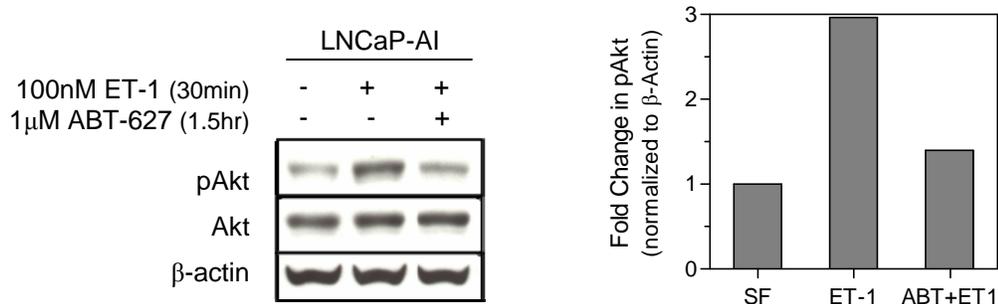
Figure 18. Specificity of Akt phosphorylation via ET-1 activation ET$_A$ in LNCaP-AI cells.
pAkt (60kD) expression normalized for β-actin and quantified via densitometric analysis relative to serum-free (SF) treated cells, and was also compared with total Akt. Data are representative of three separate experiments.

It was previously demonstrated that ET-1 treatment of ET$_A$-expressing PPC-1 prostate cancer cells protects against Taxol (paclitaxel)-induced cell death via Akt induction. It was shown that Akt phosphorylation occurs through PI3K recruitment, and that blocking PI3K with Wortmannin or LY294002 resulted in complete abrogation of pAkt (63). In this study, ET-1 treatment of prostate cancer cells achieved robust Akt activation, and with ET$_A$ blockade Akt phosphorylation is significantly but incompletely reduced, suggesting the presence of additional PI3K-activating mechanisms contributing to Akt activation.

Importantly, these results demonstrate an ET$_A$-specific mechanism of Akt activation, and that, at elevated levels, ET$_A$ signaling can affect the timing and duration of Akt activation in prostate cancer cells; a process that likely contributes to prostate cancer cell survival during androgen withdrawal. These data also show that LNCaP-AI cells likely express elevated levels of functional ET$_A$ receptor, capable of affecting Akt activation upon ET-1 stimulation. If androgen-independent prostate cancer cells maintain higher ET$_A$ receptor levels, as illustrated by the ligand binding, qPCR and pAkt immunoblot analyses, ET$_A$ receptor signaling can potentially contribute to a more therapy-resistant prostate cancer cell phenotype.

2.2.7 ET$_A$ receptor signaling in human prostate cancer cell invasion.

As mentioned earlier, several previous studies have demonstrated a role for ET-1 signaling in cell migration and invasion. In particular, one study demonstrated that ET-1 stimulation of prostate cancer cells induces focal adhesion kinase (FAK) phosphorylation leading to cell motility, and that expressing enzymatically active NEP, which cleaves ET-1, reduced cell migration due to the loss of FAK activation (103). Also, Zheng *et al.* showed that G-protein activation of RhoA is required for neuropeptide-induced prostate cancer cell migration (104). However, it remains unclear as to whether ET$_A$ signaling is a major contributor to the invasive capacity of prostate cancer cells.

To explore further the contribution of ET$_A$ activation in prostate cancer cell invasion, LNCaP, LNCaP-ET$_A$, PPC-1, and PPC-1-ET$_A$ cells were plated on a matrigel transwell membrane and treated with ET-1 with or without ABT-627. Initial results suggested that ET$_A$ activation, and not ET$_B$, might potentially affect LNCaP cell invasion. Based on these observations the effect of ET$_A$ signaling on PPC-1 cell invasion was also examined; it was found that ET$_A$-overexpression did significantly enhance PPC-1 invasion (Fig. 19A, B).
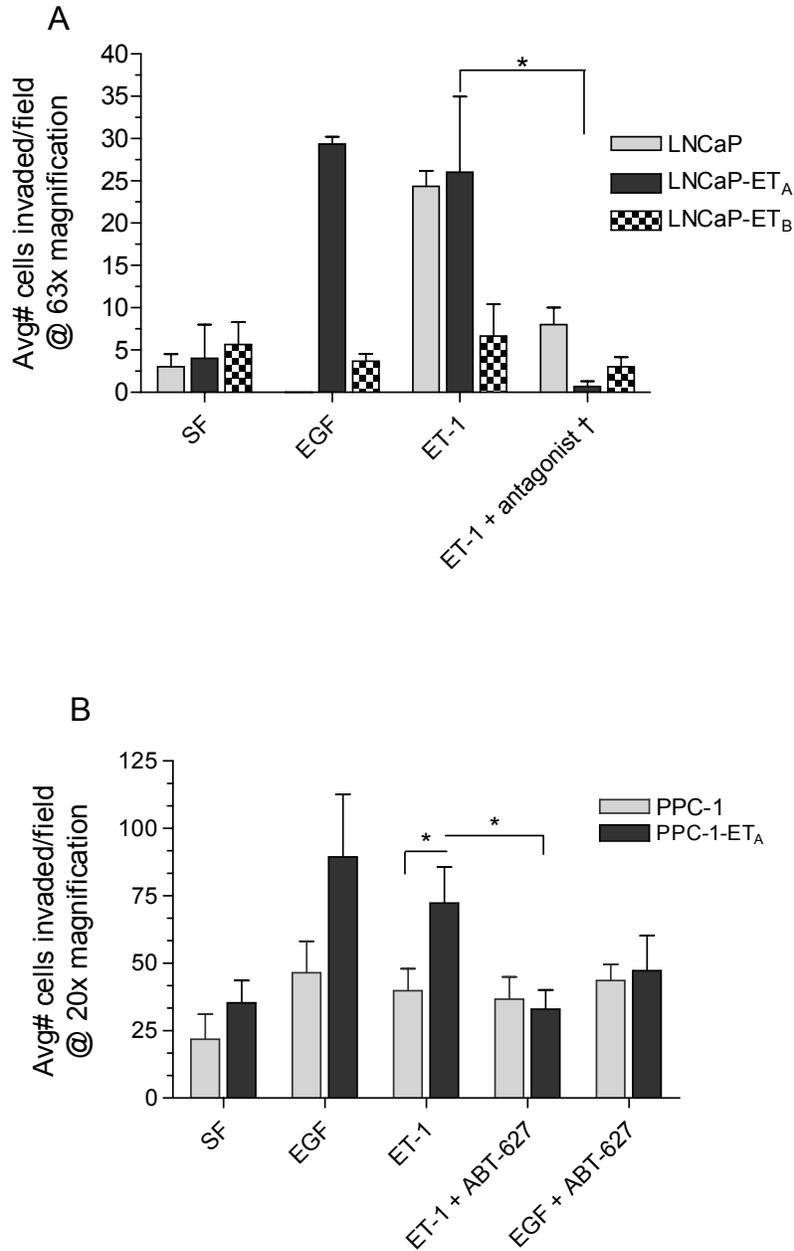
Figure 19. Prostate cancer cell invasion as measured by matrigel invasion chamber assay.
25,000 cells plated per invasion transwell and incubated for 20-22 hours at 37ºC. 100ng/ml EGF used as positive control, 100nM ET-1, 1μM antagonist (ABT-627 or ABT-621). †LNCaP cells received both ABT-627 and ABT-621, LNCaP-ET$_A$ received ABT-627, and LNCaP-ET$_B$ received ABT-621. Reduction in EGF-induced PPC-1-ET$_A$ invasion with ABT-627 pretreatment not significant. Assays performed in triplicate. Error bars indicate ± SEM. *, *P* < 0.05.

However, upon repeating this protocol several times with both cell types and various ET_A-overexpressing clones, the results were inconsistent and irreproducible. Inconsistencies in the numbers of invading cells can be related to poor adhesion in a serum free environment, especially when working with LNCaP cells. Also, the distribution of cells that successfully invaded through the matrigel to the other side of the porous membrane was highly variable and not uniform, suggesting that further optimization of the assay is needed. Although technical challenges likely contributed to inconsistencies in performing this assay, these results suggest that ET_A signaling may influence prostate cancer cell motility, but much work remains to determine the extent of this effect and whether endothelin-induced invasion is an ET_A direct or indirect mechanism. Some studies have demonstrated that ET-1 activation of the ET_A receptor leads to transactivation of the EGF-receptor (EGFR) (105,106). EGFR activation has been well documented in driving cellular motility and therefore the potential link between ET_A and EGFR signaling cannot be ignored.

2.2.8 ET receptor expression in androgen ablated human prostate tissue.

To validate the *in vitro* findings of increased ET_A expression following androgen deprivation and provide relevance for ET_A receptor survival signaling in human prostate cancer, the effect of AAT on ET_A receptor expression in human prostate tissue was investigated. Formalin fixed and paraffin embedded prostate tissue sections were obtained from radical prostatectomy and transurethral resection (TURP) specimens that have undergone hormone therapy. Immunohistochemical analysis of ET_A expression in human prostate tissue from patients receiving acute, three month androgen ablation (Lupron depot), prior to prostatectomy, compared

51

with hormone intact patients or healthy donors, demonstrated decreased levels of ET_A expression in cancer regions (Fig. 20B-E, I). However, patients with advanced prostate cancer that received long-term ablation (>6 months) followed by channel TURP were separately evaluated: in tumor bearing regions, ET_A receptor expression was significantly elevated compared to intact patients or donor tissue (Fig. 20A, F-H, I).
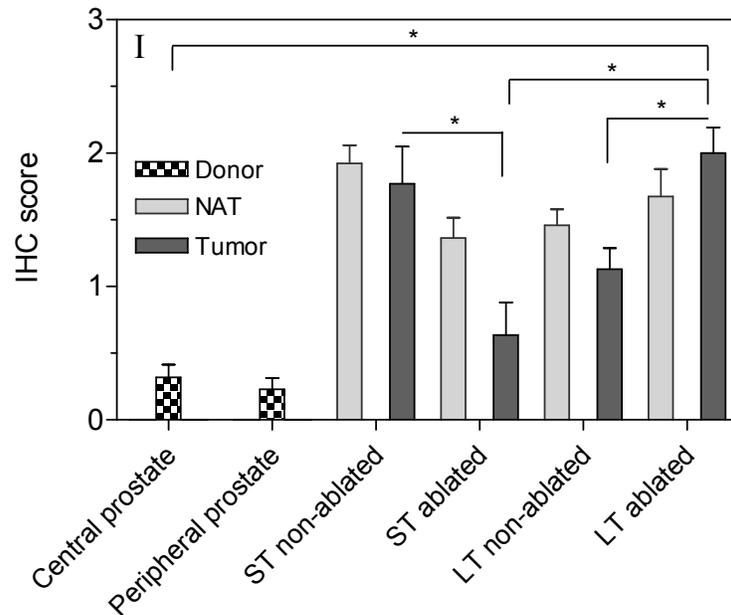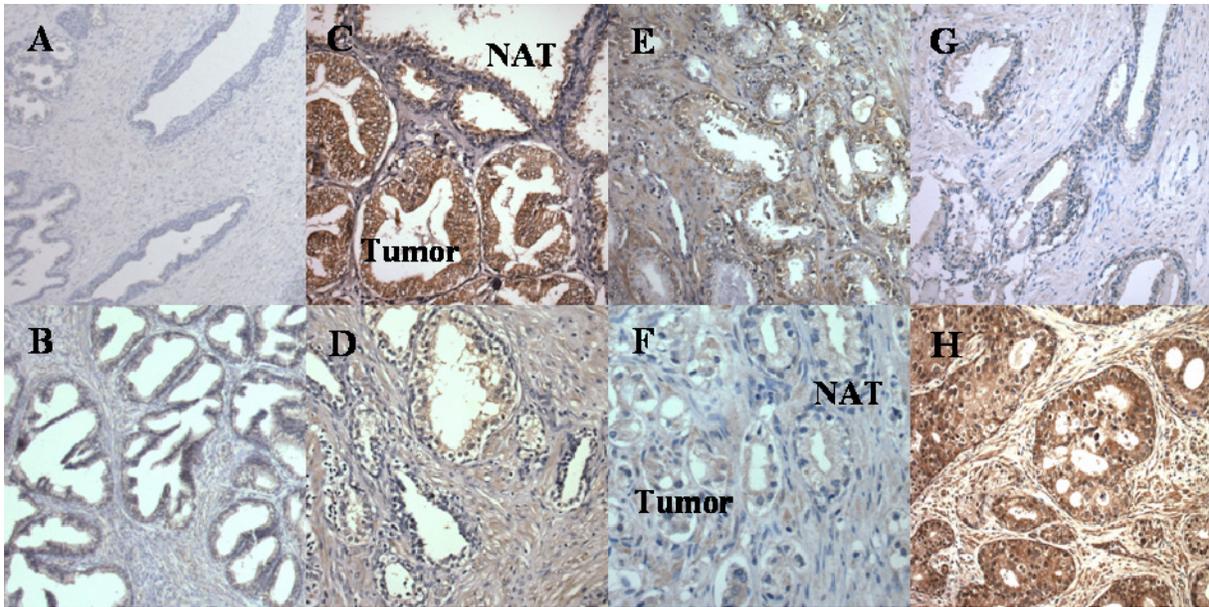
Figure 20. Immunohistochemical analysis of ET$_A$ receptor expression in human prostate tissue.

A, donor central, 0; *B*. donor peripheral, 1+; *C*. ST non-ablated NAT, 2+; tumor, 2+; *D*. ST ablated NAT, 1+; *E*. ST ablated tumor, 1+; *F*. LT non-ablated NAT, 1+; tumor, 2+; *G*. LT ablated NAT, 1+; *H*. LT ablated tumor, 3+. *C-E*, from radical prostatectomy (RP); *F-H*, from transurethral resection of the prostate (TURP). Donor sections photographed at 10x, NAT and tumor sections at 20x. *I*. Quantitative assessment of ET$_A$ expression for indicated prostate cancer tissue specimens. Receptor immunostaining was blindly scored by a pathologist on a scale of 0 to 3+. (NAT), normal adjacent to tumor. Quantitative results shown are the mean ± SEM *, $P < 0.05$.

For clinical relevance, it was critical to examine ET_A receptor expression in androgen ablated human prostate cancer tissue. The decreased ET_A expression in short-term ablated tissues was unanticipated, but is consistent with the qPCR analysis of time-course androgen deprived LAPC4 cells shown in figure 6. In patients receiving at least six months of AAT ET_A receptor expression was elevated in the epithelial compartment of tumor-bearing regions. Combined with the qPCR analysis of androgen deprived LNCaP cells in figure 3, which show increased ET_A expression between one and five months, these observations provide an approximate timeline of ET_A induction in response to androgen ablation, thus indicating a potential time for clinical intervention.

All patient tissue samples were also stained for the ET_B receptor; ET_B expression in short- and long-term androgen ablated tissues decreased significantly relative to the respective donor tissues (Fig. 21). Comparing ablated tumor bearing tissue sections, ET_B expression was significantly greater in the long-term ablated tumor tissues than in the short-term ablated tissues, results that support the increased ET_B expression shown by the qPCR analysis in figure 4. These results demonstrate that ET_B expression is reduced during early stage hormone treatment but returns to near normal expression levels following longer term therapy.
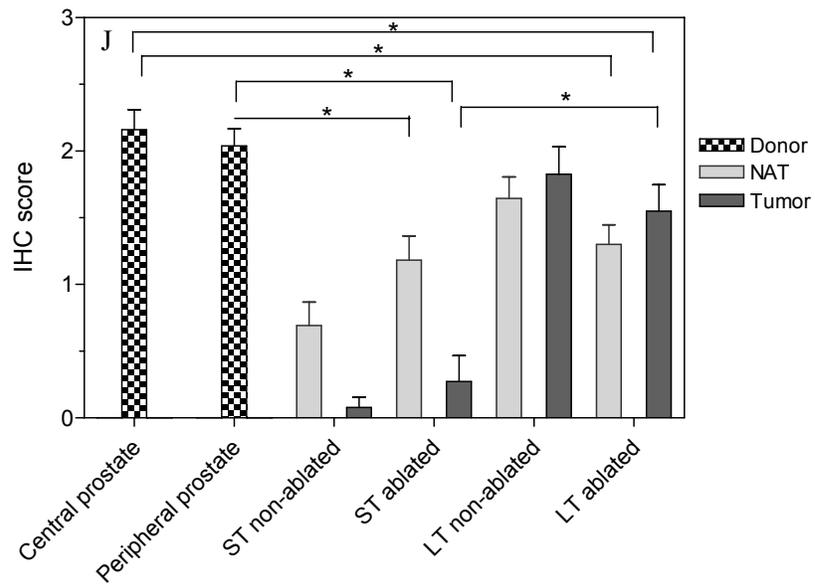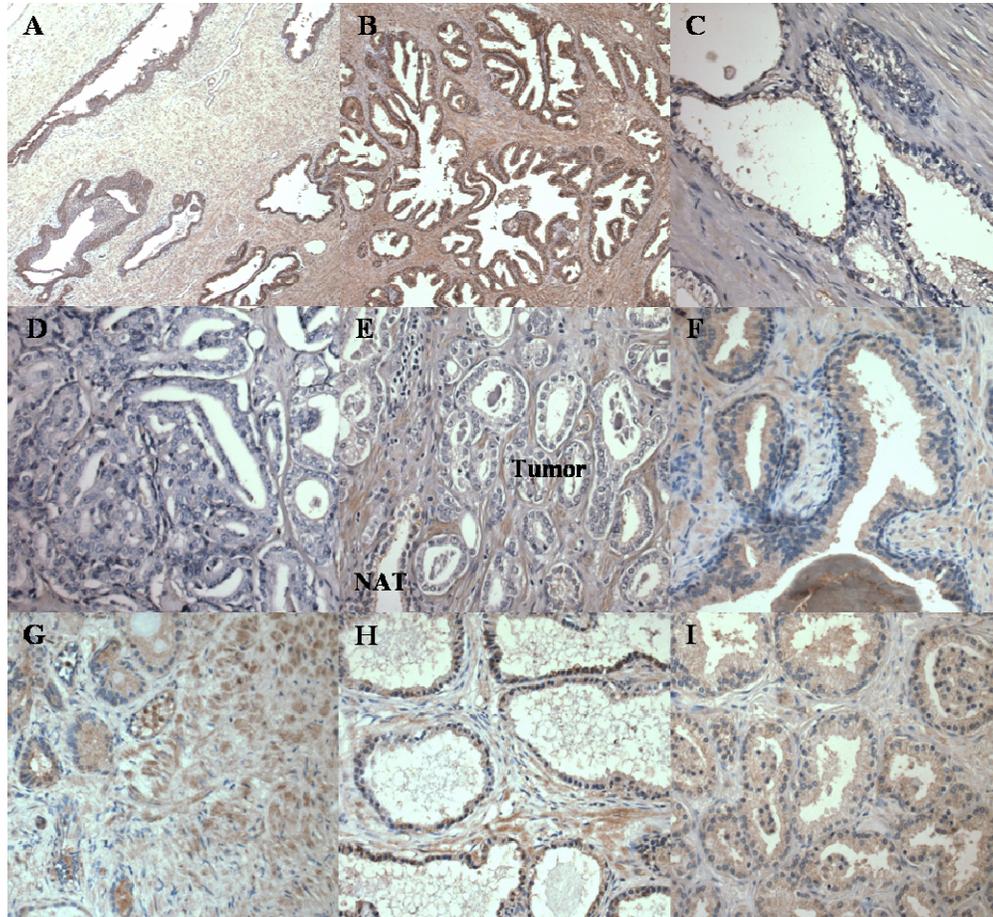
Figure 21. Immunohistochemical analysis of ET$_B$ expression in human prostate tissue.

A, donor central, 2+; *B*. donor peripheral, 3+; *C*. ST non-ablated NAT, 1+; *D*. ST non-ablated tumor, 0; *E*. ST ablated NAT 1+; tumor, 0; *F*. LT non-ablated NAT, 1-2+; *G*. LT non-ablated tumor, 2+; *H*. LT ablated NAT, 1+; *I*. LT ablated tumor 1+. *C-E*, from radical prostatectomy (RP); *F-H*, from transurethral resection of the prostate (TURP). Donor sections photographed at 10x, NAT and tumor sections at 20x. *J*. Quantitative assessment of ET_A expression for indicated prostate cancer tissue specimens. Receptor immunostaining was blindly scored by a pathologist on a scale of 0 to 3+. (NAT), normal adjacent to tumor. Quantitative results shown are the mean ± SEM *, $P < 0.05$.

It is important to mention that, as anticipated, ET_B receptor expression in normal, donor tissues (IHC score ~2.1) is greater than that of ET_A (~0.3). Also, in the short-term non-ablated tumor and NAT tissues, ET_A expression increases whereas ET_B decreases. A result not anticipated was the increase in ET_B in long-term ablated tissues relative to short-term tumor tissues, nearly restoring ET_B expression levels back to that of donor tissues. This suggests that, as a result of androgen deprivation, there is a release of the inhibitory mechanism responsible for repressing ET_B expression in prostate cancer. Whether the increase in ET_B expression following androgen deprivation has a functional consequence, and therefore implications for ablated patients, remains to be determined.

Having demonstrated in this study that ET-1 signaling results in ET_A-specific Akt activation, and that Akt induction inhibits apoptosis in prostate cancer cells (63), these results implicate increases in ET_A receptor expression, and not ET_B, in promoting prostate cancer cell survival during AAT and progression to androgen-independent disease.

2.2.9 *In vitro* analysis of combination ET$_A$ blockade (ABT-627) plus Taxotere (docetaxel) treatment of prostate cancer cells.

Docetaxel is a cytostatic agent that binds to the β-subunit of tubulin, resulting in irreversible polymerization of microtubules. Stabilization of microtubules, which comprise the mitotic spindles, effectively paralyzes the cell in mitosis, leading to initiation of the apoptotic cascade. Using the MTT viability assay and Flow cytometry, the question of whether ET$_A$ antagonism could enhance prostate cancer cell sensitivity to docetaxel treatment in both control (LNCaP and PPC-1) and ET$_A$-overexpressing (LNCaP-ET$_A$ and PPC-1-ET$_A$) cells was tested. Cells were treated in serum-free media with 1) vehicle, 2) 100nM ET-1, 3) 10nM docetaxel, 4) ET-1 + docetaxel, 5) 1μM ABT-627 + ET-1 + docetaxel. For both MTT and Flow, ABT-627 and ET-1 pretreatments were for 1 hour. For MTT cell viability analysis, docetaxel treatment varied from 24 to 72 hours. Based on previous studies and time course determination, earlier docetaxel time points (4 hour – LNCaP; 24 hour – PPC-1) were analyzed by Flow analysis of Annexin V-FITC binding because flipping of phosphatidylserine to the outer membrane is an early stage event in apoptosis.

In most of the MTT experiments, single regime docetaxel treatment as well as combination ABT-627 + ET-1 prior to docetaxel treatment resulted in decreased cell viability compared to control or ET-1 treated cells, regardless of cell type. However, ET-1 pretreatment never generated a protective effect leading to increased cell viability compared to docetaxel alone or combination ABT-627 + ET-1 + docetaxel (Fig. 22).
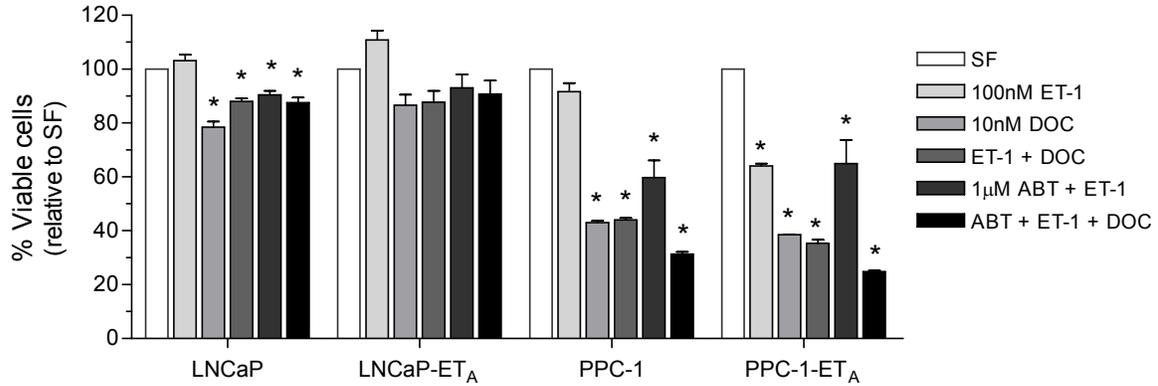
Figure 22. Effect of combination docetaxel + ABT-627 treatment on prostate cancer cell viability.
(Representative MTT assays. Docetaxel treatment: LNCaP – 24hrs; PPC-1 – 48hrs). Results shown are the mean ± SEM. Analyzed by one-way analysis of variance (ANOVA) *, $P < 0.05$.

However, after running two-way ANOVA on the raw O.D. values to compare between cell types, the LNCaP-ET_A cells typically showed lower viability compared to parental LNCaP cells, whereas PPC-1-ET_A cells typically demonstrated greater viability compared to PPC-1 cells (Fig. 23).
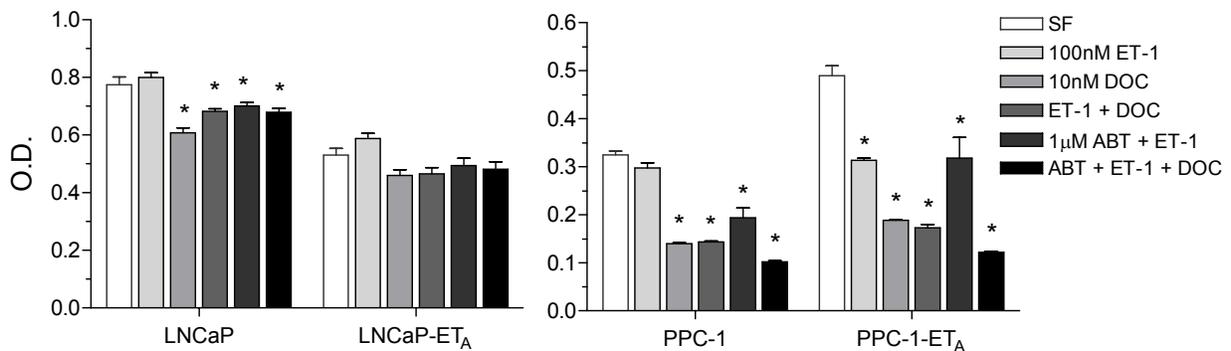
Figure 23. Prostate cancer cell viability following treatment with 10nM docetaxel, as indicated.
Results were analyzed by two-way ANOVA and shown are the mean ± SEM *, $P < 0.05$.

After several attempts of varying the docetaxel treatment times, observations were irreproducible and therefore inconclusive.

Suspecting poor cell adhesion during pretreatments as a source of limitation for using the MTT assay platform, Flow cytometric analysis was applied to this line of investigation. Furthermore, various ET_A-overexpressing LNCaP clones were employed in addition to PPC-1-ET_A cells. As described in the methods, ABT-627 and ET-1 pretreatments were for 1 hour before docetaxel treatments. The hypothesis was that ET_A-overexpression would provide a survival advantage; however, neither LNCaP-ET_A nor PPC-1-ET_A cells were more resistant to docetaxel treatments compared to the respective parental control cells. Moreover, ET-1 pretreatment prior to docetaxel never demonstrated a rescuing effect compared to docetaxel-only treated cells. Regardless of treatment times and independent of cell type or ET_A clone used, the results from the Flow cytometric analysis demonstrated a very high degree of variability and inconsistency and therefore are inconclusive.

2.2.10 Castration plus ET_A blockade increases prostate cancer cell doubling time *in vivo*.

To address the efficacy of combination androgen ablation (castration) plus ET receptor blockade *in vivo*, tumor progression of prostate cancer cell xenografts grown in male nude mice was examined. Three groups of 40 mice received subcutaneous injection of either parental LNCaP, LNCaP-ET_A or ET_B-overexpressing LNCaP (LNCaP-ET_B) cells. Following randomization, mice were separated into 4 treatment subgroups: 1) intact + vehicle, 2) intact + antagonist, 3) castrate + vehicle, and 4) castrate + antagonist. At the time of castration LNCaP bearing mice received ET_A antagonist ABT-627 and LNCaP-ET_B bearing mice received the ET_B antagonist

ABT-621. Due to tumor progression, LNCaP mice were followed for a total of 7 weeks and LNCaP-ET_B mice were followed for 10 weeks.

For the LNCaP mice, linear growth curve analysis of tumor volume progression was not applicable because at the time of randomization there was too much variation in tumor size within each subgroup: some mice had large tumors while some had no tumor at all. Also, several mice did not survive the duration of the seven week period, making it impossible to incorporate their tumor progression data into a standard growth curve analysis. This variability would be a reason for using nonlinear mixed effects in the context of the exponential growth model. The growth pattern served as a template for determining growth parameters by nonlinear mixed effects modeling analysis and therefore was able to incorporate every data point regardless of whether a given mouse reached the study endpoint. As a result, the nonlinear analysis demonstrated that when mice were treated with combination therapy compared to either treatment alone, or vehicle, there was a significant delay in tumor cell doubling time (Table 1). In other words, ET_A blockade significantly enhanced the effects of androgen deprivation in decreasing tumor cell growth, which equates to increased time to disease progression.

Table 1. Castration plus ABT-627 significantly inhibits LNCaP prostate cancer cell growth, compared to either treatment alone.

Treatment group (n)	Approx. doubling time (days)	Avg PSA velocity (ng/ml/week)
Intact + vehicle (5)	15	5.32
Intact + ABT-627 (4)	55	0.57
Castrate + vehicle (8)	23	-3.40
Castrate + ABT-627 (8)	156[†]	-0.95

[†] *P*, <0.0001

These results demonstrate the therapeutic advantage of combination therapy, and provide support for the targeted use of ET_A receptor antagonism in treating advanced prostate cancer. Additional study endpoints were evaluated to assess the impact of treatment in mice. Castrated mice demonstrated negative PSA velocities indicating that castration effectively suppressed LNCaP cell AR transcriptional activity (Table 1). Also, tumors were removed, fixed and analyzed for ET_A and ET_B expression via immunohistochemistry; however, no significant differences were observed between treatment groups in terms of ET receptor expression (Fig. 24).
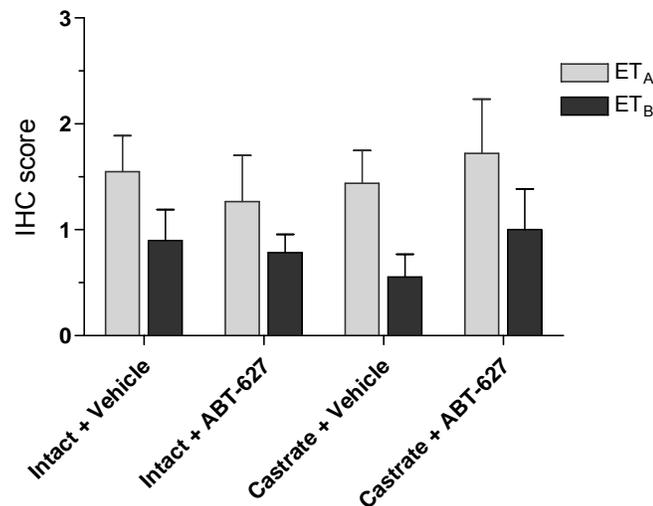
Figure 24. IHC analysis of ET_A and ET_B receptor expression in LNCaP cell xenografts following 7 weeks of treatment.
Staining intensities were blindly scored by a UPMC pathologist on a scale of 0 to 3+. Quantitative results shown are the mean ± SEM.

In prostate cancer ET_B primarily functions in sequestration of ET-1 but is suspected to play a role in inducing apoptosis. In a parallel study, the effect of ET_B blockade on tumor progression was examined by using the selective ET_B receptor antagonist, ABT-621, in

combination with castration. However, LNCaP cells express very low levels of endogenous ET$_B$; therefore, a second set of 40 mice were injected with LNCaP-ET$_B$ cells. Due to tumor regression in several mice, there remained only enough mice bearing tumors to constitute two treatment groups: castrate + vehicle and castrate + ABT-621. After 10 weeks of treatment there was a trend towards a decrease in tumor progression in the mice treated with combination castration plus ABT-621 treatment, but the differences in the end were not significant due to small sample size with large variation in tumor burdens within each treatment group (Fig. 25).
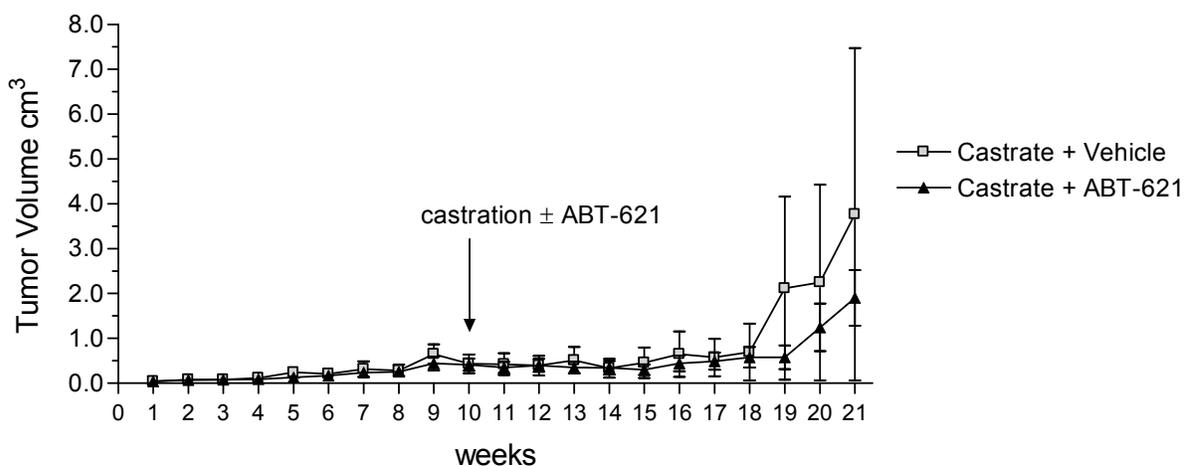
Figure 25. Analysis of LNCaP-ET$_B$ xenograft tumor progression.
Two-way ANOVA performed to examine the influence of treatment on tumor growth.

Lastly, the ultimate goal of this line of *in vivo* investigation was to examine the effect of ET$_A$ blockade, in combination with castration, in the context of ET$_A$-overexpression. The third set of 40 athymic male mice received injections of LNCaP-ET$_A$ cells. After three weeks, tumors developed in 25 of the 40 mice (62.5%) and two weeks later 36 (90%) had palpable tumors. However, over the next four weeks every mouse bearing a LNCaP-ET$_A$ tumor experienced

complete regression of their tumor burden; therefore, no treatment regime was ever initiated in these mice.

One possible explanation for the spontaneous regression of the LNCaP-ET$_A$ tumors is that the LNCaP-ET$_A$ clone used for the study was not able to survive in the subcutaneous murine environment, whereas the heterogeneous population of wild-type LNCaP cells more likely possessed a rare tumor cell capable of growing in athymic mice. Alternatively, it was possible that the exogenous ET$_A$-overexpression may have created an environment hostile to angiogenesis through its vasoconstrictive properties, effectively starving the developing tumor mass. As mentioned earlier, ET$_A$ and ET$_B$ receptor signaling in the human body are predominantly associated with regulating blood vessel physiology. ET$_A$ signaling promotes contraction of the surrounding smooth muscle leading to vasoconstriction, whereas ET$_B$ activation at the site of the endothelial cells provokes the opposing effect of vasodilation through nitric oxide production. While there was probably no developed smooth muscle in the tumors, it does not rule out ET$_A$ signaling affecting the development of new blood vessels. In support of this hypothesis, tumors removed from the LNCaP mice demonstrated extensive necrosis particularly in the center of the tumor masses where no blood vessels were detected by CD31 staining (Fig. 26). However, tumors removed from LNCaP-ET$_B$ mice showed consistent vascularization throughout the entire tumor mass as demonstrated by more robust CD31 staining (Fig. 26).
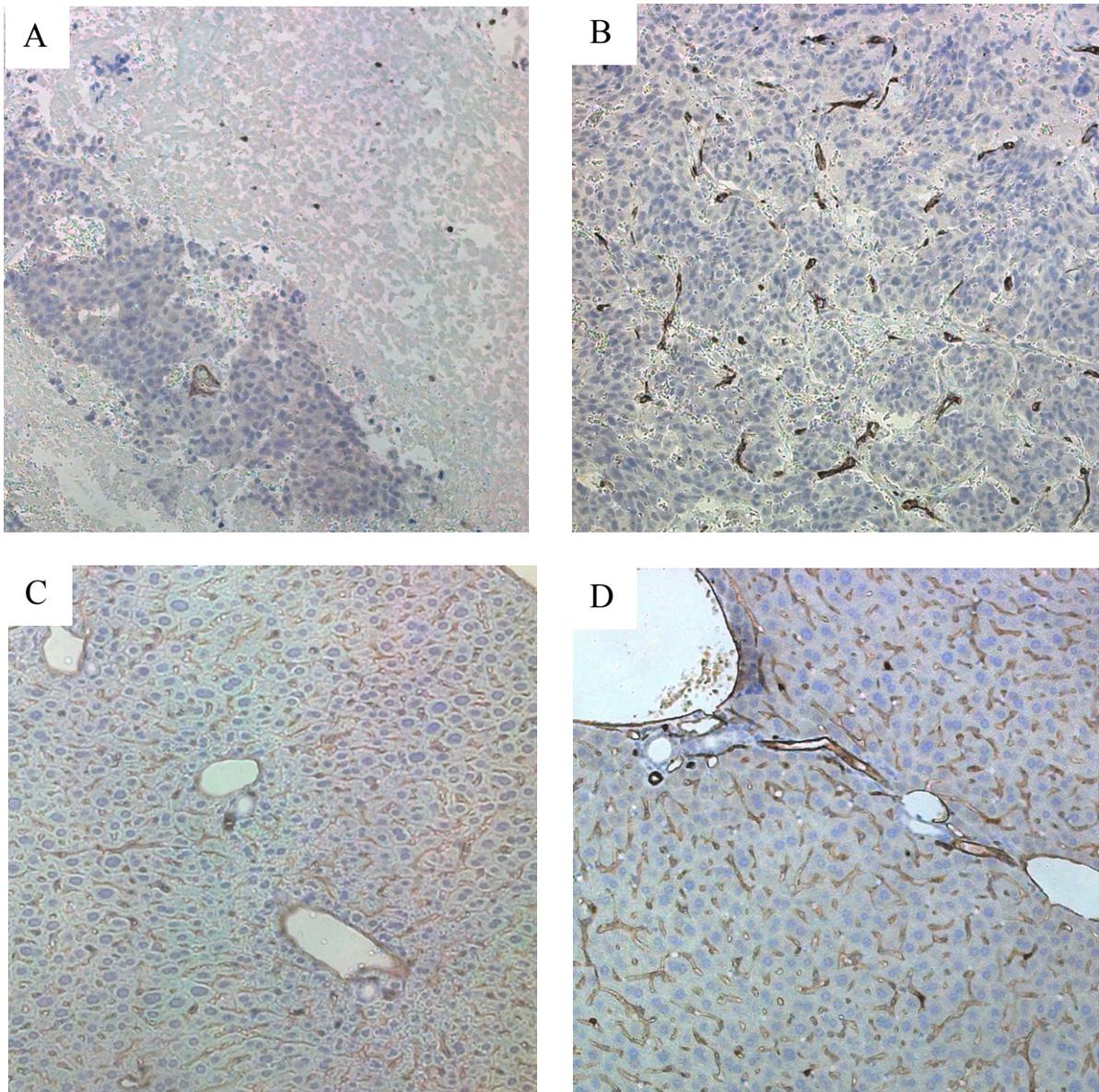
Figure 26. CD31 immunostaining in LNCaP and LNCaP-ET$_B$ xenograft sections.
CD31 expression in representative *A*. LNCaP and *B*. LNCaP-ET$_B$ xenograft sections, and in *C-D*. positive control liver sections from respective mice. Note the predominance of necrotic tissue in the LNCaP xenograft section compared to the more highly vascularized LNCaP-ET$_B$ xenograft. All sections photographed at 10x.

These observations suggest that if ET$_B$-overexpression somehow supported angiogenesis and that blocking the pro-angiogenic response with ABT-621 resulted in a trend towards reduced tumor burden (Fig. 25), ET$_A$-overexpression might be generating an anti-angiogenic response in

64

the LNCaP-ET$_A$ xenograft tumors. To test this hypothesis, mice with established LNCaP-ET$_A$ tumors could be treated with ABT-627 to determine if ET$_A$-blockade promotes blood vessel development and subsequent tumor progression.

2.3 CONCLUSIONS

In this series of experiments it was shown that long-term androgen deprivation of prostate cancer cells significantly affected the gene expression of ET-1, ET$_A$, ET$_B$, AR and NEP. In short-term deprived prostate cancer cells, expression of ET$_A$ and ET$_B$ were reduced, but ET-1 expression increased rapidly. These results suggest that up-regulation in the ligand occurs as a more immediate response, and only after sustained deprivation is there an increase in the receptors to aid in maintaining endothelin signaling.

Long-term androgen deprivation of LNCaP prostate cancer cells resulted in the emergence of a highly proliferative clone, LNCaP-AI, characterized by decreased AR expression and accelerated androgen-independent growth. However, LNCaP-AI cells are not insensitive to androgens as treatment with DHT induced the expression of AR, ET$_A$ and ET-1, and affected cell viability at more extended periods of treatment.

At the protein level, it was shown that fluctuations in androgens as well as complete disruption of AR signaling resulted in rapid secretion of ET-1. But the response of ET-1 secretion is not a simple linear process that increased with increasing DHT. When ET$_A$-expressing prostate cancer cells were treated with ET-1, it was also shown that, at elevated levels of expression, ET$_A$ activation specifically leads to the phosphorylation of Akt. In terms of

65

prostate cancer cell invasiveness, the matrigel invasion studies establish a potential link between ET-1, ET_A and motility. Although not conclusive, these results are encouraging and provide additional rationale to pursue future studies.

Performing immunohistochemical staining on androgen ablated human prostate tissue established that, at the protein level, ET_A receptor expression was elevated only in those patients treated with AAT at least six months. ET_B expression, like ET_A, was decreased in the short-term ablated patient tissues, but ET_B expression never surpassed that of donor or non-ablated control tissue samples in the long-term. This, in light of the qPCR data in figure 4, suggests some mechanism, such as mRNA instability, responsible for the dramatic increase in ET_B mRNA that does not equate into increased receptor protein.

To test the hypothesis that ET-1 can protect ET_A-overexpressing prostate cancer cells from apoptosis-inducing effects of docetaxel, both MTT and Flow cytometry platforms were employed. Previous work by Nelson *et al*. established a clear link between endothelin and prostate cancer cell survival (63). However, the results in figures 22 and 23 illustrate the difficulty in demonstrating the endothelin survival response in the isolation of *in vitro* experimental systems. Incorporating ABT-627 prior to ET-1 and docetaxel treatment appears to result in increased cell death; however, ET-1 pretreatment prior to docetaxel failed to demonstrate a protective effect. Clinically, these results are still relevant as ET_A blockade increased prostate cancer cell sensitivity to a chemotherapeutic agent, but it remains unclear as to whether or not exogenous ET_A-overexpression is functioning as expected. Additionally, these assays likely need fine tuning with respect to timing of the treatments and use of the Flow cytometer.

Moving into an athymic mouse model to test the efficacy of combination ET_A blockade plus castration, a significant reduction in the rates of LNCaP xenograft tumor cell proliferation was demonstrated, but only with combination therapy. The reduced tumor take exhibited by LNCaP-ET_B tumor bearing mice bore out only enough mice for two treatments groups: intact + ABT-621 and castrate + ABT-621; however, there was not statistical difference between the effects of these treatments on tumor progression. Most striking of all the observations was the complete regression of every LNCaP-ET_A xenograft tumor in the athymic mice. Having witnessed and measured palpable tumors for up to six weeks, poor tumor take is likely not the reason for the disappearance of these tumors.

Induction of Akt phosphorylation in LNCaP and LNCaP-ET_A cells was also examined; however, both LNCaP and LNCaP-ET_A cells exhibit very high basal levels of pAkt indicative of constitutive activation, thus it was difficult to demonstrate further increased Akt activation following ET-1 treatment. The use of charcoal-stripped FBS in androgen depriving prostate cancer cells has been used extensively because of its specificity for filtering out only low molecular weight aromatic compounds such as testosterone. Unfortunately, molecules such as 17β estradiol, parathyroid hormone, follicle-stimulating hormone, and luteinizing hormone, and several vitamins, including folate, retinol, and vitamin D are also reduced. Nonetheless, this does not devalue these findings because one can still appreciate the impact of increased ET_A and ET-1 expression: the withdrawal of testosterone in addition to these other compounds represents a cell stress that appears to induce the endothelin expression. Additionally, the endothelin survival advantage was not demonstrated in ET_A-overexpressing prostate cancer cells when treated with ET-1 + docetaxel compared to docetaxel alone, suggesting that up-regulation of exogenous ET_A may lack the functional capacity that is observed *in vivo*.

67

Overall, the results discussed in this section support the concept of ET-1 functioning through the ET_A receptor in promoting prostate cancer cell survival. They also establish the notion that ET_A signaling likely contributes to cell survival during androgen deprivation, and thus have very important implications for patients with advanced prostate cancer receiving AAT: if ET-1 in fact promotes Akt activation via ET_A, then it is reasonable to conclude that increased ET_A signaling contributes to prostate cancer cell survival during AAT.

3.0 LONGITUDINAL ANALYSIS OF ANDROGEN DEPRIVATION OF PROSTATE CANCER CELLS IDENTIFIES PATHWAYS TO ANDROGEN INDEPENDENCE

(Adapted from manuscript Jason M. D'Antonio, Changqing Ma, Federico A. Monzon, and Beth R. Pflug, Departments of Urology and Pathology, University of Pittsburgh School of Medicine, *submitted for publication*)

3.1   INTRODUCTION

Prostate cancer is the most widely diagnosed cancer in American men with an approximate 27,350 deaths expected annually. The interaction between testosterone and the AR is essential for prostate development. Because AR signaling has also been shown to play a key role in prostate carcinogenesis, AAT is a commonly used form of treatment, particularly for advanced disease. While AAT leads to significant levels of prostate cancer cell apoptosis, the effect is short-lived and ultimately not curative as most patients develop androgen-independent disease. In the late 80's, Isaacs and Coffey proposed a stem cell model for the organization of the prostate. In this model, pluripotent stem cells reside within the basal cell compartment and give rise to terminally differentiated luminal epithelial cells (107). It has since been postulated that while the luminal epithelial cells undergo apoptosis, the putative androgen-independent, basal-layer epithelial stem cells survive AAT, proliferate in a less restrictive environment, resulting in

recurrent androgen-independent disease (108-111). Recognizing this, it was proposed that treatment failure occurs, in part, due to androgen-sensitive, AR positive, luminal epithelial cells adapting to a low-hormone environment by promoting survival and suppressing apoptosis.

Focusing on the role of the ET-1 signaling through the ET_A receptor in prostate cancer progression, it was previously established that ET-1 treatment of ET_A-expressing prostate cancer cells induces Akt activation resulting in cell survival (63), and that ET_A blockade using the selective, high-affinity ET_A inhibitor, ABT-627, restores sensitivity to paclitaxel and docetaxel treatment (87). Additionally, it has been demonstrated that chronically androgen deprived LNCaP clones are significantly more tumorigenic than wild-type LNCaP cells when injected into both castrated and intact male nude mice. More importantly, when injected with androgen deprived LNCaP clones, 87% of castrated mice formed tumors compared to 47% of intact mice, signifying that chronically androgen deprived LNCaP cells have adapted to grow in a low androgen environment, but retain sensitivity to androgens as AR expression remains (98).

The most common means of androgen depriving AR-expressing prostate cancer cells for extended periods of time, *in vitro*, is by culturing in charcoal-dextran stripped fetal bovine serum (csFBS)-supplemented media because the filtering process is highly specific for removing only low molecular weight aromatic compounds such as testosterone. In the context of AAT, laboratory studies focused on how endothelin survival signaling contributes to treatment failure and progression to androgen-independent prostate cancer. Therefore, early-passage LNCaP cells were grown in 10% csFBS for 12 months and ET-1, ET_A and AR mRNA expression changes

were quantified, relative to control cells, using real time quantitative RT-PCR[2]. The LNCaP tumor cell line was selected because it expresses AR, responds to androgens, secretes PSA yet was derived from a lymph node metastasis. At time points reflecting critical growth and phenotypic changes, Affymetrix expression array analysis was performed to examine the effects of androgen deprivation during the acute response, during the period of apparent quiescence, and during the emergence of highly proliferative, androgen-independent prostate cancer cells (LNCaP-AI). Here it is demonstrated that, in addition to ET-1 and the ET_A receptor, a variety of other important genes undergo significant expression changes, thereby painting an even more vivid picture of prostate cancer cell adaptation to an androgen depleted environment.

3.2   RESULTS

3.2.1   Alterations in ET_A, ET-1, AR and GR expression during androgen deprivation.

To investigate the effects of long-term androgen deprivation, LNCaP cells were cultured in media containing either FBS or csFBS and analyzed gene expression by Affymetrix GeneChip HG-U133 Plus 2 expression arrays. From the 20,646 genes examined by microarray Table 2

---

[2] Jason M. D'Antonio, Geeta Godara, Drazen M. Jukic, Robert R. Bies, and Beth R. Pflug. ET_A Receptor Signaling as a Key Mechanism in the Emergence of Androgen-Independent Prostate Cancer. *Under Review*.

outlines a subset of genes that were found to have altered expression, many of which are relevant to prostate cancer cell growth, proliferation, survival, or apoptosis signaling.

Table 2. Genes analyzed for altered expression in long-term androgen deprived LNCaP cells.

†Change: early (e) = 3 week – 1 month; middle (m) = 5 month; late (l) = 12 month; (s) = sustained; (ns) = not significant. Changes in gene expression at each androgen deprivation time point, compared to control levels, were analyzed for statistical significance by one-way analysis of variance.

Symbol	Gene group and description	†Change
	Endothelin and nuclear hormone receptors	
EDNRA	Endothelin receptor type A (ET$_A$)	increase (m)
EDN1	Endothelin 1 (ET-1)	increase (e, m)
AR	Androgen receptor	increase (e)
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	increase (m, l)
	Stem cell, basal cell and epithelial cell markers	
NANOG	Nanog homeobox	unchanged (undetectable)
POU5F1	POU domain, class 5, transcription factor 1 (Oct-3) (Oct-4)	unchanged (undetectable)
CD44	CD44 antigen (homing function and Indian blood group system)	unchanged (undetectable)
KRT5	Keratin 5	unchanged (undetectable)
KRT14	Keratin 14	unchanged (undetectable)
TP73L	Tumor protein p73-like (p63)	unchanged (undetectable)
KRT8	Keratin 8	unchanged
KRT18	Keratin 18	unchanged
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	unchanged
	Growth and survival	
ERBB2	Human epidermal growth factor receptor (Her2/*neu*)	increase (e)
MET	Met proto-oncogene (hepatocyte growth factor receptor)	increase (m, l)
TGFBR1	Transforming growth factor, beta receptor I	increase (m, l)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	increase (m)
JAG1	Jagged 1 (Alagille syndrome)	increase (l)
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	unchanged
SHH	Sonic hedgehog homolog (Drosophila)	increase (m, l)
MMP-7	Matrix metalloproteinase 7	increase (m)
	Cell cycle and apoptosis	
TP53	Tumor protein p53 (Li-Fraumeni syndrome)	decrease (e, m)
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	decrease (e, l)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	decrease (l)
CDK2	Cyclin-dependent kinase 2	decrease (e, m)
CDK4	Cyclin-dependent kinase 4	decrease (s)
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	decrease (e, m)
BCL2	B-cell CLL/lymphoma 2	unchanged
BCL-XL	BCL2-like 1	unchanged
BAD	BCL2-antagonist of cell death	unchanged
BAX	BCL2-associated X protein	decrease (s)

BAK1	BCL2-antagonist/killer 1 (BCL2-like 7 pseudogene 1)	decrease (e)
FAS	Fas (TNF receptor superfamily, member 6)	decrease (s)
CYCS	Cytochrome c, somatic	decrease (e, m)
AR co-regulator, AR-regulated and androgen metabolism		
NCOA1	Nuclear receptor coactivator 1, (SRC1)	increase (e, m)
NCOA2	Nuclear receptor coactivator 2, (TIF2/SRC2)	increase (s)
NCOA3	Nuclear receptor coactivator 3, (AIB1/SRC3)	increase (s)
EP300	E1A binding protein p300	increase (e, l)
CREBBP	CREB binding protein (Rubinstein-Taybi syndrome), (CBP)	increase (e)
GAK	Cyclin G associated kinase	increase (l)
GSN	Gelsolin (amyloidosis, Finnish type)	increase (s)
NCOA4	nuclear receptor coactivator 4 (ARA70)	unchanged
HTATIP	HIV-1 Tat interacting protein, 60kDa (TIP60)	unchanged
NCOR1	Nuclear receptor co-repressor 1	unchanged
NCOR2	nuclear receptor co-repressor 2	unchanged
KLK3	Kallikrein 3, (prostate specific antigen), PSA	decrease (s)
KLK2	Kallikrein 2, prostatic	decrease (s)
NKX3-1	NK3 transcription factor related, locus 1 (Drosophila)	decrease (s)
AKR1C3	Aldo-keto reductase family 1, member C3	increase (s)
AKR1C2	Aldo-keto reductase family 1, member C2	increase (m)
AKR1C1	Aldo-keto reductase family 1, member C1	increase (m, l)
SRD5A1	Steroid-5-alpha-reductase, alpha polypeptide 1	decrease (e); increase (l)
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2	decrease (ns)
Neuroendocrine		
NTS	Neurotensin	increase (m, l)
ENO2	Enolase 2 (gamma, neuronal)	increase (s)
CHGB	Chromogranin B (secretogranin 1)	increase (l)
SCGN	Secretagogin, EF-hand calcium binding protein	increase (m, l)
DDC	Dopa decarboxylase (aromatic L-amino acid decarboxylase)	increase (s)
CHGA	Chromogranin A (parathyroid secretory protein 1)	unchanged
GRP	Gastrin-releasing peptide (bombesin)	unchanged
ET$_B$ and DNA methyltransferases		
EDNRB	Endothelin receptor type B (ET$_B$)	increase (m)
DNMT1	DNA (cytosine-5-)-methyltransferase 1	decrease (e, m)
DNMT3a	DNA (cytosine-5-)-methyltransferase 3 alpha	decrease (m, l)
DNMT3b	DNA (cytosine-5-)-methyltransferase 3 beta	decrease (e, m)

Not surprisingly, ET-1 (*EDN1*), ET$_A$ (*EDNRA*), and AR are among these genes. As previously observed via quantitative PCR analysis (Fig. 3), ET$_A$ receptor expression started increasing by one month of androgen deprivation and was elevated more than 8-fold at five months, ET-1 gene expression increased as early as three weeks after androgen withdrawal, with

more than a 3.5-fold increase at five months, and AR expression increased almost 2-fold at one month, compared to untreated, zero time point control cells (Fig. 27).
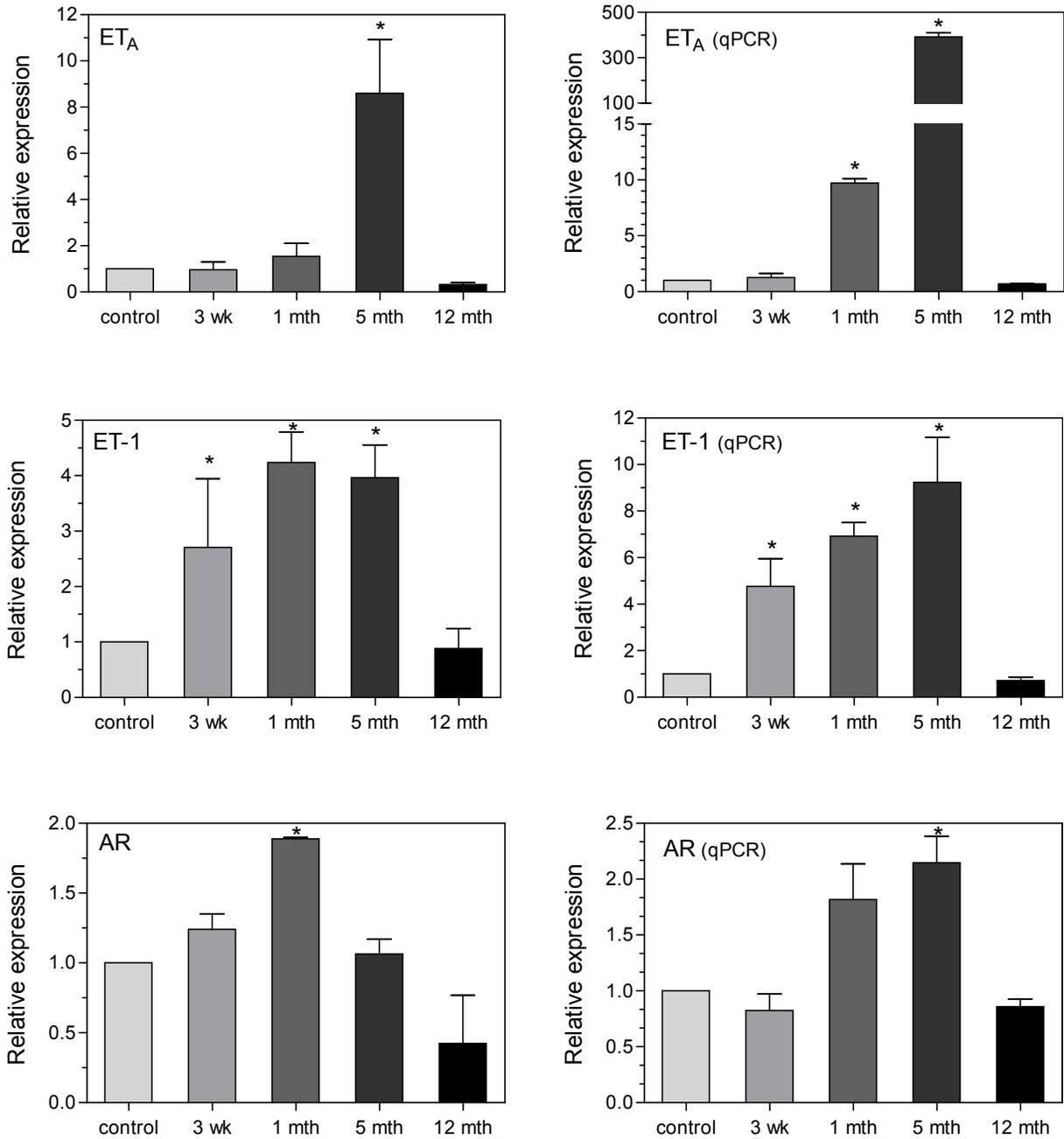
Figure 27. Affymetrix array and qPCR analyses of ET$_A$, ET-1, and AR gene expression in long-term androgen deprived LNCaP prostate cancer cells[3].

Related to endothelin biology and metastatic prostate cancer to the bone, it was discovered that ET-1 stimulates the proliferation of human osteoblasts, cells that constitutively express ET$_A$. Knowing that glucocorticoids promote bone metabolism, a 1998 study by Borcsok *et al.* showed that glucocorticoids increase both *in vitro* and *in vivo* levels of ET-1 and ET$_A$ specifically in human osteoblasts, with a more than 2-fold increase in total ET-1 binding capacity per osteoblast (72). With these results suggesting a relationship between glucocorticoid and endothelin signaling, expression of the glucocorticoid receptor (GR), *NR3C1*, was examined in the androgen deprived LNCaP cells. Compared to control cells, GR expression levels increased 172-fold at five months and remained elevated 37-fold at 12 months (Fig. 28), signifying that increased GR may be an important factor in prostate cancer cell survival. GR up-regulation may also function in promoting ET$_A$ expression, therefore enhancing endothelin survival signaling during AAT.

------

[3] X-axis indicates time of androgen deprivation and error bars indicate $\pm$ SEM (*, $P < 0.05$) for all bar graphs presented in this chapter. Expression relative to zero time point, untreated control cells.
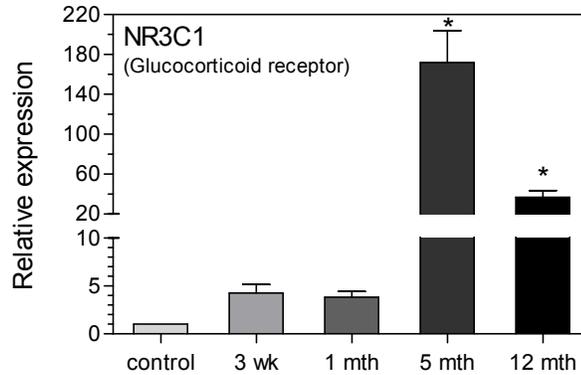
Figure 28. Affymetrix analysis of glucocorticoid receptor (NR3C1) expression.

By 12 months expression of ET-1, ET$_A$, and AR returned to levels equivalent to control cells, and the remaining cells that survived deprivation, characterized as androgen-independent (LNCaP-AI), had not only re-entered exponential growth, fully adapted to androgen depleted media, but demonstrated a significantly accelerated growth rate. In comparison to control LNCaP cells, which exhibited a doubling rate of approximately 41 hours, LNCaP-AI cells doubled every 22 hours (Fig. 7). Further supporting their classification as androgen-independent, LNCaP-AI cells expressed lower levels of AR (Fig. 8) and exhibited steady growth, unlike parental LNCaP cells (95), when exposed to short-term increasing doses of androgen[1] (Fig. 9). Moreover, with extended exposure to androgens, LNCaP-AI cells demonstrate an increase in the percent of dead cells as seen in figure 10, suggesting they retain sensitivity to androgens. In support of the increased ET$_A$ expression via qPCR and microarray analyses, previous immunohistochemical analysis demonstrated ET$_A$ receptor up-regulation in human prostate cancer following at least six months of AAT (Fig. 20). These results lend direct support to the hypothesis that, during androgen deprivation, prostate cancer cells acclimate to a low

androgen environment partly through up-regulating ET-1 and ET_A expression, in addition to increasing expression of AR and GR.

3.2.2   Affymetrix expression analysis of prostate stem cell, basal cell, and epithelial cell markers demonstrates retention of epithelial phenotype.

During fetal development, embryonic progenitor/stem cells of the UGE co-express both basal cytokeratins (CK5, CK14) and p63, and luminal cytokeratins (CK8, CK18). Following terminal differentiation in the adult prostate, luminal epithelial cells maintain CK8 and CK18 while losing CK5, CK14 and p63, whereas mature basal cells retain CK5, CK14 and p63, and shed CK8 and CK18. Additionally, a small population of cells in the basal epithelial compartment contain the full line-up of basal and luminal markers, suggesting there exists a subpopulation of cells that maintains the embryonic profile of epithelial cell differentiation markers (112).

To address concerns that this protocol selected for androgen-independent prostate stem cells, the expression of specific stem cell markers in addition to basal cell and epithelial cell markers was examined. The stem cell markers Nanog homeobox, POU domain 5 transcription factor 1 (POU5F1/Oct-4), and CD44, and basal cell markers CK5, CK14 and p63 were essentially undetectable in control cells and in all androgen deprived cells. Additionally, epithelial cell markers CK8, CK18 and E-cadherin were expressed at high levels in control cells and remained stably expressed at high levels in LNCaP-AI cells (Fig. 29), strongly suggesting LNCaP-AI cells were not derived from stem or basal cell precursors.

77
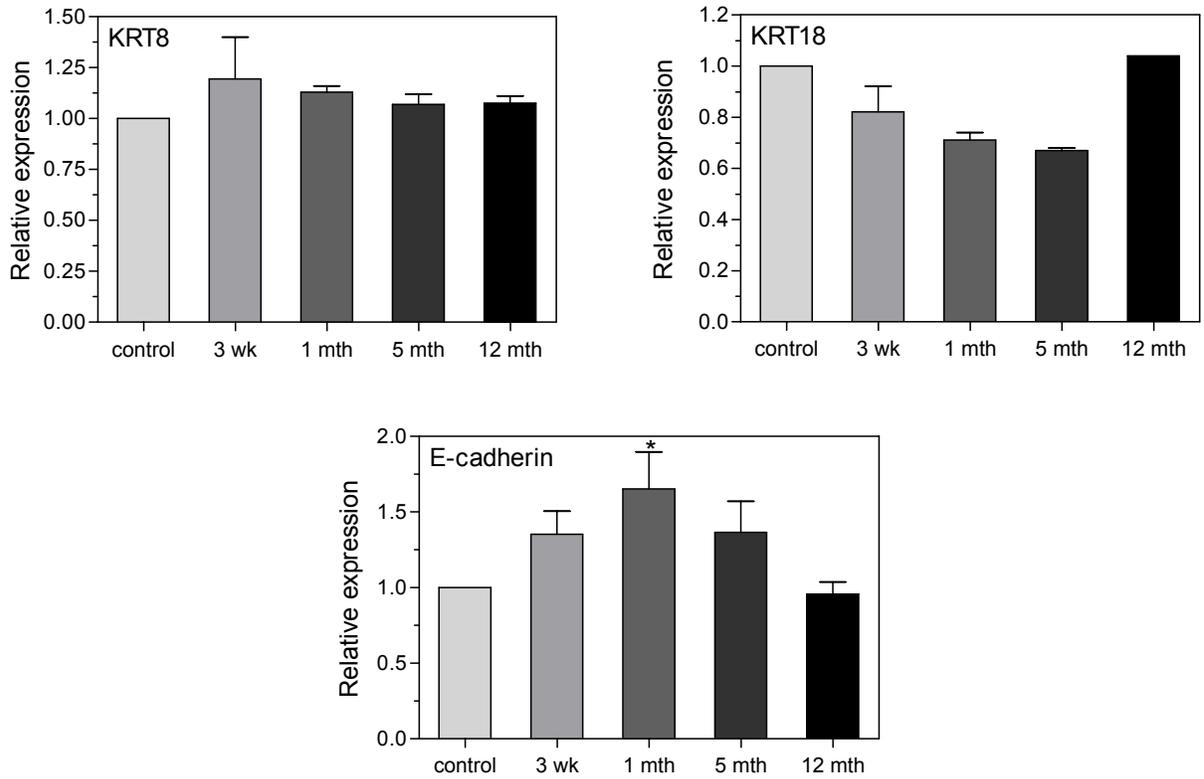
Figure 29. Affymetrix analysis of epithelial markers in androgen deprived LNCaP cells.

3.2.3 Genes linked to growth and survival exhibit marked changes in gene expression during androgen deprivation.

The cellular response to a severe insult, such as androgen starvation, likely involves more than the simple up-regulation of ET_A or AR; thus, efforts were made to identify additional genes affected by androgen deprivation in order to further dissect the AAT-resistant prostate cancer cell phenotype. Upon examination of the expression array data for other known survival- and growth-associated genes, remarkable alterations in gene expression that correlate with the observed changes in ET_A and ET-1 expression were discovered (Table 2). Elevated levels of the receptor tyrosine kinase erb-B2 (Her2/neu) has been documented in a variety of cancers,

including breast, ovarian, and prostate (113,114). Erb-B2, which lacks a ligand binding domain, induces receptor dimerization to affect cellular proliferation, survival and metastasis (113), processes shown to be enhanced through ET_A receptor transactivation of EGFR (105,106). Another study demonstrated a role for Her2/erb-B3 signaling in stabilizing AR protein expression and optimizing AR binding to promoter regions of androgen-regulated genes, which effectively enhances AR transcriptional activity in human prostate cancer (115). This study demonstrates that erb-B2 expression increased almost 4-fold at one month, but returned to near control levels by 12 months (Fig. 30).
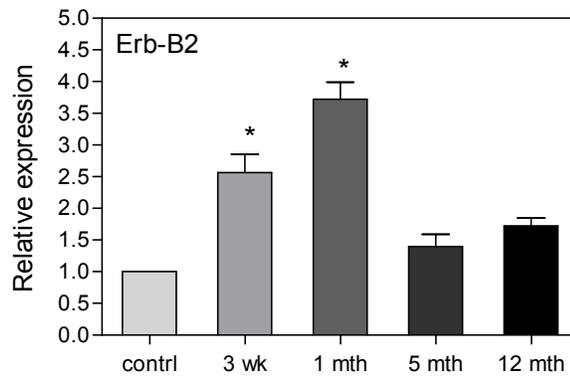
Figure 30. Affymetrix analysis of Erb-B2 (Her2/*neu*) expression.

Another growth factor receptor intimately associated with promoting cancer cell growth and proliferation, Met (hepatocyte growth factor receptor), exhibited dramatically increased expression. However, unlike many of the other genes analyzed, Met remained elevated (8.5-fold) in LNCaP-AI cells compared to control cells (Fig. 31), representing a potentially more permanent change characteristic of androgen-independent prostate cancer.
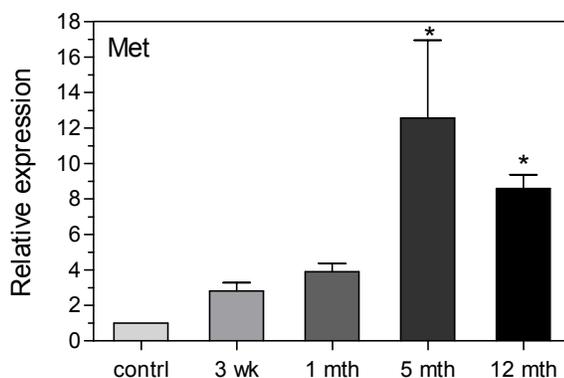
Figure 31. Affymetrix analysis of Met (hepatocyte growth factor receptor) expression.

Androgen deprivation also affected expression of the transforming growth factor-beta 1 receptor (TGFβR1). In the normal prostate gland, luminal epithelial cell turnover is carefully regulated by apoptosis through stromal-secreted TGF-beta activating epithelial-expressed TGFβR1 (116). However, previous studies have found that once tumor cells escape the growth-inhibitory effects of TGF-beta, often they constitutively activate the TGF-beta signaling pathway to promote tumor progression by suppressing anti-tumor immunity in addition to enhancing angiogenesis, invasion and metastasis (117,118). Additionally, a mouse model study found that when stromal fibroblasts lose TGF-beta responsiveness, Met was identified as a potential inducer of epithelial cell malignant transformation (119). In this study, TGFβR1 expression decreased slightly through one month, but increased more than 2-fold by five months and remained elevated in LNCaP-AI cells (Fig. 32). These results suggest TGF-beta-induced apoptosis may be inhibited early on but that increased TGFβR1 levels in the surviving cells supports the hypothesis of altered TGFβR1 function as previously documented.
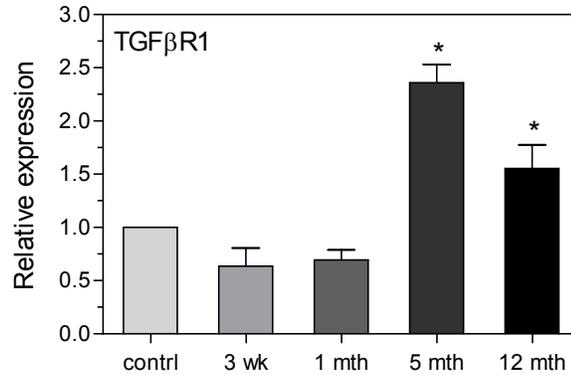
Figure 32. Affymetrix analysis of TGFβR1 expression.

Nuclear factor kappa B (NFκB), a nuclear transcription factor widely known for its role in a variety of cellular processes, including cell survival, also exhibited altered gene expression during the 12 months of androgen deprivation. Showing a 2.3-fold increase in expression over control cells at one month, NFκB levels followed a similar pattern of gene expression compared to that of ET-1 (Fig. 33). Eventually normalizing to control levels by 12 months, fluctuations in NFκB mRNA expression suggest that prostate cancer cells may exploit NFκB survival signaling during androgen deprivation, but not necessarily as a means to maintaining androgen independence.
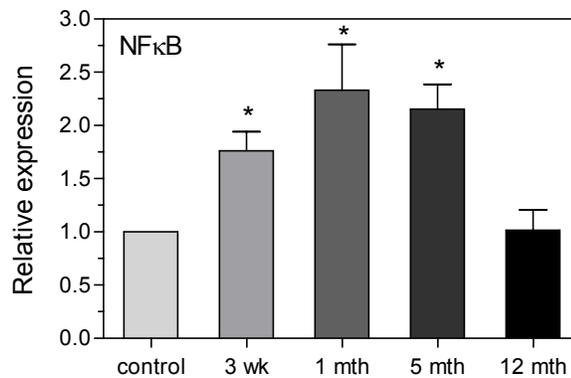
Figure 33. Affymetrix analysis of NFκB expression.

Previous studies have documented expression for Jagged-1, the Notch receptor ligand involved in cell fate determination and differentiation, to be significantly up-regulated in metastatic prostate cancer compared to local disease or benign prostate tissue (120). Furthermore, reduced Jagged-1 expression appears to inhibit cell growth through S-phase cell cycle arrest (121). With a dramatic increase in Jagged-1 expression, reaching greater than 14-fold at 12 months, these results strongly suggest that Jagged-1 signaling is not only up-regulated to aid in cell cycle regulation during early months of deprivation, but harnessed to promote androgen-independent prostate cancer cell growth (Fig. 34). Additionally, Notch-1 receptor expression levels were not significantly affected throughout the time course (Fig. 34).
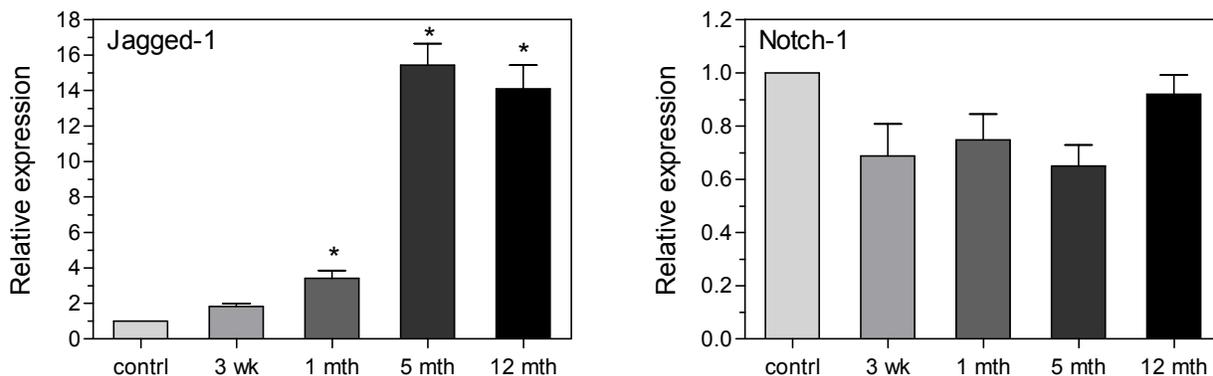
Figure 34. Affymetrix analysis of Jagged-1 and Notch receptor expression.

Sonic hedgehog (Shh) is another protein well known for its role in cell proliferation and differentiation during development, and has recently been found to be up-regulated in advanced prostate cancer (122,123). Shown to correlate with tumor severity, autocrine Shh signaling is essential for prostate cancer cell proliferation and invasiveness (123). Shh expression increased

45-fold at five months with greater than a 13-fold increase in Shh expression in LNCaP-AI cells (Fig. 35), suggesting that elevated Shh, like Met, TGFβR1, and Jagged-1, potentially contributes to cell survival and the highly proliferative phenotype seen in LNCaP-AI cells.
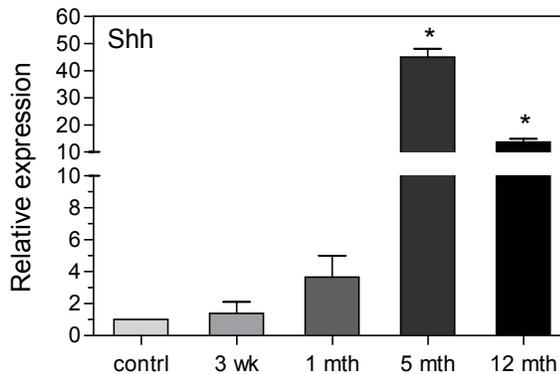
Figure 35. Affymetrix analysis of Sonic Hedgehog expression.

Matrix metalloproteinases (MMPs) are essential for maintenance of the normal extracellular matrix (ECM). In doing so, MMPs release factors from the ECM that promote cell growth, motility, apoptosis and differentiation. In cancer, the implications are obvious as MMP activity functions to mobilize growth and survival factors from the ECM, promoting proliferation, angiogenesis and metastasis (124). Of the more widely characterized MMPs, MMP-7 showed the most significant change in expression, reaching an almost 10-fold increase at five months (Fig. 36), whereas expression of MMP-2 and MMP-9 remained unaffected during the 12 months of androgen deprivation (Fig. 37).
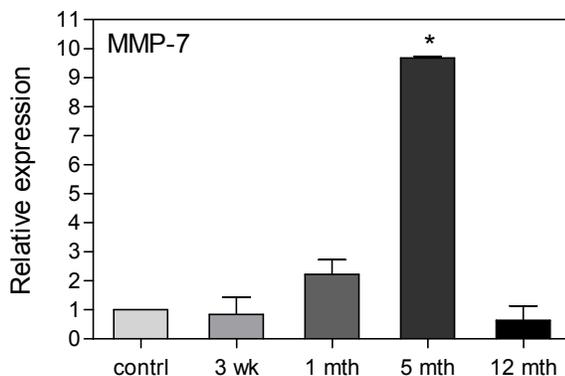
Figure 36. Affymetrix analysis of MMP-7 expression.
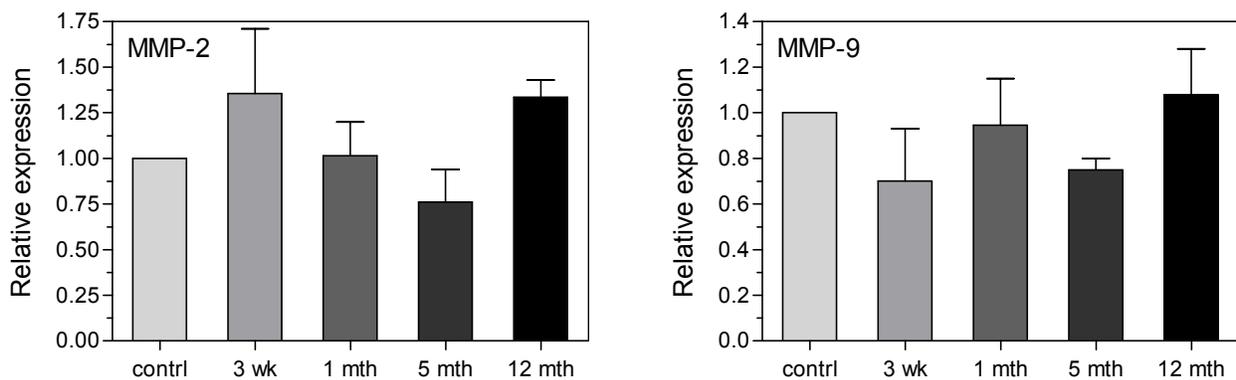
Figure 37. Affymetrix analysis of MMP-2 and MMP-9 expression.

Previously, MMP-7 expression in prostate cancer has been shown to increase during an altered hormone state (125), and in light of these results, it is highly likely that LNCaP cells up-regulate MMP-7 expression as part of a programmed response to liberate growth and survival factors from the surrounding milieu when androgens fall below a critical level. However, the lack of a surrounding ECM in our experimental conditions would not necessitate the continued increase in MMP-7 expression, as illustrated at 12 months.

3.2.4 Changes in cell cycle and apoptosis regulation genes during androgen deprivation.

Androgen deprivation also affected the expression of genes central to the regulation of the cell cycle and apoptosis. The master tumor suppressor gene p53, which is wild-type in LNCaP cells, is essential in mediating apoptosis and cell cycle regulation. p53 exhibited a gene expression pattern inverse to ET-1, decreasing 3.6-fold by one month but returning to control levels by 12 months (Fig. 38), suggesting that androgen deprivation may cause LNCaP cells to restrain p53-induced apoptosis primarily during the period of acclimation to reduced androgens.
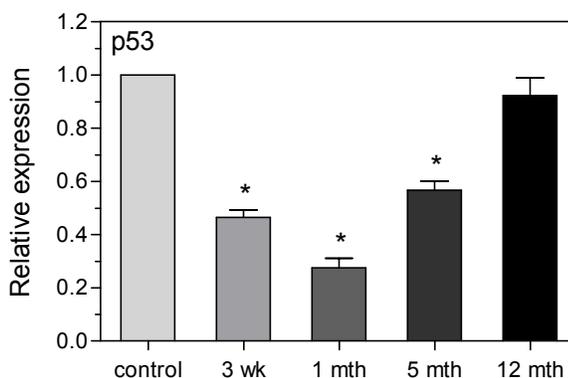
Figure 38. Affymetrix analysis of p53 expression.

p21$^{(Cip1)}$, a transcriptional target of p53, regulates cell cycle progression via binding G_1/S cyclin dependent kinases to arrest cells at various stages in the cell cycle. p21 expression was significantly reduced early on, normalized by five months, but decreased again at 12 months (Fig. 39). In light of the fact that LNCaP-AI cells grow at a much higher rate than control LNCaP cells, a possible interpretation of these observations is that LNCaP cells initially down-regulate p21 to stimulate cell growth in an androgen-depleted environment, but as time

progresses cells are forced into cell cycle arrest to survive. However, once these cells re-enter exponential growth nearing 12 months, p21 expression is reduced to promote accelerated growth of the now androgen-independent cancer cells. Additionally, it was discovered that expression of the cyclin D1-Cdk4 complex inhibitor p16$^{(INK4)}$ was significantly reduced in LNCaP-AI cells (Fig. 39), a result that potentially contributes to further de-regulated androgen-independent cell proliferation.
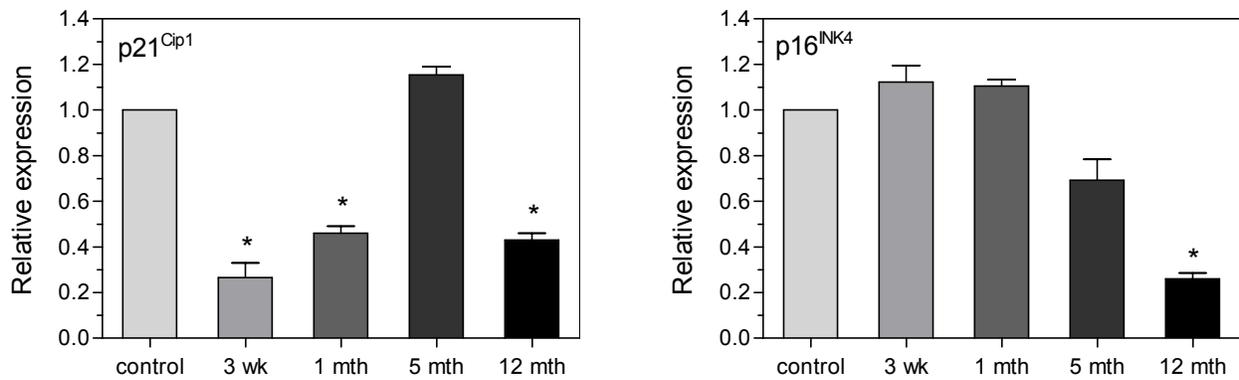
Figure 39. Affymetrix expression analysis of cell cycle regulators p21$^{(Cip1)}$ and p16$^{(Ink4)}$.

In association with the changes in p21 and p16, the reduced expression of their target cyclin-dependent kinases, CDK2 and CDK4, respectively, observed during the initial five months of androgen deprivation might function in promoting cell cycle arrest. But at 12 months, with reduced p21 and p16 expression, both CDK2 and CDK4 expression are restored (Fig. 40), potentiating an environment of significantly accelerated cell cycle progression as exhibited by LNCaP-AI cells.
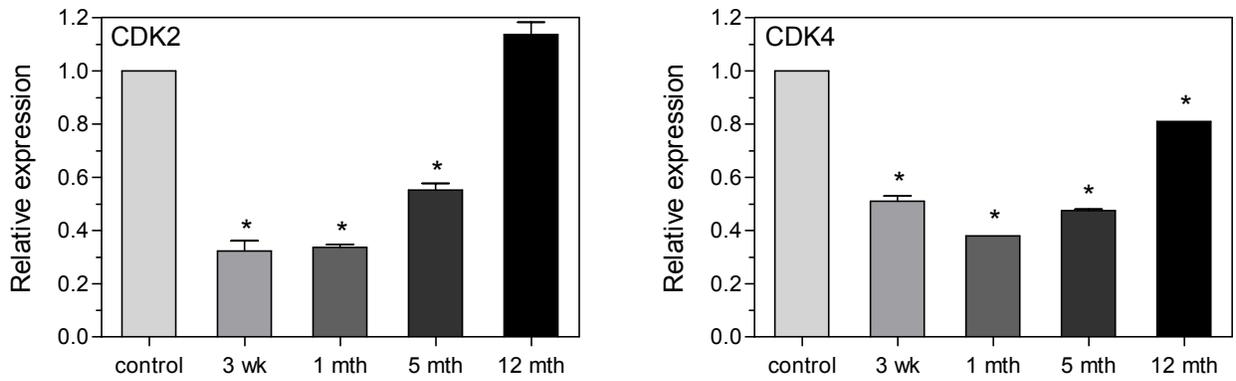
Figure 40. Affymetrix expression analysis of cell cycle promoters CDK2 and CDK4.

Survivin is a gene, like p53, which plays a critical role in regulating cell cycle progression as well as apoptosis in both normal and cancer cells (126). In androgen deprived LNCaP cells, survivin expression decreased over 70-fold, but then returned to control expression levels by 12 months (Fig. 41). In view of these findings, there exist two possible interpretations for altered survivin expression: First, survivin repression during androgen deprivation may function to inhibit cell cycle progression aiding in the cell survival response. Alternatively, early survivin repression raises the possibility that these cells decrease survivin expression in an effort to undergo apoptosis as a result of the stress of androgen withdrawal, and expression only returns to control levels once cells have developed the ability to flourish in an androgen-reduced environment via mechanisms such as those proposed in this paper.
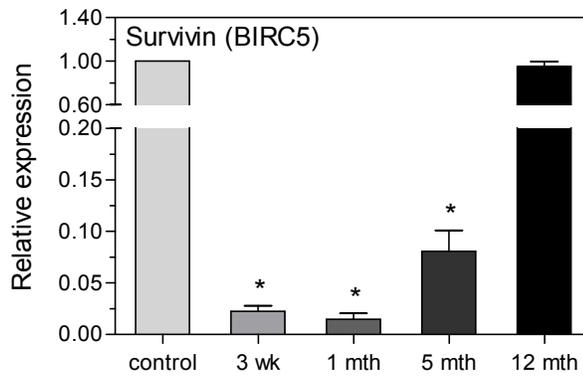
Figure 41. Affymetrix analysis of survivin expression.

Several of the more well studied apoptotic proteins, including Bcl-2, Bcl-X$_L$, and Bad exhibited mRNA expression patterns unaltered from control cells (Fig. 42); however, Bax, Bak, and Fas-receptor genes all associated with promoting cell stress-induced apoptosis, demonstrated significant decreases in expression (Fig. 43).
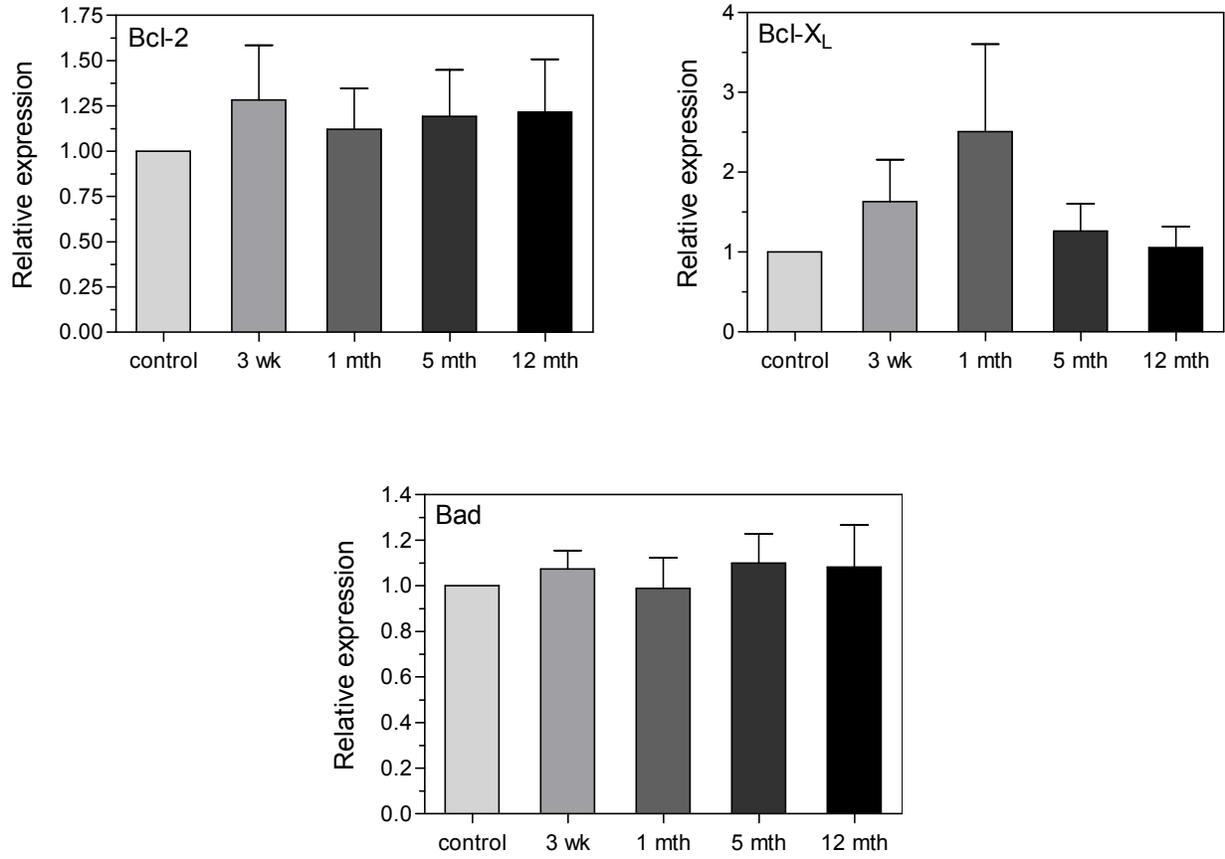
Figure 42. Affymetrix analysis of Bcl-2, Bcl-X$_L$ and Bad expression.
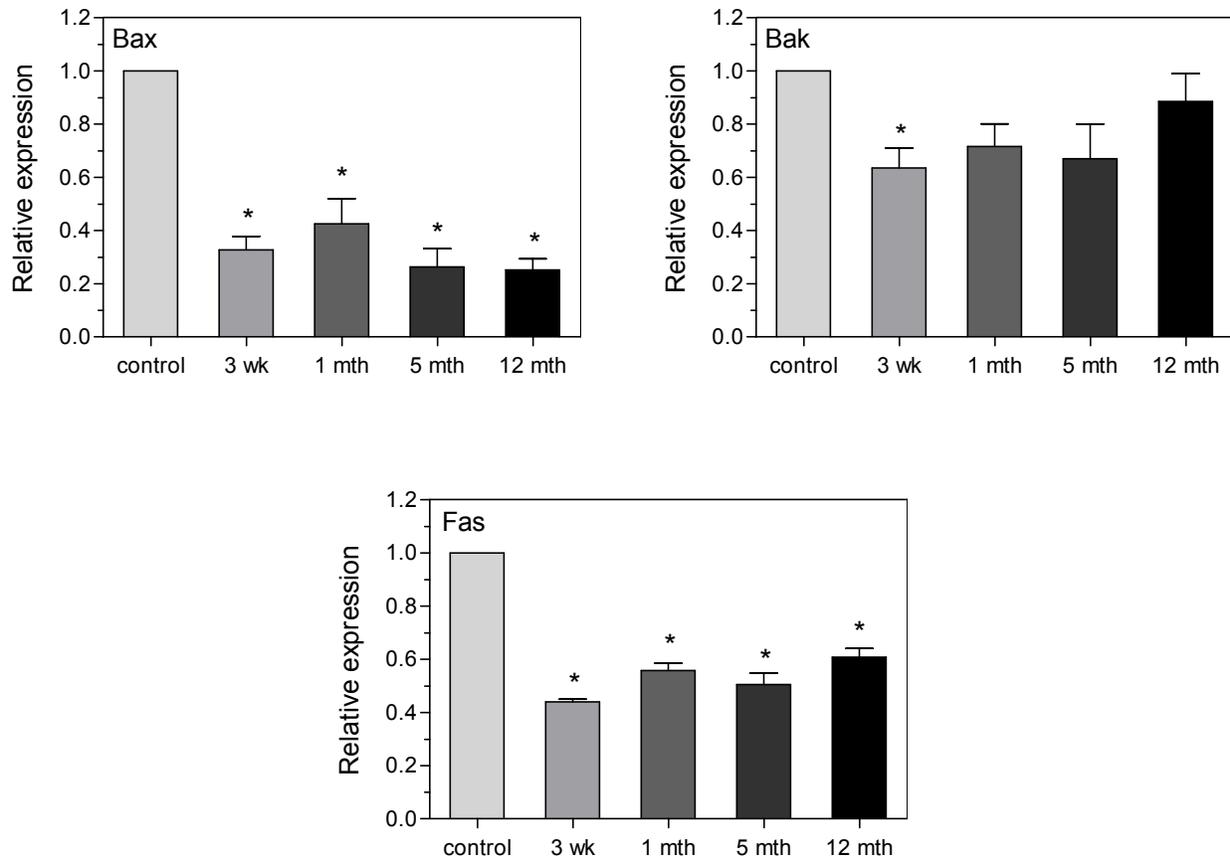
Figure 43. Affymetrix analysis of Bax, Bak and Fas expression.

Of note, expression of Bak normalized by 12 months, but Fas-receptor levels remained decreased almost 2-fold and expression of Bax remained decreased 4-fold in LNCaP-AI cells. Reduction in the expression of these pro-apoptotic factors likely contributes to enhanced prostate cancer cell survival, further promoting the transition to androgen-independence.

3.2.5 AR co-regulators, AR regulated genes and genes associated with androgen metabolism demonstrate expression changes indicative of modified AR function in the development of androgen-independent prostate cancer.

AR, a member of the nuclear hormone receptor family, functions as a DNA-binding transcription factor (127). AR transcriptional activity is essential for the growth and development of the normal prostate gland and plays a crucial role in prostate carcinogenesis. Upon entering the nucleus, AR transcriptional activity is tightly regulated by the recruitment and interaction with multiple transcription factor co-regulators, including steroid receptor co-activator (SRC) family, cyclic adenosine monophosphate response element binding protein (CBP), and p300 (128). The SRC family proteins are the best characterized AR co-activators; they possess intrinsic histone acetyl transferase activity essential in promoting AR transactivation, and also serve by recruiting additional chromatin remodeling proteins such as p300 and CBP. It has previously been reported that expression of all three SRC proteins (SRC-1, TIF2/SRC-2, and AIB1/SRC-3) is increased in recurrent, androgen-independent prostate cancers or in tissue from patients who failed AAT (129). Of the AR co-regulators analyzed in this longitudinal analysis, the three SRC proteins were up-regulated the most, with all three reaching maximum expression at one month. However, by 12 months, SRC-1 expression returned to control levels whereas TIF2 and AIB1 levels remained increased (Fig. 44).
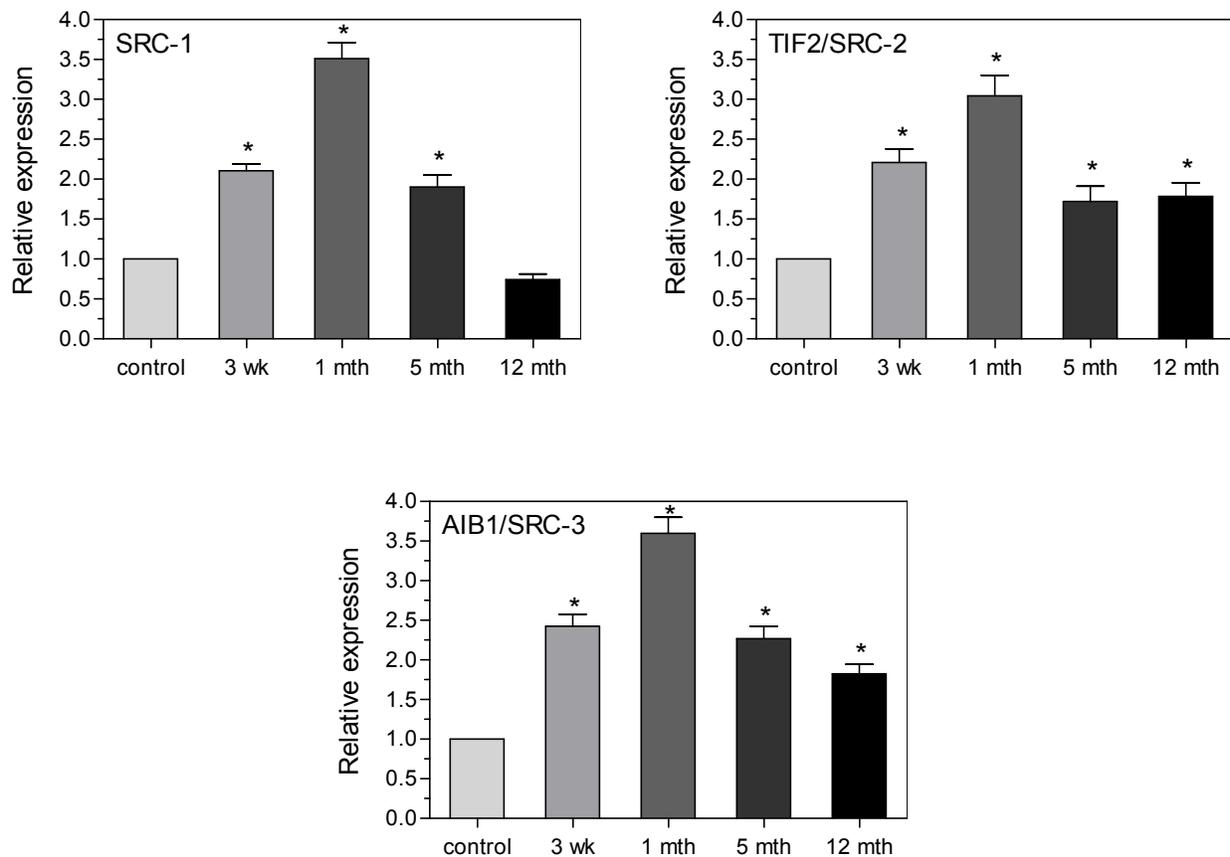
Figure 44. Affymetrix expression analysis of the SRC family of AR co-regulators.

A study by Heemers *et al.*, published earlier this year, found that p300 expression is closely tied to the presence of functional AR in prostate cancer cells: p300 levels decrease with the addition of androgens while androgen starvation increases p300 expression. It was seen that elevated p300 during androgen deprivation was essential for AR function and cell proliferation, whereas the loss of p300 reduced expression of cyclins A, B, and D1 resulting in decreased DNA synthesis and cell proliferation (130). This study shows that both p300 and CBP expression increased during the 12 months of androgen deprivation with the most significant increases seen at one month (Fig. 45).
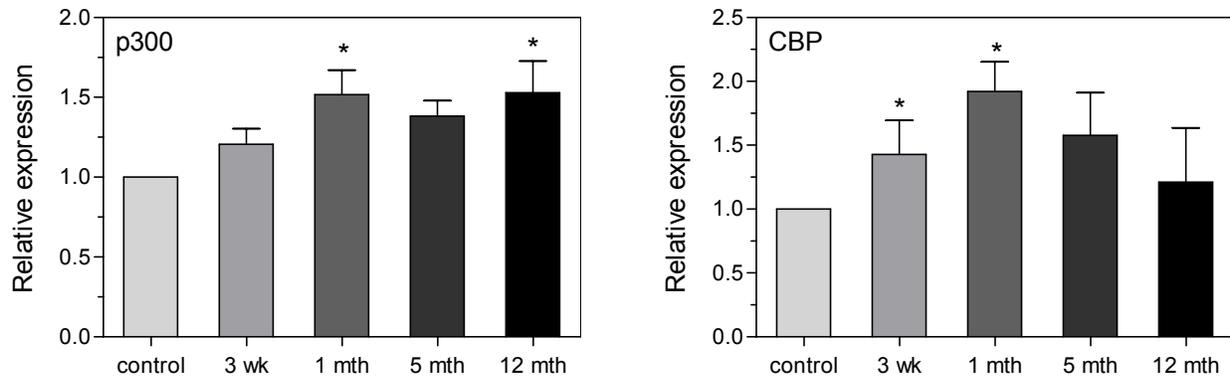
Figure 45. Affymetrix expression analysis of DNA binding AR co-activators p300 and CBP.

Elevated levels of cyclin G-associated kinase (GAK), a co-factor that interacts with AR in a ligand-independent manner to enhance AR transcriptional activity, have been found in patients with refractory disease or those under prolonged AAT (129). There was a linear increase in GAK expression which achieved significance at 12 months in LNCaP-AI cells (Fig. 46), suggesting that increased GAK expression may be more characteristic of the androgen-independent phenotype than in cell survival during AAT.
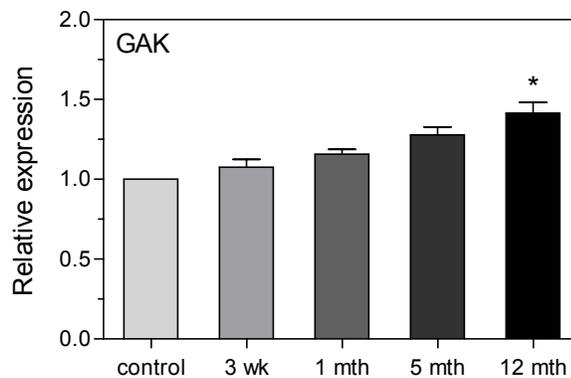
Figure 46. Affymetrix analysis of G-associated kinase (GAK) expression.

Gelsolin (GSN) is a recently identified AR co-factor that binds to AR in a ligand-dependent manner and translocates into the nucleus where it acts to promote AR transcriptional activity (131). GSN levels increased 2.5-fold by five months of androgen deprivation and remained elevated in LNCaP-AI cells (Fig. 47), corroborating previous findings that GSN expression is enhanced in LNCaP cells, LNCaP xenografts and human prostate cancers following androgen withdrawal (131).
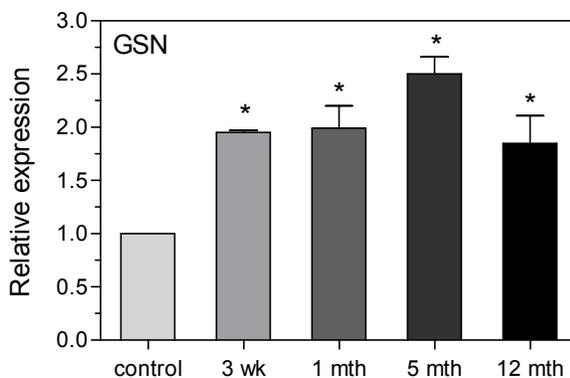
Figure 47. Affymetrix analysis of Gelsolin expression.

Expression of additional AR co-activators, such as AR-associated protein 70 (ARA70) and Tat interactive protein 60 (TIP60), and co-repressors, such as nuclear co-repressors (NCoR1 and NCoR2), were also examined but found to be unaltered (Fig. 48).
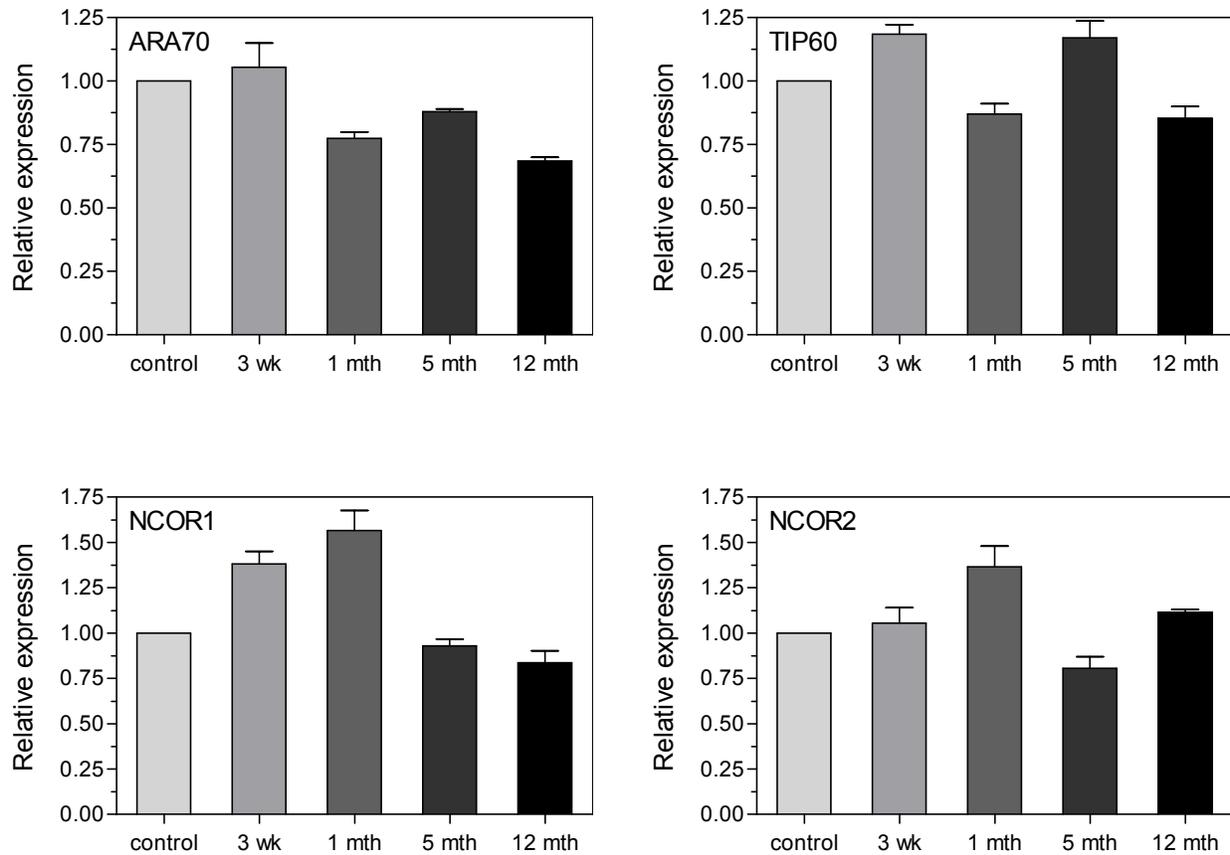
Figure 48. Affymetrix expression analysis of AR co-activators ARA70 and TIP60 and AR co-repressors NCOR1 and NCOR2.

To confirm that this protocol reduced the levels of androgens to effectively distress the AR signaling axis, expression of specific AR-regulated genes, prostate specific antigen (PSA/KLK3), kallikrein 2 (KLK2), and NK3 transcription factor related, locus 1 (NKX3.1) were examined. All three genes exhibited significant reductions in expression throughout the study and remained significantly decreased in LNCaP-AI cells (Fig. 49), signifying that traditional AR transcriptional activity was in effect reduced.
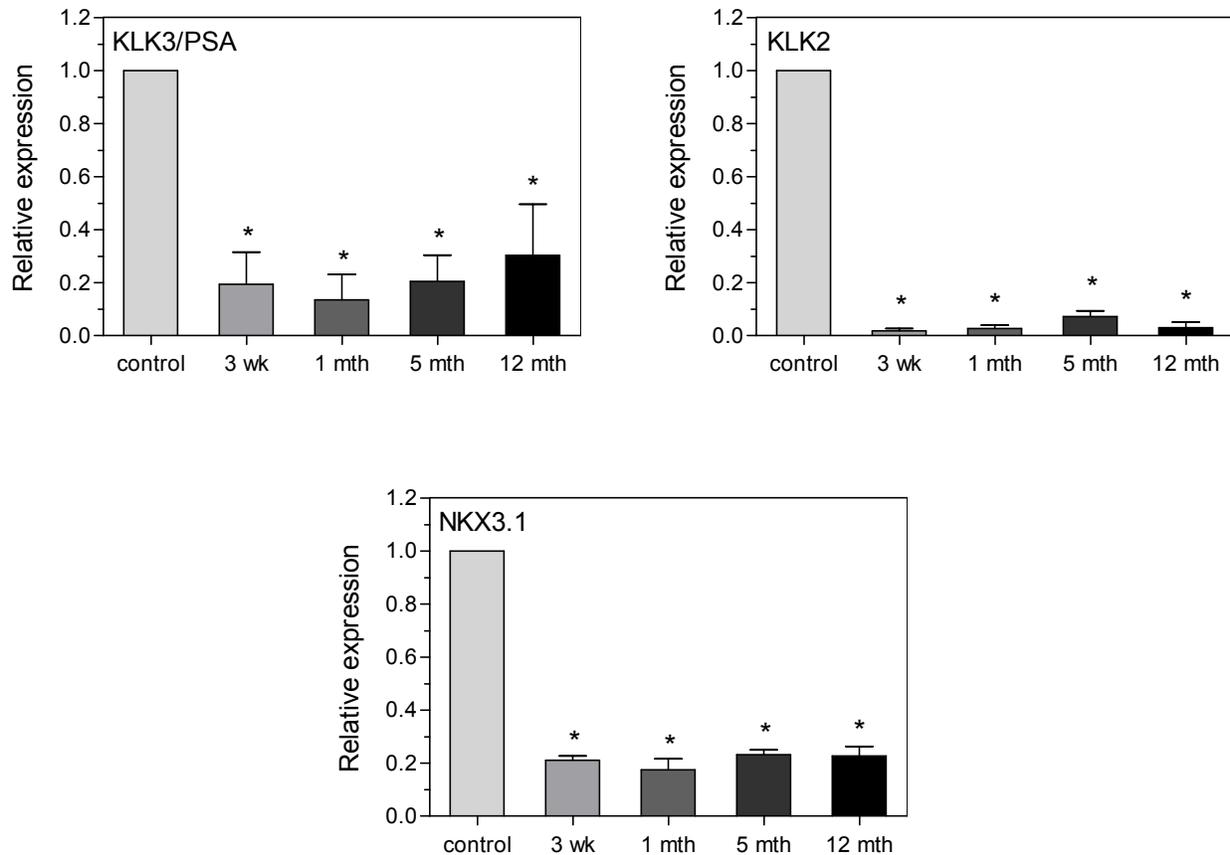
Figure 49. Affymetrix expression analysis of AR-regulated genes KLK3/PSA, KLK2 and NKX3.1.

In the field of prostate cancer therapeutics, androgen ablation remains the most widely used form of therapy for advanced disease. However, the scientific community is just beginning to uncover the molecular ramifications of AAT to better understand the biology underlying treatment failure. Alongside increased AR expression, AR mutation and AR co-regulator modifications, recent studies have examined mechanisms that aid in prostate cancer cell survival during androgen ablation via conversion of adrenal androgens to testosterone. The enzymes primarily responsible for androgen metabolism, AKR1C1, AKR1C2, and AKR1C3, were found to be significantly increased in androgen-independent bone marrow metastases compared to primary prostate cancer samples, results that are illustrative of an adaptive mechanism (132). All

three AKR1C enzymes exhibited significantly increased expression in androgen deprived LNCaP cells at five months (Fig. 50), suggesting that the behavior of these cells, even in the isolation of *in vitro* cell culture, resembles that of cells adapting to a low androgen environment, *in vivo*.
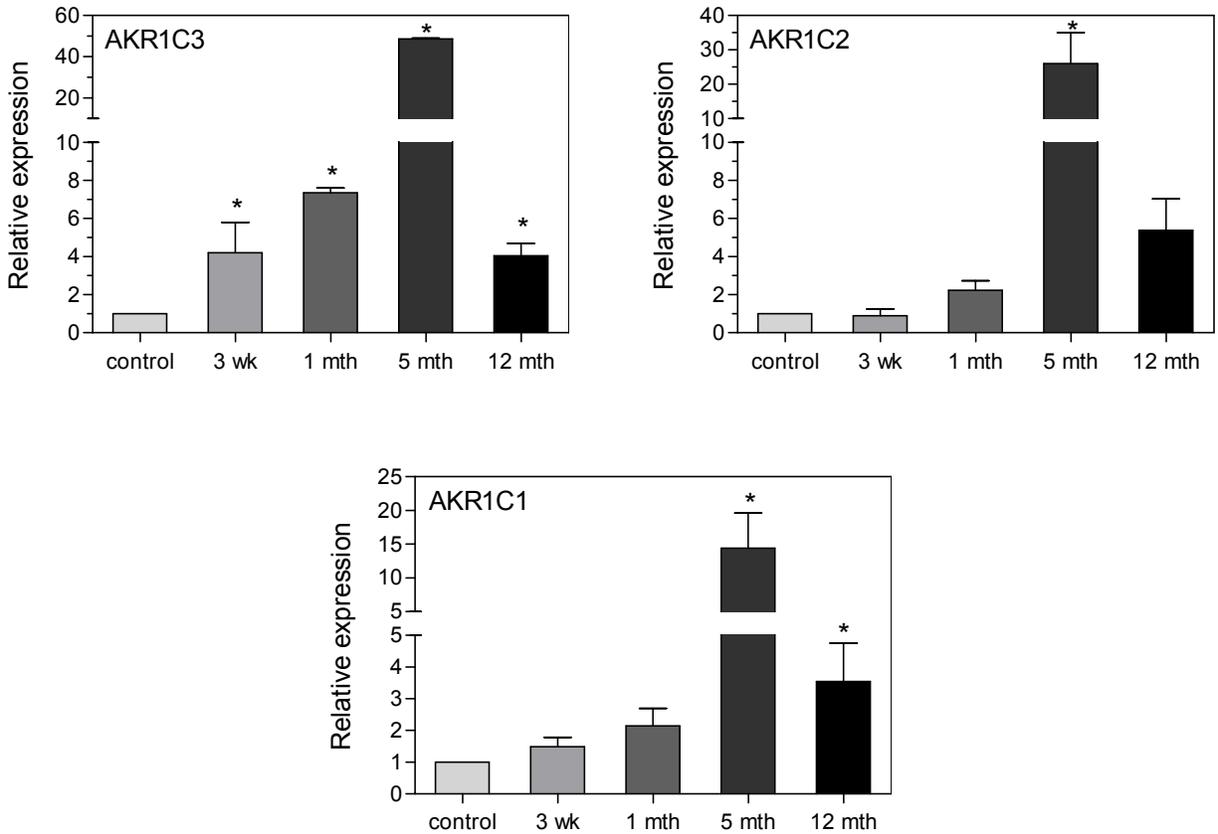
Figure 50. **Affymetrix expression analysis of androgen metabolism enzymes AKR1C3, AKR1C2 and AKR1C1.**

Type 1 5α-reductase (SRD5A1) and type 2 5α-reductase (SRD5A2, the major isoform expressed in normal prostate) function in the conversion of testosterone to the higher affinity 5α-dihydrotestosterone. In the same study that examined AKR1C enzyme expression, Stanbrough and colleagues also found that SRD5A2 was decreased by approximately 50% but SRD5A1 was

increased 2.1-fold in the metastatic samples (132). The expression of SRD5A2 decreased initially but then returned to control levels by 12 months; however, SRD5A1 increased significantly (almost 2-fold) at 12 months (Fig. 51), supporting recent evidence that there is a shift from SRD5A2 to SRD5A1 expression in prostate cancer (133-135).
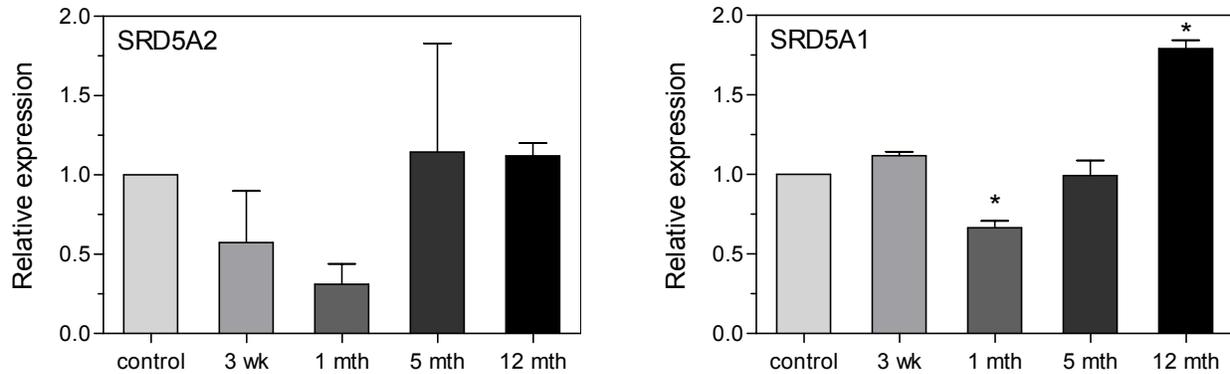
Figure 51. Affymetrix expression analysis of type 2 5α-reductase and type 1 5α-reductase.

3.2.6 Gene expression changes in neuroendocrine markers implicate a potential neuroendocrine-like morphological transition in progression to androgen independence.

Neuroendocrine (NE) cells are present in nearly all prostate cancer cases; however, the prognostic value of NE differentiation remains enigmatic (136). Nonetheless, increased detection of NE cells following AAT has been documented in human prostate cancer, suggesting a role in disease progression to androgen independence (137,138). In support of these findings, culturing LNCaP cells in csFBS media has previously been shown to induce a NE-like morphology (139-141). In this study, cells undergoing androgen deprivation over the 12 month period were photographed periodically and dramatic alterations in morphology suggestive of NE

differentiation were observed (Fig. 52). Between one and ten months cells appeared quiescent as they developed widespread dendritic extensions. At eleven months, cells began to withdraw from this neuronal-like morphology and, soon after, resumed proliferating at a much accelerated rate.
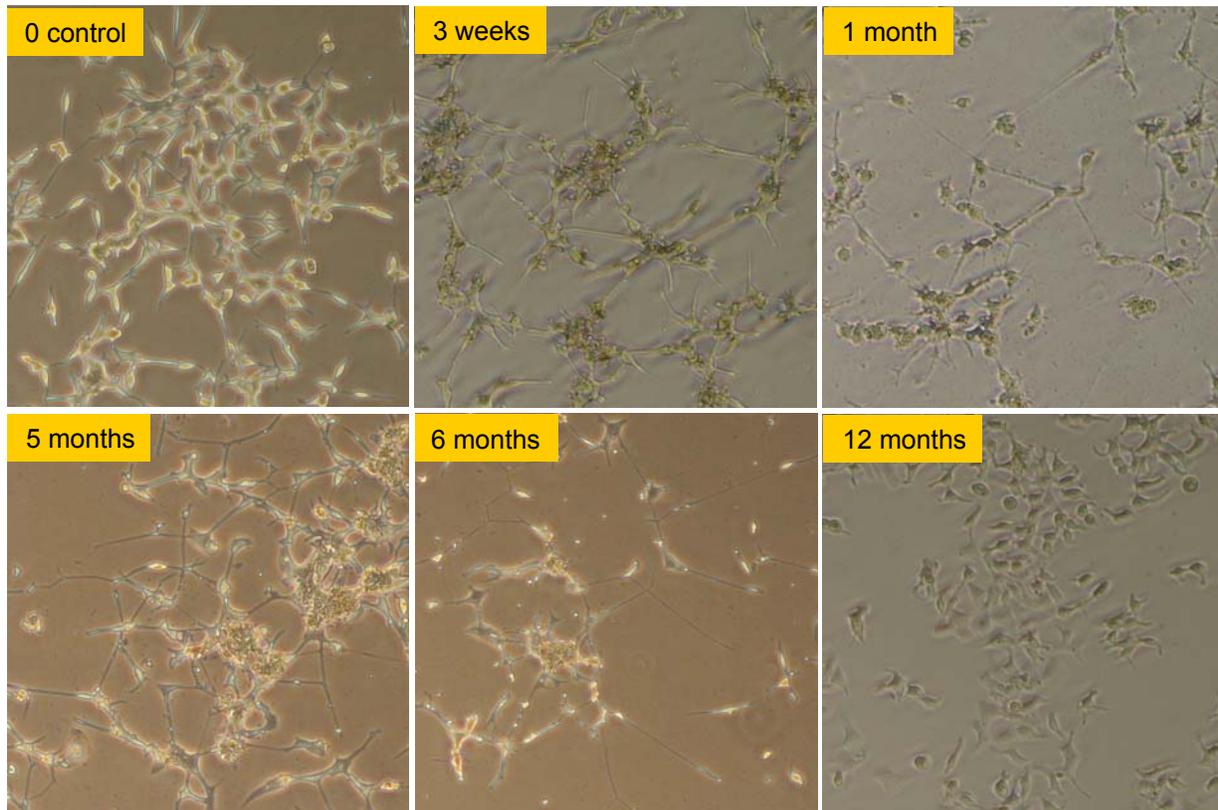
Figure 52. Phenotypic changes in androgen deprived LNCaP cells undergoing a transient neuroendocrine-like morphological transition.

The expression of several specific NE markers not only increased throughout the androgen deprivation protocol, but remained elevated in the LNCaP-AI cells. Previous reports have shown increased expression of neurotensin (NTS), neuronal enolase (ENO2), and chromogranin B (CHGB) in androgen deprived LNCaP cells (139,141) and one study

demonstrated that NTS functions as an autocrine growth factor in response to androgen deprivation in LNCaP cells (142). It was found that NTS, ENO2, and CHGB levels were up-regulated 440-fold, 8.6-fold, and 3.8-fold, respectively, in LNCaP-AI cells (Fig. 53).
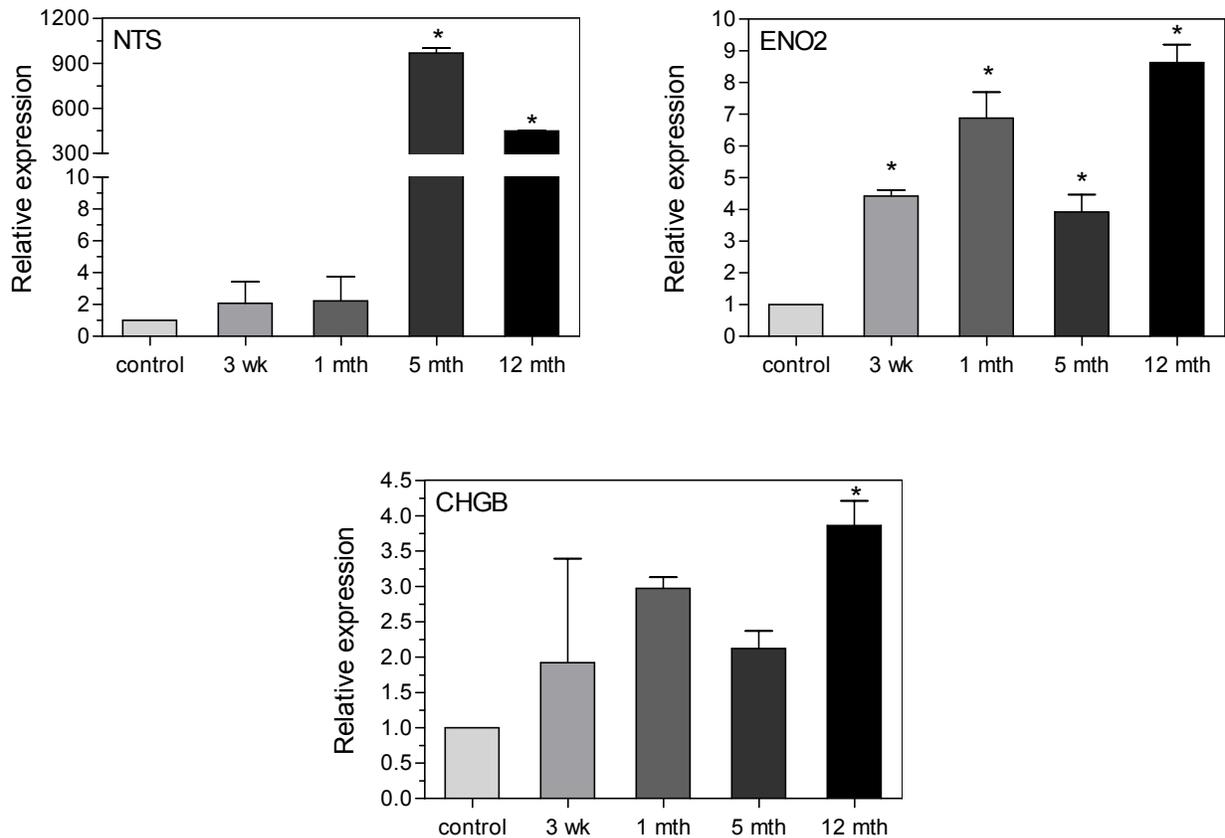
Figure 53. Affymetrix expression analysis of neuroendocrine markers neurotensin, neuronal enolase and chromogranin B.

There was also a 175-fold increase in expression of secretagogin (SCGN) in LNCaP-AI cells (Fig. 54), a molecule recently reported as a powerful indicator of NE differentiation in prostate cancer (143). Additionally, a very recent study by Wafa *et al.* found that the extent of NE transdifferentiation in AR-expressing epithelial cells depended on the duration of AAT, a conclusion based on the extensive expression analysis of NE marker L-dopa decarboxylase

(DDC) (144). These results show that DDC expression increased significantly by three weeks reaching a maximum expression of 28-fold at 12 months (Fig. 54).
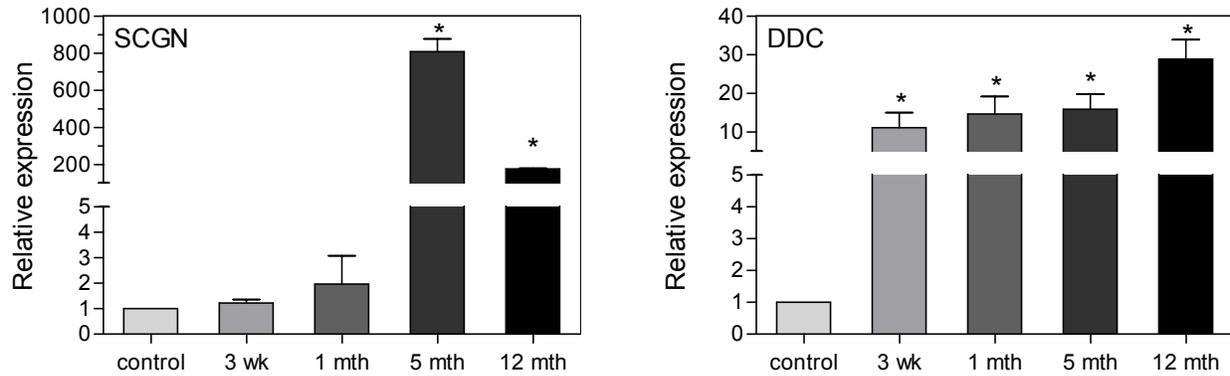
Figure 54. **Affymetrix expression analysis of neuroendocrine markers secretagogin and L-dopa decarboxylase.**

It is hard to ignore or discount the changes in gene expression for these select neuroendocrine markers. There is growing debate over the contribution of neuroendocrine cells in prostate cancer progression, many studies suggesting they may play an important role in promoting the growth of surrounding epithelial cells by providing growth signals lost during therapeutic manipulation. If indeed neuroendocrine cells are truly androgen-independent and can provide mitogenic support for the surrounding androgen-dependent luminal epithelial cells in an androgen depleted environment, neuroendocrine cells likely pose a serious threat to patients undergoing hormone therapy.

3.2.7 ET_B receptor expression analysis via Affymetrix expression array.

In efforts to understand why ET_B receptor expression increased so dramatically in the time course androgen deprived LNCaP cells, as demonstrated by the qPCR analysis presented earlier (Fig 4), ET_B expression was also examined in the Affymetrix array data. According to the longitudinal analysis, ET_B exhibited significantly increased expression at five months, but like many genes, including ET_A and ET-1, ET_B expression returned to control levels by 12 months (Fig 55).
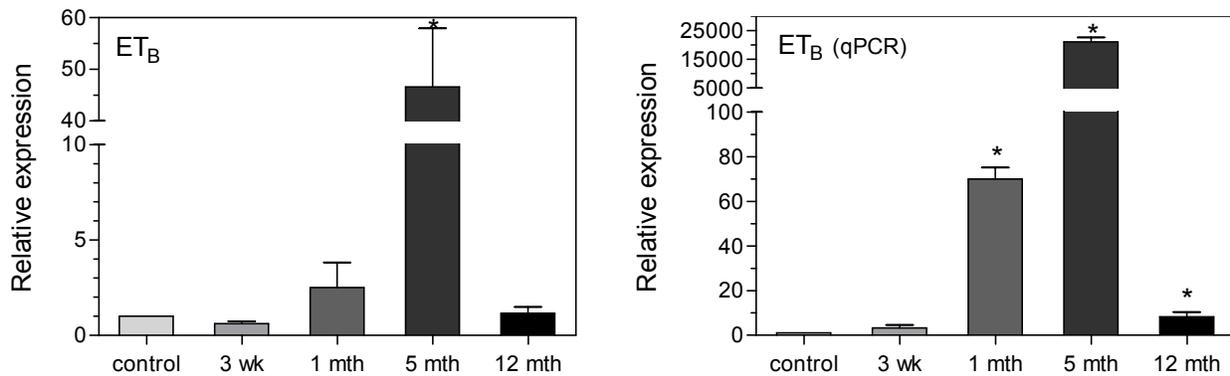
Figure 55. Affymetrix array and qPCR expression analyses of ET_B.

Having shown such large increases in expression via qPCR and Affymetrix analyses, yet demonstrating decreased protein expression following short-term androgen ablation and no change following long-term androgen ablation in human prostate tissue, compared to donor tissues, it remains unclear as to how ET_B receptor expression is regulated during androgen withdrawal. Taking into consideration that ET_B receptor expression is significantly decreased in prostate cancer cells and human prostate cancer tissue due to hypermethylation of the *EDNRB*

102

promoter, these conflicting results suggest the possibility of alterations in the methylation status of *EDNRB*.

Epigenetic modification of mammalian DNA, including DNA methylation and covalent modifications of histone proteins, play an essential role in regulating gene expression, differentiation, embryonic development, genomic imprinting, and inactivation of the X chromosome in females (145). Methylation at the 5'-position of a cytosine residue within a cytosine-guanine dinucleotide residue (CpG) produces a 5-methylcytosine (m^5C) (146), rendering the DNA inaccessible by RNA polymerase II and transcription co-factors. During embryogenesis and tissue development, DNA methyltransferase enzyme 1 (DNMT1) is responsible for maintaining DNA methylation, whereas DNMT3a and DNMT3b are responsible for generating *de novo* DNA methylation patterns. Disruption in the maintenance of DNA methylation, primarily at the promoter region and exon 1, can facilitate aberrant expression of protooncogenes or lead to repression of tumor suppressor gene expression (147). To examine the possibility that ET_B expression increased during androgen deprivation due to altered *EDNRB* promoter methylation, expression of DNMT enzymes was examined by Affymetrix analysis (Fig. 56).
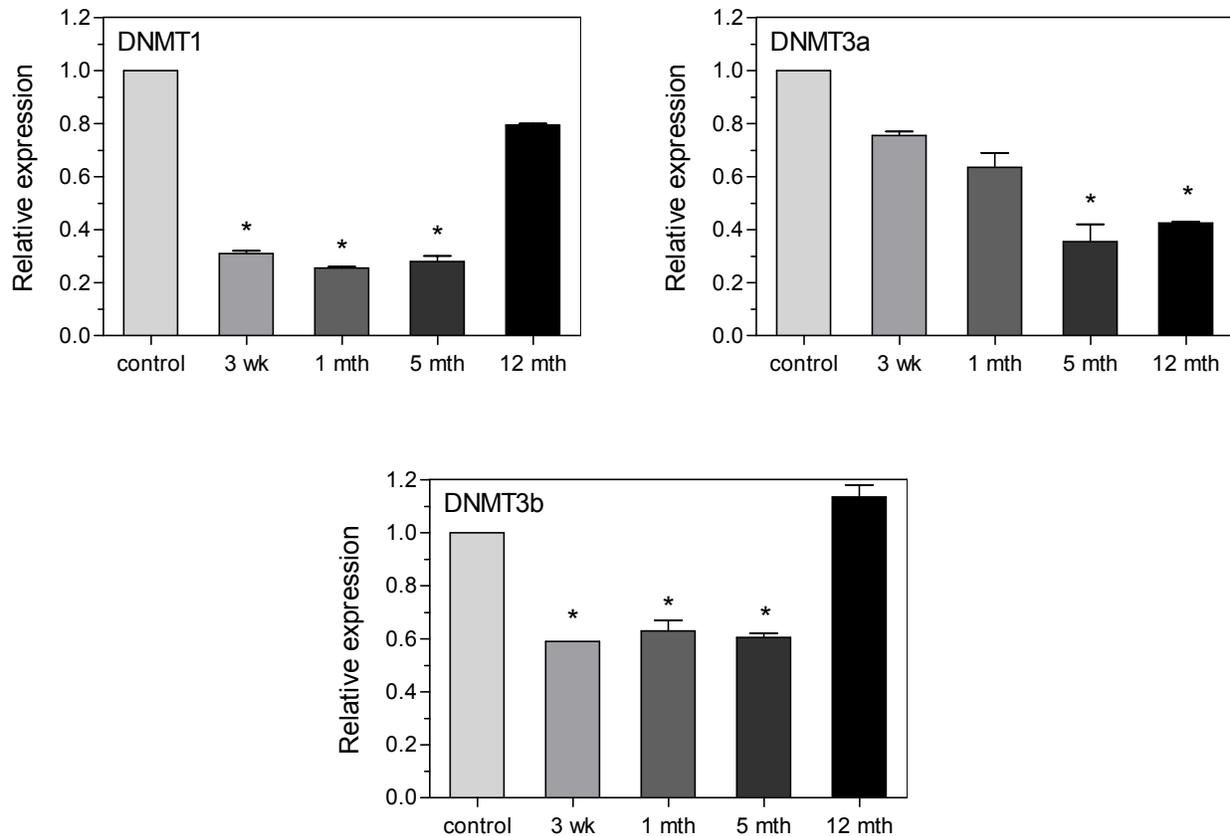
Figure 56. Affymetrix expression analysis of DNMT1, DNMT3a, and DNMT3b.

All three DNMT enzymes exhibited significantly reduced expression during the 12 months of androgen deprivation. Looking at the qPCR (Fig. 4) and Affymetrix (Fig. 55) ET_B data, it is reasonable to speculate that loss of DNA methylation, due to the reduction in DNMT1, could have resulted in active transcription of *EDNRB*. From 8-10 months, ET_B expression decreases, but remains elevated in LNCaP-AI cells (Fig. 5). Although DNMT3b levels stabilized by 12 months, reduced DNMT3a in LNCaP-AI cells might result in incomplete *de novo EDNRB* promoter methylation. The overall gene expression patterns of the DNMT enzymes provide a possible explanation for the observed alterations in ET_B expression.

3.3   CONCLUSIONS

The value of this longitudinal technique is evident not only in the vast body of information generated, but in the nature of the information itself. The findings from this Affymetrix expression array analysis describe changes in gene expression, at various time points, over the duration of a therapeutic intervention that resulted in the generation of therapy resistant prostate cancer cells. It was not a simple comparison of benign versus hyperplastic, benign versus malignant, or local versus metastatic, where a causal relationship is assumed and the dimension of time is lost. Rather, the longitudinal approach examines gene expression before, during and after the development of androgen independence. It is a measure of transformation and progression from one stage to an intermediate stage resulting in yet an entirely different cell type. At each of these stages in the progression to androgen independence, changes in gene expression were unique, thus defining distinctive cytological and phenotypic states.

Efforts focused initially on examining the changes in endothelin expression in the hope of confirming the qPCR results. The microarray gene expression changes for ET_A, ET-1, ET_B, and AR agree with the qPCR results, and show remarkable similarities in the patterns of expression. Affymetrix analysis also demonstrated altered gene expression of growth factors and growth factor receptors, as well as MMP-7, which is responsible for remodeling of the extracellular matrix, releasing growth and survival factors into the cellular milieu. Through modulating the expression of key cell cycle enzymes and controlling the function of pro-apoptotic factors, LNCaP cells appear to regulate cell cycle progression while suppressing apoptosis to survive and eventually acclimate to castrate levels of androgen.

It has been well established that the AR is a vital component to the development of prostate cancer and progression to advanced disease (111); however, AR cannot act alone. This study discovered changes in gene expression of AR co-factors that are essential in promoting AR function by stabilizing the AR/DNA interaction. Other co-factors promote epigenetic modification of the DNA as well as recruit additional co-factors such as p300 and CBP, which facilitate additional epigenetic modification.

It is becoming evident that prostate cancer cells are not just reactive to AAT, but proactive in the sense that they harvest adrenal androgens to aid in maintaining AR function (132). With the up-regulation of AKR1C enzymes both the conversion of adrenal androgens to testosterone and the metabolism of DHT are accelerated, allowing for increased AR activity in an environment deplete of testicular androgens.

Lastly, the morphological transformation that androgen deprived cells underwent reflects a neuroendocrine-like phenotype, and these observations were supported by significantly elevated expression of several neuroendocrine markers. This neuroendocrine morphology lasted approximately ten months and then essentially disappeared; however, the expression of neurotensin, enolase, chromogranin B, L-dopa decarboxylase and secretagogin remained elevated in the LNCaP-AI cells. This suggests that although the cells may not have terminally differentiated into neuroendocrine cells, they still exhibit molecular neuroendocrine characteristics that likely function in promoting the androgen-independent phenotype.

The value of this longitudinal approach lies in the ability to examine gene expression changes throughout the cellular response to androgen deprivation; it provides a more dynamic illustration of those genes which contribute to disease progression in addition to specific genes which constitute a malignant androgen-independent phenotype (Fig 57). In conclusion, it is of

great importance that new approaches, such as the one proposed here, are employed to continue exploring the cellular mechanisms of therapy resistance and identify promising targets to improve cancer therapeutics.

AR co-factors
SRC1
SRC2/TIF2
SRC3/AIB1
p300
CBP
GSN
GAK

Survival factors
ET_A
ET-1
AR
NFκB

Growth factors and receptors
erb-B2 (Her-2/*neu)*
Met
TGFβR1
Jagged-1
Shh
MMP-7
AR*
GR

Testosterone enzymes
AKR1C1
AKR1C2
AKR1C3
SRD5A1

Cell cycle factors
p53
p21
p16
CDK2
CDK4
Survivin

Apoptotic factors
Bax
Bak
Fas
cytochrome-C
p53*

Neuroendocrine factors
Neurotensin
Enolase 2
Chromogranin B
L-dopa decarboxylase
Secretagogin

Figure 57. Schematic summary of the gene expression changes discovered in 12 month androgen deprived LNCaP cells that likely play important roles in promoting prostate cancer cell survival, leading to the development of androgen-independent prostate cancer.

4.0   ET$_B$ SIGNALING IN PROSTATE CANCER CELLS

4.1   INTRODUCTION

To date, only two ET receptors have been identified in mammalian tissues, ET$_A$ and ET$_B$; the third, ET$_C$, remains to be identified in mammalian tissues and is not well characterized. The seven-transmembrane G-protein coupled ET$_B$ was originally cloned and characterized in 1990 (148) and has since been identified as an important protein in several different fields of human physiology. Unlike ET$_A$, which exhibits a greater affinity for ET-1 than ET-2 or ET-3 and shows high expression in vascular smooth muscle cells, ET$_B$ binds all three ET peptides with equal affinity and is predominantly expressed by vascular endothelial cells (61). ET$_B$ receptor signaling is essential in regulating vascular blood flow via nitric oxide-induced vasodilation (37,66) and has also been shown to affect neural crest cell migration during development (80). Through deregulated signaling, ET$_B$ has been associated with the pathobiology of several human cancers, including melanoma and prostate cancer (43,64,149,150). Although 63% similar to the ET$_A$ receptor at the protein level, ET$_B$ receptor signaling typically functions counteractive to that of ET$_A$ (61).

In the normal prostate, opposite the ET$_A$ receptor, ET$_B$ is predominantly expressed in the secretory luminal epithelial cells, with very low levels in the stromal compartment. Although luminal epithelial cell ET$_B$ expression has been shown to decrease in prostate cancer due to

promoter hypermethylation (68,151), it is hypothesized that ET_B signaling is primarily associated with ET-1 clearance (64) and potentially with the induction of apoptosis. Through sequestering ET-1, ET_B can modulate ET_A receptor signaling simply by regulating ET-1 availability. To test the extent of ET_B signaling in affecting apoptosis, ET_B null prostate cancer cells were transfected to express elevated levels of ET_B and then stimulated with ET-1. Induction of apoptosis was subsequently measured using an ELISA based assay that detects and quantifies the amount of cleaved DNA and histone fragments in apoptotic cells.

4.2   RESULTS

4.2.1   Time course serum starvation of PPC-1-ET_B cells.

To examine the potential role of ET_B signaling in prostate cancer cell apoptosis, a time course serum starvation of PPC-1 prostate cancer cells transfected to express the ET_B receptor (PPC-1-ET_B) was first performed. Using a PPC-1-ET_B clone, which expresses moderately elevated levels of ET_B, cells were cultured in serum-free media for 0, 4, 8, 12, 24 or 48 hours, lysed and examined for levels of apoptosis. Although apoptosis was significantly increased at all time points, treatment for 12 hours in serum-free media resulted in the greatest induction of apoptosis (Fig. 58). Vector-only PPC-1 cells (PPC-1-pCMV) were not used in this experiment because it was originally thought they would demonstrate no measurable induction of apoptosis given their lack of ET_B expression. However, in retrospect, they should have been used as the proper control for assessing the magnitude of apoptosis in the PPC-1-ET_B cells.
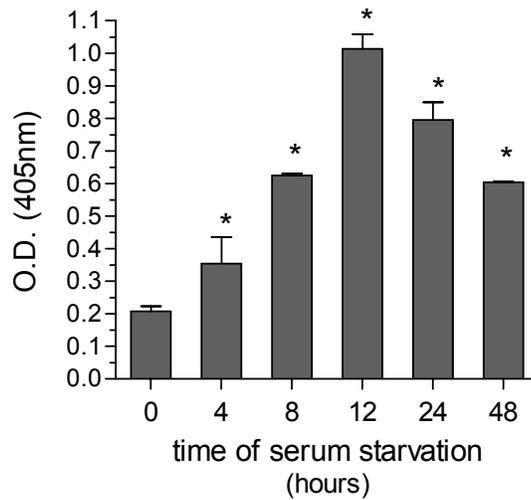
Figure 58. Time course serum starvation induction of apoptosis in PPC-1-ET$_B$ cells.
Assay performed in triplicate. Error bars indicate ± SEM. *, $P < 0.05$ relative to zero control cells.

4.2.2 Serum dose response induction of apoptosis in PPC-1-ET$_B$ cells.

Based on the time course analysis, 12 hours of treatment was determined to be the most ideal time for best quantifying the levels of apoptosis using this ELISA platform. Next, the impact of increasing doses of serum on apoptosis was examined to determine if serum aided in optimizing the assay and if so, what amount of serum was required. PPC-1-pCMV and PPC-1-ET$_B$ cells were treated for 12 hours in increasing amounts of serum, as indicated in figure 59, and examined by ELISA for the levels of apoptosis induction.
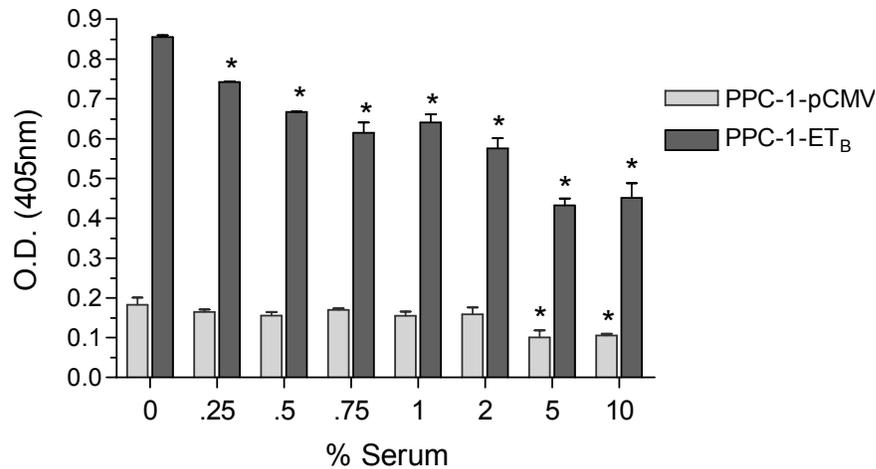
Figure 59. Serum dose response of PPC-1-pCMV and PPC-1-ET$_B$ cells.
Cells were treated for 12 hours. Assay performed in triplicate. Error bars indicate ± SEM. * $P < 0.05$ relative to respective zero control cells.

Serum-free treated PPC-1-ET$_B$ cells demonstrated the greatest amount of apoptosis, with significantly decreased levels with increasing serum. Additionally, vector-only cells demonstrated nearly unchanged levels of apoptosis, with only a slight decrease at the highest levels of serum. Importantly, PPC-1-pCMV cells exhibited consistently lower apoptosis than ET$_B$-expressing cells, supporting our hypothesis that cells lacking ET$_B$ receptors are more resistant to apoptosis. Concerned that background levels of apoptosis in PPC-1-ET$_B$ cells in serum-free media were too high for the ELISA system to accurately measure any further increase upon ET-1 stimulation, 1% serum was selected for further analyses. The expectation was that serum would maintain enough cellular stability but allow for sensitive and accurate measurement of cell death.

4.2.3 Induction of apoptosis by ET-1 signaling through the ET_B receptor.

With both treatment time and level of serum determined, we next compared the induction of apoptosis in PPC-1-pCMV and PPC-1-ET_B cells by challenging with ET-1. If ET_B receptor signaling induces apoptosis in prostate cancer cells, the hypothesis stands that elevated ET_B expression should enhance the induction of apoptosis over cells that lack ET_B. For 12 hours, PPC-1-pCMV and PPC-1-ET_B cells were treated in 1% serum with increasing amounts of ET-1 (0.01 – 100nM) or vehicle and analyzed for levels of apoptosis induction by ELISA (Fig. 60).
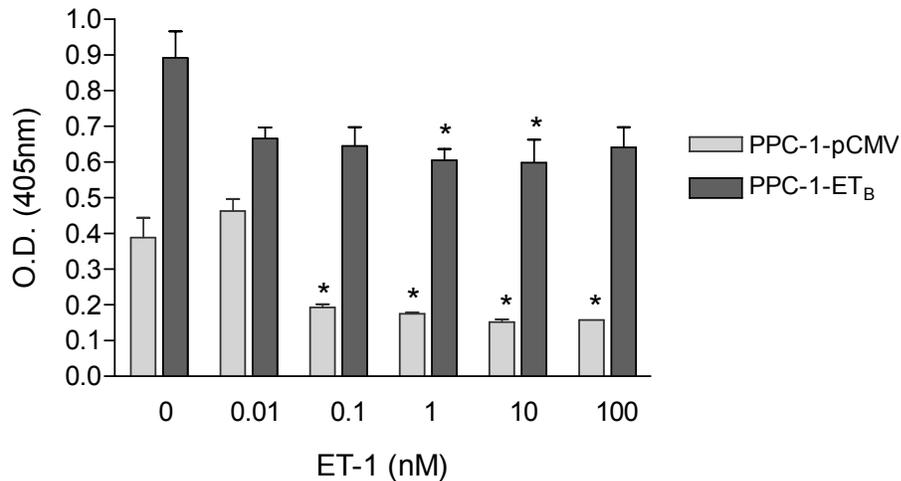
Figure 60. ET-1 induction of apoptosis in PPC-1pCMV and PPC-1-ET_B cells.
Cells were treated with ET-1 for 12 hours in 1% serum containing media. Assay performed in triplicate. Error bars indicate $\pm$ SEM. * $P < 0.05$ relative to respective zero control cells.

PPC-1 cells, like PC3 cells, express higher levels of endogenous ET_A receptor than other prostate cancer cells such as LNCaP and LAPC4. According to the results presented in figure 60, it became apparent that ET-1 treatment, which resulted in an overall decrease in apoptosis in both cell types, compared to vehicle control, might be activating ET_A survival signaling leading

112

to inhibition of apoptosis. However, the overall results were reassuring because the PPC-1-ET$_B$ cells demonstrated higher levels of apoptosis over the range of ET-1 treatment compared to vector-only cells. These results suggest that if ET$_A$ receptor blockade is used prior to ET-1 treatment, it might be possible to inhibit survival signaling and thus favor ET$_B$ activation. A likely explanation for the large difference in detection of cell death between PPC-1-pCMV and PPC-1-ET$_B$ cells could stem from the idea that, in the presence of secreted ET-1, only PPC-1-ET$_B$ cells should initiate apoptosis. The fact that PPC-1-pCMV cells lack surface ET$_B$ protein receptors could also explain why the low levels of apoptosis measured in PPC-1-pCMV cells follow a near linear trend.

Therefore, PPC-1-pCMV and PPC-1-ET$_B$ cells were first pretreated in 1% serum with 1μM ET$_A$ antagonist ABT-627 for 1 hour, then increasing amounts of ET-1 (0.01 – 100nM) or vehicle, and analyzed for levels of apoptosis induction by ELISA after 12 hours (Fig. 61).
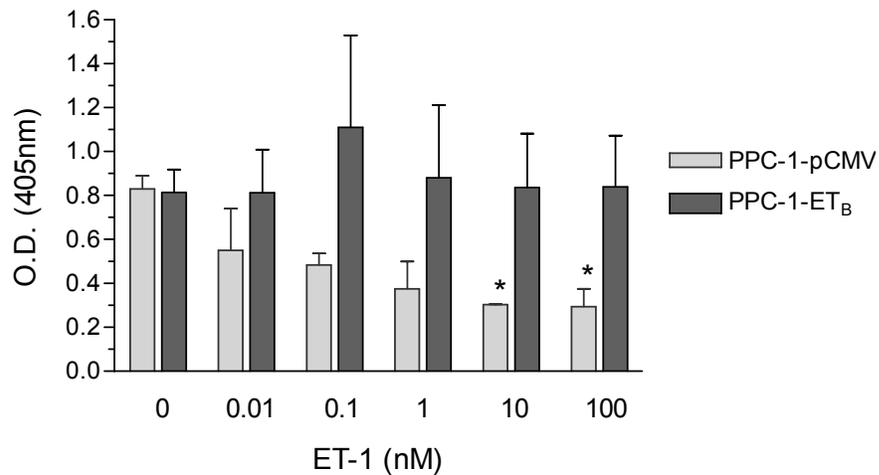
Figure 61.   ET-1 induction of apoptosis in PPC-1-pCMV and PPC-1-ET$_B$ cells with 1μM ABT-627 pretreatment.
ABT-627 pretreatment for 1 hour, ET-1 treatment for 12 hours in 1% serum. Assay performed in triplicate. Error bars indicate ± SEM. * $P < 0.05$ relative to respective zero control cells.

Our laboratory previously established that 1μM ABT-627 is highly effective in binding nearly all ET_A receptors with minimal toxicity, *in vitro* and *in vivo*. With ET_A blockade pretreatment it was anticipated that ET-1 signaling through the ET_B receptor would be favored predominantly over ET_A. Comparing the results of figures 60 and 61, it appears that ET_B signaling was favored slightly over that of ET_A, represented by the slight increase in apoptosis at 0.1nM ET-1 in PPC-1-ET_B cells; however, the changes were not significant (Fig. 61). PPC-1-pCMV cells, responding almost exactly the same as in the previous experiment, showed a steady decrease in apoptosis suggesting that by lacking any functional ET_B receptor, induction of apoptosis by ET-1 cannot be achieved. In fact, the decrease in apoptosis seen in PPC-1-pCMV cells over the ET-1 dose treatment is suggestive of intact ET_A receptor signaling, hinting that not all ET_A receptors were sufficiently inhibited.

To address concerns of ABT-627 specificity and function in this setting, PPC-1-pCMV and PPC-1-ET_B cells were pretreated with an increasing range of ABT-627 (0.1nM-1μM), then with 100nM ET-1 in 1% serum for 12 hours. Like previous assays, PPC-1-pCMV cells demonstrated no change in the levels of apoptosis. In PPC-1-ET_B cells it was hypothesized that there would be a trend of increasing apoptosis from the low to high concentrations of ABT-627 because as more ET_A receptors are inhibited, there should be more ET_B activation. However, PPC-1-ET_B cells showed no significant change in apoptosis compared to zero control cells (Fig. 62).
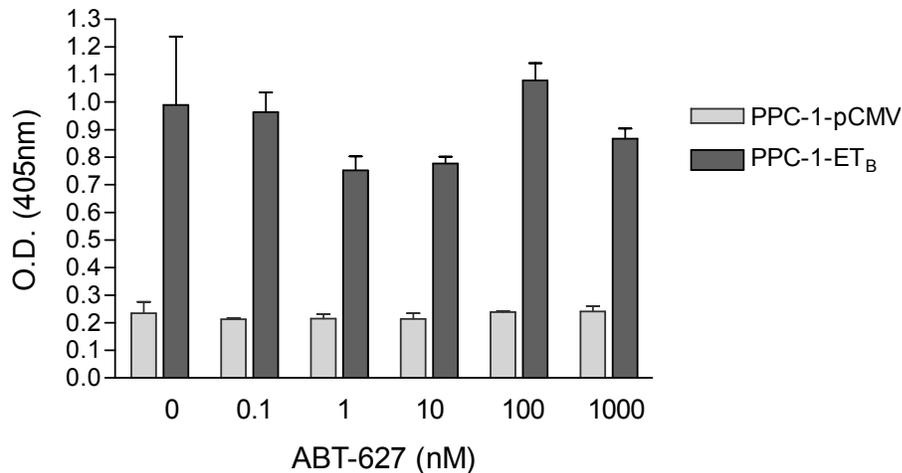
Figure 62. 100nM ET-1 induction of apoptosis in PPC-1-pCMV and PPC-1-ET$_B$ cells with dose ABT-627 pretreatment.
ABT-627 pretreatment for 1 hour, as indicated. ET-1 treatment for 12 hours in 1% serum. Assay performed in triplicate. Error bars indicate ± SEM.

Regarding the slight decrease in apoptosis in PPC-1-ET$_B$ cells from 100 to 1000nM (1μM), we suspected that 1μM ABT-627 might be interfering with ET$_B$ activation. Although not conclusive, we decided to reduce the amount of ABT-627 to 100nM in efforts to improve the induction and therefore detection of apoptosis following ET-1 treatment. It was also decided that the presence of serum might be complicating the sensitivity and accuracy of the assay, thus serum-free conditions were to be used here on out.

To get at the heart of whether ET$_B$ signaling plays a role in apoptosis in prostate cancer cells, PPC-1-pCMV and PPC-1-ET$_B$ cells were treated with 100nM ET-1, or vehicle, in serum-free media for 12 hours. However, select wells received 1 hour pretreatment of ABT-627, the selective ET$_B$ antagonist A-192621 (ABT-621), or both prior to ET-1 stimulation (Fig. 63).

115
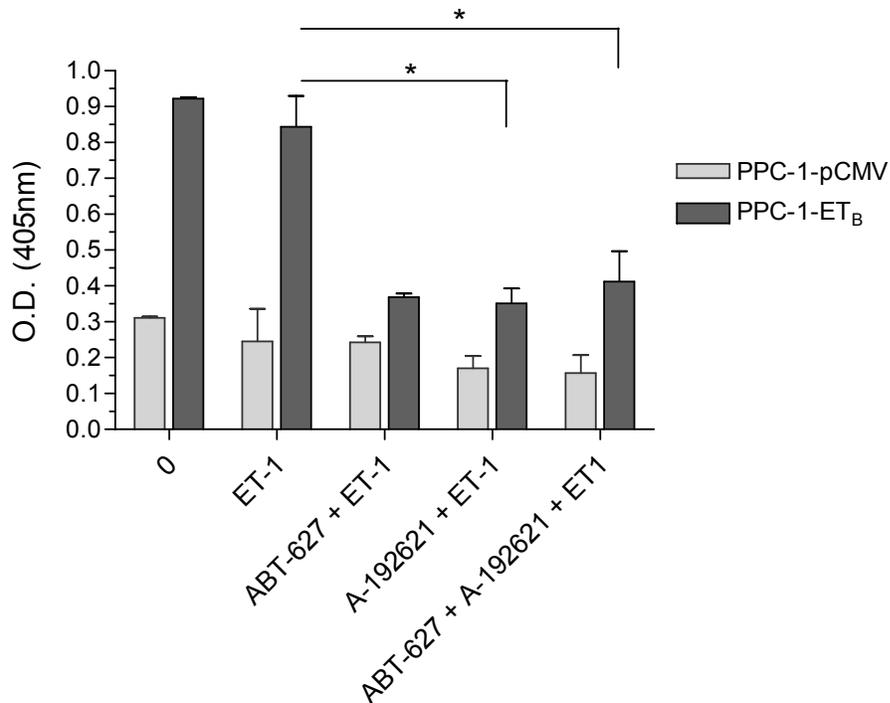
Figure 63. ET-1 induction of apoptosis in PPC-1-pCMV and PPC-1-ET_B cells with 100nM ABT-627 and/or A-192621 pretreatment.

Antagonist pretreatment for 1 hour, ET-1 treatment for 12 hours in serum-free conditions. Assay performed in triplicate. Error bars indicate $\pm$ SEM. *, $P < 0.05$.

Compared to ET-1 only treated cells, ET_B blockade in either single regime or in combination with ABT-627 prior to ET-1 stimulation resulted in significantly decreased levels of apoptosis, an indication that ET-1 signaling through the ET_B receptor might indeed be affecting apoptosis in prostate cancer cells. However, this conclusion can only be applied to those cells which exhibit expression of the ET_B receptor; it is this notion that highlights the clinical relevance of decreased ET_B expression in prostate cancer. On the contrary, the observations surrounding the use of ABT-627 and its failure to enhance apoptosis remain to be elucidated.

4.3   CONCLUSIONS

Based on the initial time course and serum dose response experiments, it appeared as if ET-1 engagement of the ET_B receptor might play a role in directly affecting prostate cancer cell apoptosis. Upon incorporation of the ET_A antagonist ABT-627, it was anticipated that levels of cell apoptosis would increase due to inhibition of survival signaling; however, this was not clearly demonstrated as ET_A antagonism had little effect on apoptosis. Even at increasing doses of ABT-627, attempts to induce apoptosis in ET_B-expressing PPC-1 cells were unsuccessful. After rethinking the serum dose response, it was decided to perform the assay in serum-free conditions to eliminate the confounding factor of serum-induced effects. With the use of A-12621 (ABT-621) prior to ET-1 stimulation, in comparison to ET-1 only treated cells, there was a significant reduction in the levels of apoptosis. Although a promising observation, it is important to recognize that the magnitude of apoptosis incurred by ET-1 treatment alone was not greater than control, serum-free treated cells.   While these results may not convincingly demonstrate an ET-1/ET_B specific mechanism, the effect of ET_B antagonism in blocking apoptosis cannot be ignored.

ET_A and ET_B share similar affinities for ET-1, so therefore it remains unclear if either receptor is favored when cells are exposed to ET-1. With the use of antagonists, a specific receptor subtype can be selectively inhibited, to a degree, but there always remains the issue of non-specific inhibition of the other receptor subtype. Further analysis with extended treatment times, both for antagonists and ET-1, should be performed to improve the specificity of this line of investigation. ET-1 induction of apoptosis should also be examined in prostate cells that endogenously express ET_B.

5.0   DISCUSSION

This work was undertaken to further our insight into the relationship between endothelin signaling and prostate cancer cell survival during androgen deprivation. The current hypothesis is that endothelin provides prostate epithelia an alternate means of survival during the stress of lost androgenic signaling by inhibiting apoptosis. The effect of androgen deprivation on the expression of ET_A had not been examined in human prostate cancer; therefore, studies were undertaken to establish the potential role of endothelin in progression to androgen-independent prostate cancer.

The vast majority of the endothelin related research centers on the role of endothelin in cardiovascular physiology. ET-1 is secreted predominantly by vascular endothelial cells and functions by binding either the ET_A or ET_B receptor to affect vascular tone and blood flow. Expressed predominantly by vascular smooth muscle cells, ET_A activation induces PLC production of secondary molecules DAG and IP3, which coordinate the release of intracellular calcium (92,152). Calcium mobilization is the driving force behind vascular smooth muscle contraction, resulting in vasoconstriction. Activation of the ET_B receptor, which is expressed primarily by vascular endothelial cells, triggers a cascade of intracellular molecules including cyclooxygenase, PLC, and nitric oxide synthase, resulting in the release of prostaglandins, calcium and NO/cGMP. Secretion of NO/cGMP counters the vasoconstrictive and mitogenic

118

effects of ET_A (33,153). Importantly, ET_B is essential in regulating the actions of ET-1 by binding and removing ET-1 from the cellular environment (64). Additionally, NO produced via ET_B signaling promotes the dissociation of ET-1 from ET receptors, thus curtailing the effects of ET-1 (154).

Following the identification of ET-1 and its cognate receptors in the prostate (155) and the discovery that levels of ET-1 in the human ejaculate are among the highest in the body (35), it became evident that endothelin likely played an important role in prostate physiology. In fact, it was soon discovered that levels of ET-1 and ET_A, but not ET_B, are elevated in some men with prostate cancer, particularly those patients with more advanced disease (36). Importantly, ET-1 was shown to possess mitogenic and survival properties which induce growth regulation and modulate apoptosis through the ET_A receptor (34,42,156).

Establishing the premise for this dissertation research project, our laboratory discovered that chronic androgen deprivation increased ET_A and reduced the levels of the regulatory counterpart, ET_B, in prostate cancer cells (Figs. 1 and 2). Because the majority of androgen ablated patients develop resistance to the effects of androgen withdrawal, and that ET_A signaling inhibits apoptosis in prostate cancer cells, we hypothesized that endothelin suppresses the apoptosis-inducing effects of androgen deprivation, thus promoting disease progression. However, the timing of endothelin up-regulation remained to be established in androgen ablated patients. Therefore, androgen-dependent prostate cancer cells were subjected to long-term androgen deprivation and examined for ET-1, ET_A and ET_B expression over 12 months. Within one month of treatment, all three genes exhibited increased expression. Additionally, AR and NEP expression increased in androgen deprived cells, suggestive of elevated levels of AR transcriptional activity even under conditions of reduced AR ligand. A potential explanation for

increased AR activity is likely related to the AR T877A mutation, carried by LNCaP cells, which promotes promiscuous AR activation (111). Also, epigenetic factors such as AR stabilization at the DNA level and alterations in the architecture of the AR transcriptional complex, via modifications in AR co-factor expression, function to promote AR transcriptional activity. Interestingly, NEP expression was elevated in ten month control cells, showed a decreasing trend through 12 months, but remained significantly elevated in the LNCaP-AI cells. This raises important questions about the effects of longevity in cell culture affecting gene expression independent of androgen deprivation. However, this effect was only observed in the expression of NEP, a surface metallopeptidase that functions in degrading peptides such as ET-1. NEP is transcriptionally regulated by AR but AR expression is not altered in the 10-12 month control cells, suggesting possible alterations in AR protein levels influencing AR transcriptional activities. Ambiguities surrounding the NEP expression analysis highlight one of the main setbacks in the design of this study.

Gene expression analysis provides a great deal of information regarding the effect of androgen deprivation on prostate cancer cells, but must be substantiated by protein analysis as post-transcriptional and post-translational regulations invariably impact ultimate protein levels. Surprisingly, the increase in ET_B expression was orders of magnitude greater than that of ET-1 and ET_A, suggesting a potential indirect association with up-regulation of ET_B transcription. Looking back to the Affymetrix analysis of ET_B, it was discovered that expression of the DNA methyltransferase enzymes DNMT1, DNMT3a, and DNMT3b were significantly reduced in the androgen deprived LNCaP cells. A sharp decrease in DNMT enzyme levels would lead to reduced, if not lost, maintenance of DNA methylation, potentiating the increase in transcription of genes under epigenetic control, such as ET_B. Additional studies are required to investigate the

potential causal link between androgen deprivation and epigenetic modifications, for this might have critical implication for patients undergoing AAT.

With increasing amounts of DHT, LAPC4 prostate cancer cells respond with increasing ET-1 secretion at very low and high concentrations of DHT. Upon disrupting the AR signaling axis, ET-1 secretion was increased at all concentrations of DHT, but most significantly at the range prostate cancer cells are accustomed (0.1-1nM). These results emphasize that prostate cancer cells secrete ET-1 in response to fluctuations in and the removal of androgenic stimulation. The most interesting aspect of these results is that LAPC4 ET-1 secretion did not increase linearly in response to increasing doses of DHT.

Studies looking at the effect of ET-1 stimulation of prostate cancer cells established for the first time that phosphorylation of protein kinase B/Akt is an ET_A-specific mechanism, and that, at elevated levels, ET_A receptor signaling significantly affects the induction of the survival factor Akt. The next logical study would be to determine whether activation of Akt takes place during androgen deprivation in parallel with ET-1 induction.

In regards to prostate cancer cell motility, the results from the matrigel invasion assays are preliminary at best. The invasion data establishes a correlation between ET_A signal transduction and prostate cancer cell motility, but fails to prove a direct link. Previous studies have identified NEP, RhoA, CXCL factors, and cell adhesion molecules as some of the factors associated with endothelin-induced migration in various normal and neoplastic cell types; however, there exists a preponderance of research documenting the role of ET_B receptor signaling in cell migration and invasion (79-81,83,157-161). Nonetheless, when considering recent studies in combination with these preliminary findings, which establish the foundation for ET_A involvement in cell motility regulation, it is essential to first identify factors downstream of

121

the ET_A receptor that more directly influence prostate cancer cell motility. Only then can we reasonably expect to demonstrate a causal relationship between ET-1 and prostate cancer cell invasiveness.

The immunohistochemical analysis of ET receptor expression following AAT not only supports the qPCR findings, but provides additional insight into the role of ET_A in prostate cancer cell survival. In long-term androgen ablated human prostate cancer tissue expression of the ET_A receptor, and not ET_B, was elevated. ET_A up-regulation at the protein level occurs approximately six months after treatment initiation, identifying a potential therapeutic window for the targeted use of ET_A antagonism in combination with current therapeutics.

The *in vivo* xenograft studies showed that combination castration plus ABT-627 significantly delayed LNCaP xenograft tumor growth. While parental LNCaP cells established the most numerous and stable xenograft tumors, ET_B-overexpressing cells successfully formed tumors as well; however, there was no significant effect with combination ABT-621 + castration over castration alone in affecting tumor progression. Importantly, LNCaP-ET_B tumors were highly vascularized whereas LNCaP tumors exhibited widespread necrosis with pockets of vascularized tumor cells, as visualized by CD31 immunohistochemical staining. Initially, the majority of mice injected with LNCaP-ET_A cells developed measurable tumors for several weeks; however, within a brief period of time all of the LNCaP-ET_A tumors spontaneously regressed, suggesting that something at the molecular or cellular level caused complete tumor cell death. Though undetermined, the cause is likely related to how the developing tumor microenvironment responded to ET_A-overexpression within the tumor. An interesting follow up study would be to perform a longitudinal assessment of LNCaP-ET_A tumor progression with IHC, RNA and protein analyses. Along the same lines as this *in vivo* study, previous

122

unpublished mouse studies from our lab, on rare occasion, found that ABT-627 treatment resulted in increased xenograft tumor growth potentially because ET_A blockade may have promoted vasodilation of the tumor microvasculature, thus effectively delivering more blood to the tumor. In attempts to make sense of these conflicting observations, it should be noted that Bagnato *et al*. showed that ET-1 binding ET_A promotes VEGF production leading to the formation of rudimentary vessel-like structures, suggesting a role for ET_A in angiogenesis (162). If ET_A signaling contributes to blood vessel development, spontaneous regression of LNCaP-ET_A tumors may be explained by insufficient ET_A signal transduction during the early stages of xenograft tumor development. Along the same lines, the role of ET_B in promoting various stages of angiogenesis, including endothelial cell proliferation, migration, and protease secretion, as well as neovascularization *in vivo*, has been well documented (149,163,164). Although the role of endothelin in tumor microvasculature development remains unclear from these studies, these results are extremely important in evaluating the impact and applicability of ET_A receptor antagonism clinically because the non-specific effects could outweigh the potential benefits.

To achieve the most effective use of ET_A antagonism in a therapeutic environment, it is essential to identify those patients who present with elevated levels of ET_A. Additionally, it is essential to further characterize the downstream intermediates of endothelin survival signaling. To improve upon these findings and further validate the use of ET_A antagonists clinically, additional *in vivo* studies should be conducted, which include the use of ET_A blockade in combination with anti-androgens, novel immune-based therapies, and chemotherapeutic agents. Now that the timing of ET_A receptor up-regulation in prostate cancer following AAT is better characterized, clinical investigation combining androgen ablation plus ET_A blockade is necessary.

123

Previous studies claim that androgen ablation, while killing most androgen-dependent cells (165), results in the selection for and clonal expansion of pre-existing androgen-independent cells (109,166-168). Our expression array analysis revealed that throughout the entire androgen deprivation protocol, expression of stem cell and basal cell markers were not detectable, suggesting that these cell types were not selected for during the androgen deprivation protocol. In support of cellular adaptation, the longitudinal Affymetrix studies discovered increased expression of genes linked to cell survival and proliferation, changes in critical cell cycle regulation genes, and decreased pro-apoptotic genes. Concurrently, sustained changes in Fas, Bax, p16, p21, Met, TGFβR1, Jagged-1, and Shh, may reflect more permanent alterations that inhibit apoptosis, promote accelerated cell proliferation, and contribute to the androgen-independent phenotype.

Acting to stabilize the AR/DNA interaction along with enhancing epigenetic modification, increased AR co-activators serve to sustain AR signaling in a ligand-deplete environment (129), thus contributing to the development of a more malignant phenotype. Additionally, increased expression of enzymes responsible for androgen metabolism supports the recent hypothesis that androgen-independent prostate cancer cells maintain AR signaling through harvesting adrenal androgens (132). In light of these observations, genes associated with androgen metabolism likely play a more prominent role in castrate men as the constant source of low level adrenal androgens could be exploited to sustain the prostate cancer cells, but in cell culture models the absence of this sustained androgen source would not necessitate the continued increase in expression of these genes. The ability of prostate cancer cells to up-regulate the expression of enzymes that convert adrenal androgens to testosterone exemplifies the adaptive nature of cancer cell biology, and are some of the most important and intriguing observations, in

my opinion, because they open up an entirely new approach to understanding and investigating prostate cancer. These results force us to step back from the molecular level and think about prostate carcinogenesis on the cellular and organismic level.

It has been proposed that since NE cells in the normal prostate lack AR expression and are considered post-mitotic, terminally differentiated cells, they are likely derived from resident stem cells (136). Some believe that prostate cancer epithelial cells transdifferentiate in NE cells, and that NE cells contribute to a more aggressive phenotype (169). However, it seems unlikely these androgen deprived LNCaP cells changed completely because LNCaP-AI cells retain expression of AR and epithelial markers, show no expression of basal or stem cell markers, and exhibit accelerated growth rates characteristic of aggressive prostate cancer cells. This data combined with recent studies on the NE component of prostate cancer have uncovered a potential link with AAT and, as a result, raised our awareness of how prostate cancer cells might be incorporating NE-like morphology and growth signaling in acquiring androgen independence. Though not ruling out the theory of selection, the results from this series of studies provide evidence that androgen-independent disease likely occurs, at least in part, due to adaptation (170).

The role of ET_B signaling in prostate cancer might hold important implications for understanding disease progression. The hypothesis is that ET-1 activation of ET_B induces apoptosis in prostate cancer cells. However, our results with PPC-1-ET_B cells leave open several questions regarding the nature of ET_B signaling in prostate cancer cell apoptosis. First, it remains unclear as to why there is a drop in apoptosis from the serum-free, vehicle control cells to the ABT-627 pretreated cells as seen in figure 63. In theory, ABT-627 treatment should block ET_A survival signaling and focus ET-1 signaling through ET_B, thus promoting cell death.

Second, ET-1 only treated cells demonstrated a significant increase in apoptosis compared to either single or combination antagonist treated cells, bringing into question the selectivity of ET-1 affinity in this *in vitro* system. In ET-1 only treated cells, it appears ET_B signaling might be overpowering that of ET_A, resulting in elevated levels of apoptosis compared to antagonist treated cells. However, upon examining the corresponding duplicate wells included for assessing cell counts, ET-1 only treated cells had the highest total number of cells. On the contrary, the optical density readings between serum-free and ET-1 only treated PPC-1-ET_B cells are nearly the same, suggesting that ET-1 treatment simply restored the levels of apoptosis to that of background. In the control PPC-1-ET_B cells it is possible that, because ET_A and ET_B exhibit similar high affinities for ET-1, there is signaling through both ET receptors. Simultaneous signal transduction through ET_A and ET_B could potentially generate both apoptotic-inducing and survival-inducing signals, thus reaching some sort of equilibrium. On the other hand, signaling through one ET receptor might generate a negative feedback loop to affect signaling through the other. To possibly differentiate between these two hypotheses, analysis of PPC-1-ET_B proliferation, with ET_A- or ET_B-blockade, could be employed. Concerning the control PPC-1-ET_B cells treated in serum-free conditions, it is possible that endogenous ET-1 secretion triggers a certain amount of apoptosis; however, the degree of apoptosis induction is likely affected by ET_A activation from endogenously secreted ET-1.

The fact that PPC-1-pCMV cells demonstrated no significant changes in apoptosis, regardless of treatment, is important because they demonstrate poor induction of apoptosis in cells that lack ET_B receptor expression. In PPC-1-pCMV cells, which express ET_A, there was no increase in apoptosis, even with ET_A blockade. On the other hand, the flat response of the PPC-1-pCMV cells lends no help to understanding the effects of ET-1 on the PPC-1-ET_B cells. Had

126

ABT-627 pretreatment resulted in higher levels of apoptosis in the vector-only cells, given the stress of serum-free conditions triggering non-ET apoptotic mechanisms, it would be reasonable to speculate that the ET_A receptor was functioning in the time frame of these experiments. However, as with the PPC-1-ET_B cells, ET-1 treatment alone resulted in a slight increase in the total number of PPC-1-pCMV cells, suggestive of ET_A mitogenic signaling. These results beg the question: could ET_A signaling promote cell proliferation at the same time ET_B activation induces apoptosis in the same cell population?

What is so fascinating about ET_B receptor signal transduction is that it has been so well characterized, in part, due to melanoma research, and that the role of ET_B signaling in melanoma is opposite to that which is hypothesized in prostate cancer. In fact, ET_B signaling in melanoma more or less parallels that of ET_A in prostate cancer, but ET_A signaling in prostate cancer is not as well characterized (149,150,171-173). In order to validate this hypothesis, detailed characterization of ET_B signaling in prostate cancer cells is required.

"There is little consensus regarding specific differences in receptor regulation…between ET_A and ET_B", even though both ET receptors possess putative SP-1 sites, four GATA sites, an Ebox, and an acute-phase response element (33). ET_A and ET_B share similar paths following ET-1 exposure, but the specificity of these pathways are highly site dependent. Future investigations should focus on characterizing the molecular link between AAT and altered ET-1 and ET_A expression, and seek to identify potential transcription factors responsible for regulating ET_A gene expression, particularly at times of cell stress. Future studies should also focus on expanding the characterization of the signaling intermediates downstream of ET_A to improve our understanding of survival signaling as well as identify potential factors associated with ET_A-induced cell motility. Following the expression studies discussed in chapter 2, it remains to be

shown that elevated levels of ET_A, during androgen deprivation, lead to the activation of Akt and enhanced prostate cancer cell survival. To demonstrate this, it is necessary to androgen deprive various ET_A-expressing cells, with or without ET_A inhibition, and assay for induction of Akt in surviving cells. Additionally, the role of endothelin in affecting angiogenesis, as raised by our mouse model studies showing regression of ET_A tumors and extensive vascularization of ET_B tumors, requires significant attention in order to ensure modulation of endothelin can be employed safely and effectively in the patient population.

Lastly, a very important concept in endothelin biology is the notion of ET_A GPCR transactivation of receptor tyrosine kinases (RTKs) such as EGFR. A seminal study by Daub *et al*. demonstrated that ET-1 engagement of the ET_A receptor leads to rapid phosphorylation of EGFR and Her2, activation of the MAP kinase pathway, *fos* gene expression, and DNA synthesis in rat fibroblasts (105). ET_A transactivation of EGFR mitogenic signaling has also been documented in ovarian carcinoma cells (106). These results are important because they demonstrate a role for downstream GPCR activation of RTKs in a RTK ligand-independent manner; therefore, future research should investigate the potential of this intracellular signal crosstalk in prostate cancer cells, especially during androgen deprivation.

The scientific and medical communities have known about the effects of castration and the benefits of androgen deprivation in treating prostate disease since 1895 (174). Over the past few decades, our understanding of prostate carcinogenesis has advanced dramatically; however, due to the complexity and multifactorial nature of this disease, improvements in current treatments and advances in novel therapeutic interventions must be made (175).

These studies have important implications for the treatment of advanced prostate cancer. The majority of ablated patients develop androgen-independent disease with approximately 23 to

37 months survival from time of AAT initiation (176). Efforts must focus on extending patient survival while maintaining quality of life parameters. In clinical trials, ABT-627 is well tolerated, reduces pain associated with metastatic disease, and has shown potential in inhibiting disease progression. We believe ET_A antagonism provides the opportunity to enhance the efficacy of hormone therapy while maintaining patient quality of life.

Collectively, the work presented in this thesis supports the use of ET_A antagonism, in combination with AAT, in treating advanced prostate cancer patients. In addition to the ET_A pathways associated with vasoconstriction and mitogenesis, these studies further illustrate the survival pathway as shown in figure 64. Importantly, these studies also demonstrate that ET survival signaling is not the only mechanism contributing to treatment resistance and the development of androgen independence. In the future, effective prostate cancer treatments will likely need to target multiple gene products as well as disrupt multiple intracellular and intercellular signaling mechanisms.
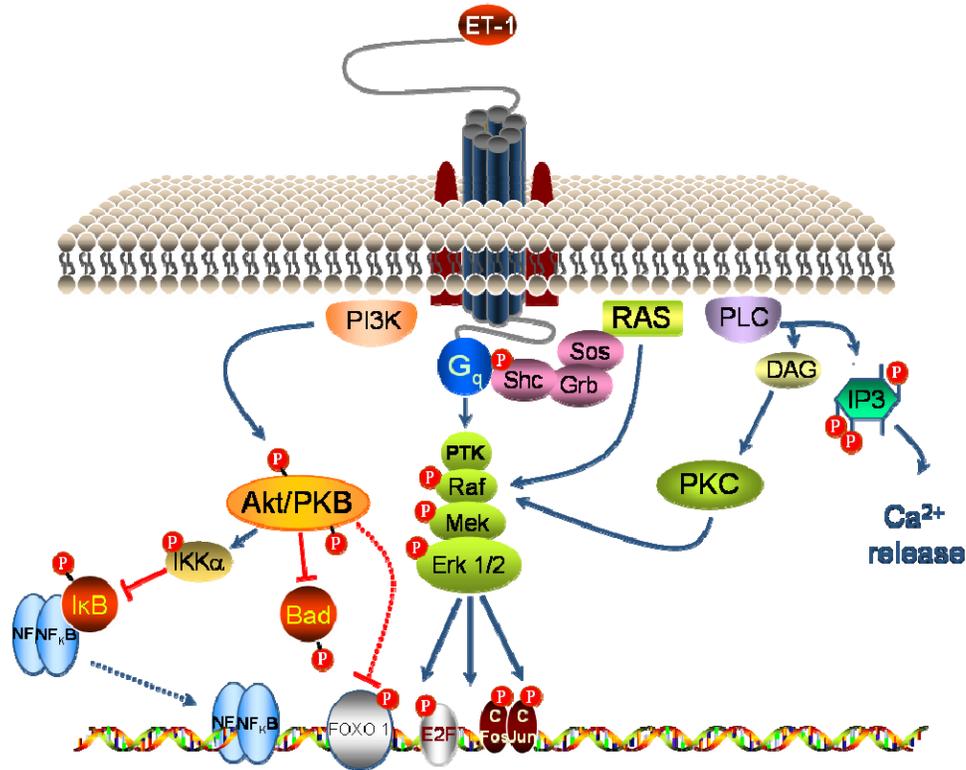
Figure 64. Schematic of the various ET_A downstream signaling pathways and signaling intermediates. Activation of PLC leading to the release of intracellular calcium drives the vasoconstrictive activities of vascular smooth muscles. The mitogenic signaling of ET_A, via induction of the MAPK pathway, has been demonstrated in various cell types, but appears not to be as major a pathway in prostate cancer cells as PI3K activation of Akt leading to inactivation of Bad. Though not shown to be ET_A specific, Akt activation is closely associated with FOXO1 inactivation as well as inactivation of IκB, thus permitting NFκB translocation to the nucleus to affect gene expression. Further studies are needed to determine if FOXO1 and NFκB are associated with ET_A signaling and/or induction of ET_A expression during AAT.

BIBLIOGRAPHY

1. Cunha GR, Ricke W, Thomson A, Marker PC, Risbridger G, Hayward SW, Wang YZ, Donjacour AA, Kurita T. Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development. J Steroid Biochem Mol Biol 2004;92(4):221-236.

2. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y. The endocrinology and developmental biology of the prostate. Endocr Rev 1987;8(3):338-362.

3. Hayashi N, Cunha GR, Parker M. Permissive and instructive induction of adult rodent prostatic epithelium by heterotypic urogenital sinus mesenchyme. Epithelial Cell Biol 1993;2(2):66-78.

4. Marker PC, Donjacour AA, Dahiya R, Cunha GR. Hormonal, cellular, and molecular control of prostatic development. Dev Biol 2003;253(2):165-174.

5. Cunha GR, Young P. Inability of Tfm (testicular feminization) epithelial cells to express androgen-dependent seminal vesicle secretory proteins in chimeric tissue recombinants. Endocrinology 1991;128(6):3293-3298.

6. Donjacour AA, Cunha GR. Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. Endocrinology 1993;132(6):2342-2350.

7. Cunha GR, Hayward SW, Dahiya R, Foster BA. Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. Acta Anat (Basel) 1996;155(1):63-72.

8. Cunha GR, Cooke PS, Kurita T. Role of stromal-epithelial interactions in hormonal responses. Arch Histol Cytol 2004;67(5):417-434.

9. Dong JT. Prevalent mutations in prostate cancer. J Cell Biochem 2006;97(3):433-447.

10.   Abate-Shen C, Shen MM. Molecular Genetics of Prostate Cancer. Genes & Development 2000;14:2410-2434.

11.   Serman A, Vlahovic M, Serman L, Bulic-Jakus F. DNA methylation as a regulatory mechanism for gene expression in mammals. Coll Antropol 2006;30(3):665-671.

12.   Kiefer JC. Epigenetics in development. Dev Dyn 2007;236(4):1144-1156.

13.   Gan Q, Yoshida T, McDonald OG, Owens GK. Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells 2007;25(1):2-9.

14.   Robertson KD. DNA methylation and human disease. Nat Rev Genet 2005;6(8):597-610.

15.   Galm O, Herman JG, Baylin SB. The fundamental role of epigenetics in hematopoietic malignancies. Blood Rev 2006;20(1):1-13.

16.   Hilakivi-Clarke L, de Assis S. Fetal origins of breast cancer. Trends Endocrinol Metab 2006;17(9):340-348.

17.   Flanagan JM. Host epigenetic modifications by oncogenic viruses. Br J Cancer 2007;96(2):183-188.

18.   Dobosy JR, Roberts JL, Fu VX, Jarrard DF. The expanding role of epigenetics in the development, diagnosis and treatment of prostate cancer and benign prostatic hyperplasia. J Urol 2007;177(3):822-831.

19.   Kerr KM, Galler JS, Hagen JA, Laird PW, Laird-Offringa IA. The role of DNA methylation in the development and progression of lung adenocarcinoma. Dis Markers 2007;23(1-2):5-30.

20.   Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM. Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci 2007;12:4254-4266.

21.   Orr JA, Hamilton PW. Histone acetylation and chromatin pattern in cancer. A review. Anal Quant Cytol Histol 2007;29(1):17-31.

22.   Rothhammer T, Bosserhoff AK. Epigenetic events in malignant melanoma. Pigment Cell Res 2007;20(2):92-111.

23.   Weidman JR, Dolinoy DC, Murphy SK, Jirtle RL. Cancer susceptibility: epigenetic manifestation of environmental exposures. Cancer J 2007;13(1):9-16.

24.   Wirth MP, Hakenberg OW, Froehner M. Optimal treatment of locally advanced prostate cancer. World J Urol 2007;25(2):169-176.

25.   Walczak JR, Carducci MA. Prostate cancer: a practical approach to current management of recurrent disease. Mayo Clin Proc 2007;82(2):243-249.

26.   Brand TC, Tolcher AW. Management of high risk metastatic prostate cancer: the case for novel therapies. J Urol 2006;176(6 Pt 2):S76-80; discussion S81-72.

27.   Fong L, Small EJ. Immunotherapy for prostate cancer. Curr Oncol Rep 2007;9(3):226-233.

28.   Nelson JB, Carducci MA. The role of endothelin-1 and endothelin receptor antagonists in prostate cancer. BJU Int 2000;85(Suppl 2):45-48.

29.   Nelson JB. Endothelin inhibition: novel therapy for prostate cancer. J Urol 2003;170(6 Pt 2):S65-67; discussion S67-68.

30.   Bhandari MS, Crook J, Hussain M. Should intermittent androgen deprivation be used in routine clinical practice? J Clin Oncol 2005;23(32):8212-8218.

31.   Ryan CJ, Small EJ. Early versus delayed androgen deprivation for prostate cancer: new fuel for an old debate. J Clin Oncol 2005;23(32):8225-8231.

32.   Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A 1989;86(8):2863-2867.

33.   Hunley TE, Kon V. Update on endothelins - biology and clinical implications. Pediatr Nephrol 2001;16(9):752-762.

34.   Battistini B, Chailler P, D'Orleans-Juste P, Briere N, Sirois P. Growth regulatory properties of endothelins. Peptides 1993;14(2):385-399.

35.   Langenstroer P, Tang R, Shapiro E, Divish B, Opgenorth T, Lepor H. Endothelin-1 in the human prostate: tissue levels, source of production and isometric tension studies. J Urol 1993;150(2 Pt 1):495-499.

36.   Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, Simons JW. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat Med 1995;1(9):944-949.

37.   Remuzzi G, Benigni A. Endothelins in the control of cardiovascular and renal function.[see comment]. Lancet 1993;342(8871):589-593.

38.   Casey ML, Byrd W, MacDonald PC. Massive amounts of immunoreactive endothelin in human seminal fluid. J Clin Endocrinol Metab 1992;74(1):223-225.

39.   Muldoon LL, Rodland KD, Forsythe ML, Magun BE. Stimulation of phosphatidylinositol hydrolysis, diacylglycerol release, and gene expression in response to endothelin, a potent new agonist for fibroblasts and smooth muscle cells. J Biol Chem 1989;264(15):8529-8536.

40.   Takuwa N, Takuwa Y, Yanagisawa M, Yamashita K, Masaki T. A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. J Biol Chem 1989;264(14):7856-7861.

41.   Sugawara F, Ninomiya H, Okamoto Y, Miwa S, Mazda O, Katsura Y, Masaki T. Endothelin-1-induced mitogenic responses of Chinese hamster ovary cells expressing human endothelinA: the role of a wortmannin-sensitive signaling pathway. Mol Pharmacol 1996;49(3):447-457.

42.   Wu-Wong JR, Chiou WJ, Dickinson R, Opgenorth TJ. Endothelin attenuates apoptosis in human smooth muscle cells. Biochem J 1997;328(Pt 3):733-737.

43.   Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. Nat Rev Cancer 2003;3(2):110-116.

44.   Pflug BR, Udan MS, Nelson JB. Endothelin-1 promotes survival in prostate and renal carcinoma cell lines by inhibition of apoptosis involving the AKT pathway.; 2000. Proc Am Assoc Ca Res. p 825.

45.   Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells.[see comment]. Nature 1988;332(6163):411-415.

46.   Bloch KD, Friedrich SP, Lee ME, al e. Strucural organization and chromosomal assignment of the gene encoding endothelin. J Biol Chem 1989;264(18):10851-10857.

134

47.   Inoue A, Yanagisawa M, Takuwa Y, Mitsui Y, Kobayashi M, Masaki T. The human preproendothelin-1 gene. Complete nucleotide sequence and regulation of expression. J Biol Chem 1989;264(25):14954-14959.

48.   Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell 1994;78(3):473-485.

49.   Kimura S, Kasuya Y, Sawamura T, Shinimi O, Sugita Y, Yanagisawa M, Goto K, Masaki T. Conversion of big endothelin-1 to 21-residue endothelin-1 is essential for expression of full vasoconstrictor activity: structure-activity relationships of big endothelin-1. J Cardiovasc Pharmacol 1989;13(Suppl 5):S5-7; discussion S18.

50.   Pirtskhalaishvili G, Nelson JB. The Endothelin Receptor; A novel target for anticancer therapy. Am J Cancer 2002;1(2):81-91.

51.   Oliver FJ, de la Rubia G, Feener EP, Lee ME, Loeken MR, Shiba T, Quertermous T, King GL. Stimulation of endothelin-1 gene expression by insulin in endothelial cells. J Biol Chem 1991;266(34):23251-23256.

52.   Skolovsky M, Galron R, Kloog Y, Bdolah A, Indig FE, Blumberg S, Fleminger G. Endothelins are more sensitive than sarafotoxins to neutral endopeptidase: possible physiological significance. Proc Natl Acad Sci U S A 1990;87(12):4702-4706.

53.   Papandreou CN, Usmani B, Geng Y, Bogenrieder T, Freeman R, Wilk S, Finstad CL, Reuter VE, Powell CT, Scheinberg D, Magill C, Scher HI, Albino AP, Nanus DM. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. Nat Med 1998;4(1):50-57.

54.   Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor.[see comment]. Nature 1990;348(6303):730-732.

55.   Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor.[see comment]. Nature 1990;348(6303):732-735.

56.   Weizmann Institute of Science. http://bioinfo.weizmann.ac.il/cards-bin/carddisp?EDNRA&search=ednra&suff=txt. GeneCard for gene EDNRA GC04P148979. Volume 2004; Copyright © 1997-2001.

57.   Weizmann Institute of Science. http://bioinfo.weizmann.ac.il/cards-bin/carddisp?EDNRB&search=ednra&suff=txt. GeneCard for gene EDNRB GC13M076267. Volume 2004; Copyright © 1997-2001.

58.   Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. Trends Pharmacol Sci 1992;13(3):103-108.

59.   Chun M, Lin HY, Henis YI, Lodish HF. Endothelin-induced endocytosis of cell surface ETA receptors. Endothelin remains intact and bound to the ETA receptor. J Biol Chem 1995;270(18):10855-10860.

60.   Yamaguchi T, Murata Y, Fujiyoshi Y, Doi T. Regulated interaction of endothelin B receptor with caveolin-1. Eur J Biochem 2003;270(8):1816-1827.

61.   Sokolovsky M. Endothelin Receptor Subtypes And Their Role In Transmembrane Signaling Mechanisms. Pharmac Ther 1995;68(3):435-471.

62.   Simonson MS, Herman WH. Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signaling by endothelin-1. Cross-talk between G protein-coupled receptors and pp60c-src. J Biol Chem 1993;268(13):9347-9357.

63.   Nelson JB, Udan MS, Guruli G, Pflug BR. Endothelin-1 inhibits apoptosis in prostate cancer. Neoplasia 2005;7(7):631-637, 2005 Jul.

64.   Fukuroda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M, Nishikibe M. Clearance of circulating endothelin-1 by ETB receptors in rats. Biochem Biophys Res Commun 1994;199(3):1461-1465.

65.   Yohn JJ, Smith C, Stevens T, Morelli JG, Shurnas LR, Walchak SJ, Hoffman TA, Kelley KK, Escobedo-Morse A, Yanagisawa M, et al. Autoregulation of endothelin-1 secretion by cultured human keratinocytes via the endothelin B receptor. Biochim Biophys Acta 1994;1224(3):454-458.

66.   Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, Marumo F. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. J Clin Invest 1993;91(4):1367-1373.

67.   Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS, Simons JW. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res 1996;56(4):663-668.

68. Nelson JB, Lee WH, Nguyen SH, Jarrard DF, Brooks JD, Magnuson SR, Opgenorth TJ, Nelson WG, Bova GS. Methylation of the 5' CpG island of the endothelin B receptor gene is common in human prostate cancer. Cancer Res 1997;57(1):35-37.

69. O'Reilly G, Charnock-Jones DS, Davenport AP, Cameron IT, Smith SK. Presence of messenger ribonucleic acid for endothelin-1, endothelin-2, and endothelin-3 in human endometrium and a change in the ratio of ETA and ETB receptor subtype across the menstrual cycle. J Clin Endocrinol Metab 1992;75(6):1545-1549.

70. Kubota T, Taguchi M, Kamada S, Imai T, Hirata Y, Marumo F, Aso T. Endothelin synthesis and receptors in human endometrium throughout the normal menstrual cycle. Hum Reprod 1995;10(8):2204-2208.

71. Polderman KH, Stehouwer CD, van Kamp GJ, Dekker GA, Verheugt FW, Gooren LJ. Influence of sex hormones on plasma endothelin levels. Ann Intern Med 1993;118(6):429-432.

72. Borcsok I, Schairer HU, Sommer U, Wakley GK, Schneider U, Geiger F, Niethard FU, Ziegler R, Kasperk CH. Glucocorticoids regulate the expression of the human osteoblastic endothelin A receptor gene. J Exp Med 1998;188(9):1563-1573.

73. Granchi S, Brocchi S, Bonaccorsi L, Baldi E, Vinci MC, Forti G, Serio M, Maggi M. Endothelin-1 production by prostate cancer cell lines is up-regulated by factors involved in cancer progression and down-regulated by androgens. Prostate 2001;49(4):267-277.

74. de Matteis A, Guidi A, Di Paolo B, Franco G, Revoltella RP. Endothelin-1 in human prostatic carcinoma treated with androgen withdrawal: an immunohistochemical study. Cancer 2001;91(10):1933-1939.

75. Padley RJ, Dixon DB, Wu-Wong JR. Effect of castration on endothelin receptors. Clin Sci (Colch) 2002;103(Suppl 48):442S-445S.

76. Takahashi W, Afiatpour P, Jr Foster HE, Ikeda K, Wada Y, Weiss RM, Latifpour J. The effect of castration on endothelins, their receptors and endothelin converting enzyme in rat prostate. Naunyn Schmiedebergs Arch Pharmacol 2002;366(2):166-176.

77. Shen R, Sumitomo M, Dai J, Hardy DO, Navarro D, Usmani B, Papandreou CN, Hersh LB, Shipp MA, Freedman LP, Nanus DM. Identification and characterization of two androgen response regions in the human neutral endopeptidase gene. Mol Cell Endocrinol 2000;170(1-2):131-142.

78.   Usmani BA, Harden B, Maitland NJ, Turner AJ. Differential expression of neutral endopeptidase-24.11 (neprilysin) and endothelin-converting enzyme in human prostate cancer cell lines. Clin Sci (Lond) 2002;103 Suppl 48:314S-317S.

79.   Lee HO, Levorse JM, Shin MK. The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Developmental Biology 2003;259(1):162-175.

80.   Berry P, Burchill S. Endothelins may modulate invasion and proliferation of Ewing's sarcoma and neuroblastoma. Clin Sci (Lond) 2002;103 Suppl 48:322S-326S.

81.   Rosano L, Spinella F, Di Castro V, Nicotra MR, Albini A, Natali PG, Bagnato A. Endothelin receptor blockade inhibits molecular effectors of Kaposi's sarcoma cell invasion and tumor growth in vivo. Am J Pathol 2003;163(2):753-762.

82.   Pedram A, Razandi M, Hu RM, Levin ER. Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion. J Biol Chem 1997;272(27):17097-17103.

83.   Dawson LA, Maitland NJ, Turner AJ, Usmani BA. Stromal-epithelial interactions influence prostate cancer cell invasion by altering the balance of metallopeptidase expression. Br J Cancer 2004;90(8):1577-1582.

84.   Nelson JB, Nabulsi AA, Vogelzang NJ, Breul J, Zonnenberg BA, Daliani DD, Schulman CC, Carducci MA. Suppression of prostate cancer induced bone remodeling by the endothelin receptor A antagonist atrasentan. J Urol 2003;169(3):1143-1149.

85.   Carducci MA, Padley RJ, Breul J, Vogelzang NJ, Zonnenberg BA, Daliani DD, Schulman CC, Nabulsi AA, Humerickhouse RA, Weinberg MA, Schmitt JL, Nelson JB. Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. J Clin Oncol 2003;21(4):679-689.

86.   Pecher S, Pflug BR, Brink AK, Nelson JB. Endothelin A receptor blockade does not alter PSA secretion in prostate cancer cell lines. Prostate 2004;60(3):175-177.

87.   Godara G, Cannon GW, Cannon GM, Jr., Bies RR, Nelson JB, Pflug BR. Role of endothelin axis in progression to aggressive phenotype of prostate adenocarcinoma. Prostate 2005:27-34.

88.   Akhavan A, McHugh KH, Guruli G, Bies RR, Zamboni WC, Strychor SA, Nelson JB, Pflug BR. Endothelin receptor A blockade enhances taxane effects in prostate cancer. Neoplasia 2006;8(9):725-732.

89.   Banerjee S, Hussain M, Wang Z, Saliganan A, Che M, Bonfil D, Cher M, Sarkar FH. In vitro and in vivo molecular evidence for better therapeutic efficacy of ABT-627 and taxotere combination in prostate cancer. Cancer Res 2007;67(8):3818-3826.

90.   Ma C, Lyons-Weiler M, Liang W, LaFramboise W, Gilbertson JR, Becich MJ, Monzon FA. In vitro transcription amplification and labeling methods contribute to the variability of gene expression profiling with DNA microarrays. J Mol Diagn 2006;8(2):183-192.

91.   Yanagisawa M, Kurihara H, Kimura S, Goto K, Masaki T. A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels. J Hypertens Suppl 1988;6(4):S188-191.

92.   Takuwa Y, Ohue Y, Takuwa N, Yamashita K. Endothelin-1 activates phospholipase C and mobilizes Ca2+ from extra- and intracellular pools in osteoblastic cells. Am J Physiol 1989;257(6 Pt 1):E797-803.

93.   Gohji K, Kitazawa S, Tamada H, Katsuoka Y, Nakajima M. Expression of endothelin receptor a associated with prostate cancer progression. Journal of Urology 2001;165(3):1033-1036.

94.   Godara G, Pecher S, Jukic DM, D'Antonio JM, Akhavan A, Nelson J, Pflug BR. Distinct patterns of endothelin axis expression in primary prostate cancer. Urology 2007;70(1):218-224.

95.   Veldscholte J, Berrevoets CA, Mulder E. Studies on the human prostatic cancer cell line LNCaP. J Steroid Biochem Mol Biol 49(4-6) 1994:341-346.

96.   Lee C, Sutkowski DM, Sensibar JA, Zelner D, Kim I, Amsel I, Shaw N, Prins GS, Kozlowski JM. Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. Endocrinology 1995;136(2):796-803.

97.   Kokontis JM, Hay N, Liao S. Progression of LNCaP prostate tumor cells during androgen deprivation: hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. 1998:941-953, 1998 Jul.

98.   Pflug BR, Reiter RE, Nelson JB. Caveolin expression is decreased following androgen deprivation in human prostate cancer cell lines. Prostate 1999;40(4):269-273.

99. Litvinov IV, Vander Griend DJ, Antony L, Dalrymple S, De Marzo AM, Drake CG, Isaacs JT. Androgen receptor as a licensing factor for DNA replication in androgen-sensitive prostate cancer cells. Proc Natl Acad Sci U S A 2006;103(41):15085-15090.

100. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 2002;62(4):1008-1013.

101. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 1997;126(1):59-73.

102. Veldscholte J, Berrevoets CA, Brinkmann AO, Grootegoed JA, Mulder E. Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation. 1992:2393-2399, 1992 Mar 2393.

103. Sumitomo M, Shen R, Walburg M, Dai J, Geng Y, Navarro D, Boileau G, Papandreou CN, Giancotti FG, Knudsen B, Nanus DM. Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. 2000:1399-1407, 2000 Dec.

104. Zheng R, Iwase A, Shen R, Goodman OB, Jr., Sugimoto N, Takuwa Y, Lerner DJ, Nanus DM. Neuropeptide-stimulated cell migration in prostate cancer cells is mediated by RhoA kinase signaling and inhibited by neutral endopeptidase. Oncogene 2006;25(44):5942-5952.

105. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 1996;379(6565):557-560.

106. Vacca F, Bagnato A, Catt KJ, Tecce R. Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. Cancer Res 2000;60(18):5310-5317.

107. Isaacs JT, Coffey DS. Etiology and disease process of benign prostatic hyperplasia. Prostate - Supplement 1989;2:33-50.

108. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer.[erratum appears in Prostate 1996 Jun;28(6):414]. Prostate 1996;28(4):251-265.

109. Bui M, Reiter RE. Stem cell genes in androgen-independent prostate cancer. Cancer Metastasis Rev 1998;17(4):391-399.

110. Isaacs JT. The biology of hormone refractory prostate cancer. Why does it develop? Urol Clin North Am 1999;26(2):263-273.

111. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1(1):34-45.

112. Wang Y, Hayward S, Cao M, Thayer K, Cunha G. Cell differentiation lineage in the prostate. Differentiation 2001;68(4-5):270-279.

113. Menard S, Pupa SM, Campiglio M, Tagliabue E. Biologic and therapeutic role of HER2 in cancer. Oncogene 2003;22(42):6570-6578.

114. Okegawa T, Kinjo M, Nutahara K, Higashihara E. Pretreatment serum level of HER2/nue as a prognostic factor in metastatic prostate cancer patients about to undergo endocrine therapy. Int J Urol 2006;13(9):1197-1201.

115. Mellinghoff IK, Vivanco I, Kwon A, Tran C, Wongvipat J, Sawyers CL. HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. Cancer Cell 2004;6(5):517-527.

116. Litvinov IV, De Marzo AM, Isaacs JT. Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? J Clin Endocrinol Metab 2003;88(7):2972-2982.

117. Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. Crit Rev Oncog 1999;10(4):303-360.

118. Ge R, Rajeev V, Ray P, Lattime E, Rittling S, Medicherla S, Protter A, Murphy A, Chakravarty J, Dugar S, Schreiner G, Barnard N, Reiss M. Inhibition of growth and metastasis of mouse mammary carcinoma by selective inhibitor of transforming growth factor-beta type I receptor kinase in vivo. Clin Cancer Res 2006;12((14 Pt 1)):4315-4330.

119. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG, Moses HL. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia.[see comment]. Science 2004;303(5659):848-851.

120. Santagata S, Demichelis F, Riva A, Varambally S, Hofer MD, Kutok JL, Kim R, Tang J, Montie JE, Chinnaiyan AM, Rubin MA, Aster JC. JAGGED1 expression is associated with prostate cancer metastasis and recurrence. Cancer Res 2004;64(19):6854-6857.

121. Zhang Y, Wang Z, Ahmed F, Banerjee S, Li Y, Sarkar FH. Down-regulation of Jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells. Int J Cancer 2006;119(9):2071-2077.

122. Sheng T, Li C, Zhang X, Chi S, He N, Chen K, McCormick F, Gatalica Z, Xie J. Activation of the hedgehog pathway in advanced prostate cancer. Mol Cancer 2004;3:29.

123. Datta S, Datta MW. Sonic Hedgehog signaling in advanced prostate cancer. Cell Mol Life Sci 2006;63(4):435-448.

124. Ii M, Yamamoto H, Adachi Y, Maruyama Y, Shinomura Y. Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med (Maywood) 2006;231(1):20-27.

125. Ouyang XS, Wang X, Lee DT, Tsao SW, Wong YC. Up-regulation of TRPM-2, MMP-7 and ID-1 during sex hormone-induced prostate carcinogenesis in the Noble rat. Carcinogenesis 2001;22(6):965-973.

126. Li F, Brattain MG. Role of the Survivin gene in pathophysiology. Am J Pathol 2006;169(1):1-11.

127. Lubahn DB, Joseph DR, Sar M, Tan J, Higgs HN, Larson RE, French FS, Wilson EM. The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 1988;2(12):1265-1275.

128. Balk SP. Androgen receptor as a target in androgen-independent prostate cancer. Urology 2002;60(3 Suppl 1):132-138; discussion 138-139.

129. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 2007;120(4):719-733.

130. Heemers HV, Sebo TJ, Debes JD, Regan KM, Raclaw KA, Murphy LM, Hobisch A, Culig Z, Tindall DJ. Androgen Deprivation Increases p300 Expression in Prostate Cancer Cells. Cancer Res 2007;67(7):3422-3430.

131. Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, Chang HC, Yeh S, Miyamoto H, Shin M, Aozasa K, Okuyama A, Chang C. Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res 2003;63(16):4888-4894.

132. Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, Febbo PG, Balk SP. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Res 2006;66(5):2815-2825.

133. Luo J, Dunn TA, Ewing CM, Walsh PC, Isaacs WB. Decreased gene expression of steroid 5 alpha-reductase 2 in human prostate cancer: implications for finasteride therapy of prostate carcinoma. Prostate 2003;57(2):134-139.

134. Thomas LN, Lazier CB, Gupta R, Norman RW, Troyer DA, O'Brien SP, Rittmaster RS. Differential alterations in 5alpha-reductase type 1 and type 2 levels during development and progression of prostate cancer. Prostate 2005;63(3):231-239.

135. Titus MA, Gregory CW, Ford OH, 3rd, Schell MJ, Maygarden SJ, Mohler JL. Steroid 5alpha-reductase isozymes I and II in recurrent prostate cancer. Clin Cancer Res 2005;11(12):4365-4371.

136. Shariff AH, Ather MH. Neuroendocrine differentiation in prostate cancer. Urology 2006;68(1):2-8.

137. Abrahamsson PA, Falkmer S, Falt K, Grimelius L. The course of neuroendocrine differentiation in prostatic carcinomas. An immunohistochemical study testing chromogranin A as an "endocrine marker". Pathol Res Pract 1989;185(3):373-380.

138. Hirano D, Okada Y, Minei S, Takimoto Y, Nemoto N. Neuroendocrine differentiation in hormone refractory prostate cancer following androgen deprivation therapy. Eur Urol 2004;45(5):586-592; discussion 592.

139. Lu S, Tsai SY, Tsai MJ. Molecular mechanisms of androgen-independent growth of human prostate cancer LNCaP-AI cells. Endocrinology 1999;140(11):5054-5059.

140. Wright ME, Tsai MJ, Aebersold R. Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. Mol Endocrinol 2003;17(9):1726-1737.

141. Yuan TC, Veeramani S, Lin FF, Kondrikou D, Zelivianski S, Igawa T, Karan D, Batra SK, Lin MF. Androgen deprivation induces human prostate epithelial neuroendocrine differentiation of androgen-sensitive LNCaP cells. Endocr Relat Cancer 2006;13(1):151-167.

142. Sehgal I, Powers S, Huntley B, Powis G, Pittelkow M, Maihle NJ. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. Proc Natl Acad Sci U S A 1994;91(11):4673-4677.

143. Adolf K, Wagner L, Bergh A, Stattin P, Ottosen P, Borre M, Birkenkamp-Demtroder K, Orntoft TF, Torring N. Secretagogin is a new neuroendocrine marker in the human prostate. Prostate 2007;67(5):472-484.

144. Wafa LA, Palmer J, Fazli L, Hurtado-Coll A, Bell RH, Nelson CC, Gleave ME, Cox ME, Rennie PS. Comprehensive expression analysis of L-dopa decarboxylase and established neuroendocrine markers in neoadjuvant hormone-treated versus varying Gleason grade prostate tumors. Hum Pathol 2007;38(1):161-170.

145. Turek-Plewa J, Jagodzinski PP. The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol Biol Lett 2005;10(4):631-647.

146. Hermann A, Gowher H, Jeltsch A. Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci 2004;61(19-20):2571-2587.

147. Luczak MW, Jagodzinski PP. The role of DNA methylation in cancer development. Folia Histochem Cytobiol 2006;44(3):143-154.

148. Arai H, Nakao K, Takaya K, Hosoda K, Ogawa Y, Nakanishi S, Imura H. The human endothelin-B receptor gene. Structural organization and chromosomal assignment. J Biol Chem 1993;268(5):3463-3470.

149. Lahav R, Suva ML, Rimoldi D, Patterson PH, Stamenkovic I. Endothelin receptor B inhibition triggers apoptosis and enhances angiogenesis in melanomas. 2004:8945-8953, 2004 Dec 8915.

150. Bagnato A, Rosano L, Spinella F, Di Castro V, Tecce R, Natali PG. Endothelin B receptor blockade inhibits dynamics of cell interactions and communications in melanoma cell progression. 2004:1436-1443, 2004 Feb 1415.

151. Jeronimo C, Henrique R, Campos PF, Oliveira J, Caballero OL, Lopes C, Sidransky D. Endothelin B receptor gene hypermethylation in prostate adenocarcinoma. Journal of Clinical Pathology 2003;56(1):52-55.

152. Topouzis S, Pelton JT, Miller RC. Effects of calcium entry blockers on contractions evoked by endothelin-1, [Ala3,11]endothelin-1 and [Ala1,15]endothelin-1 in rat isolated aorta. Br J Pharmacol 1989;98(2):669-677.

153. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev 1994;46(3):325-415.

154. Goligorsky MS, Tsukahara H, Magazine H, Andersen TT, Malik AB, Bahou WF. Termination of endothelin signaling: role of nitric oxide. J Cell Physiol 1994;158(3):485-494.

155. Kobayashi S, Tang R, Wang B, Opgenorth T, Stein E, Shapiro E, Lepor H. Localization of endothelin receptors in the human prostate. J Urol 1994;151(3):763-766.

156. Eberle J, Fecker LF, Orfanos CE, Geilen CC. Endothelin-1 decreases basic apoptotic rates in human melanoma cell lines. 2002:549-555, 2002 Sep.

157. Kohno M, Yokokawa K, Yasunari K, Kano H, Minami M, Yoshikawa J. Effect of the endothelin family of peptides on human coronary artery smooth-muscle cell migration. J Cardiovasc Pharmacol 1998;31 Suppl 1:S84-89.

158. Chakraborty C, Barbin YP, Chakrabarti S, Chidiac P, Dixon SJ, Lala PK. Endothelin-1 promotes migration and induces elevation of [Ca2+]i and phosphorylation of MAP kinase of a human extravillous trophoblast cell line. Mol Cell Endocrinol 2003;201(1-2):63-73.

159. Spinella F, Rosano L, Di Castro V, Nicotra MR, Natali PG, Bagnato A. Endothelin-1 decreases gap junctional intercellular communication by inducing phosphorylation of connexin 43 in human ovarian carcinoma cells. J Biol Chem 2003;278(42):41294-41301.

160. Rosano L, Spinella F, Salani D, Di Castro V, Venuti A, Nicotra MR, Natali PG, Bagnato A. Therapeutic targeting of the endothelin a receptor in human ovarian carcinoma. Cancer Res 2003;63(10):2447-2453.

161. Mangahas CR, dela Cruz GV, Friedman-Jimenez G, Jamal S. Endothelin-1 induces CXCL1 and CXCL8 secretion in human melanoma cells. J Invest Dermatol 2005;125(2):307-311.

162. Bagnato A, Spinella F. Emerging role of endothelin-1 in tumor angiogenesis. Trends Endocrinol Metab 14(1):44-50 2003;14(4):44-50.

163. Ziche M, Morbidelli L, Donnini S, Ledda F. ETB receptors promote proliferation and migration of endothelial cells. J Cardiovasc Pharmacol 1995;26 Suppl 3:S284-286.

164. Dong F, Zhang X, Wold LE, Ren Q, Zhang Z, Ren J. Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ETB receptor, NADPH oxidase and caveolin-1. Br J Pharmacol 2005;145(3):323-333.

165.   English HF, Kyprianou N, Isaacs JT. Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. Prostate 1989;15(3):233-250.

166.   Isaacs JT, Coffey DS. Adaptation versus selection as the mechanism responsible for the relapse of prostatic cancer to androgen ablation therapy as studied in the Dunning R-3327-H adenocarcinoma. Cancer Res 1981;41(12 Pt 1):5070-5075.

167.   Craft N, Chhor C, Tran C, Belldegrun A, DeKernion J, Witte ON, Said J, Reiter RE, Sawyers CL. Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. Cancer Res 1999;59(19):5030-5036.

168.   Tso CL, McBride WH, Sun J, Patel B, Tsui KH, Paik SH, Gitlitz B, Caliliw R, van Ophoven A, Wu L, deKernion J, Belldegrun A. Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells.[see comment]. Cancer J 2000;6(4):220-233.

169.   Deeble PD, Cox ME, Frierson HF, Jr., Sikes RA, Palmer JB, Davidson RJ, Casarez EV, Amorino GP, Parsons SJ. Androgen-Independent Growth and Tumorigenesis of Prostate Cancer Cells Are Enhanced by the Presence of PKA-Differentiated Neuroendocrine Cells. Cancer Res 2007;67(8):3663-3672.

170.   So A, Gleave M, Hurtado-Col A, Nelson C. Mechanisms of the development of androgen independence in prostate cancer. World J Urol 2005;23(1):1-9.

171.   Okazawa M, Shiraki T, Ninomiya H, Kobayashi S, Masaki T. Endothelin-induced apoptosis of A375 human melanoma cells. J Biol Chem 1998;273(20):12584-12592.

172.   Vichi P, Whelchel A, Posada J. Transmembrane helix 7 of the endothelin B receptor regulates downstream signaling. Journal of Biological Chemistry 1999;274(15):10331-10338.

173.   Lahav R, Heffner G, Patterson PH. An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo. 1999:11496-11500, 11999 Sep 11428.

174.   White JW. The results of double castration in hypertrophy of the prostate. Ann Surg 1895;22:1-80.

175.   Nelson WG. Prostate cancer prevention. Curr Opin Urol 2007;17(3):157-167.

176.   Hellerstedt BA, Pienta KJ. The current state of hormonal therapy for prostate cancer.[summary for patients in CA Cancer J Clin. 2002 May-Jun;52(3):180; PMID: 12018930]. CA Cancer J Clin 2002;52(154-179):a Cancer Journal for Clinicians. 52(53):154-179, 2002 May-Jun.

176. Hellerstedt BA, Pienta KJ. The current state of hormonal therapy for prostate cancer.[summary for patients in CA Cancer J Clin. 2002 May-Jun;52(3):180; PMID: 12018930]. CA Cancer J Clin 2002;52(154-179):a Cancer Journal for Clinicians. 52(53):154-179, 2002 May-Jun.