ANDROGEN MEDIATED REGULATION OF VEGF IN PROSTATE CANCER

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CHAPTER I

INTRODUCTION

The Prostate and prostate cancer

The prostate is a small sex accessory gland in the male reproductive system located beneath the urinary bladder surrounding the urethra (Figure 1) (Dehm and Tindall, 2006). It is comprised of many fluid producing glands that produce secretions that aid in the ejaculatory function and protect sperm as they exit the seminal vesicles. As with all human malignancies, cancer of this organ occurs when genetic mutations arise leading to uncontrollable cell proliferation. Although the cause of prostate cancer still remains a mystery, androgen regulation is extensively studied since androgen signaling is vital to both normal prostate development and prostate carcinogenesis.

Worldwide, there are more than 900,000 new cases of prostate cancer each year, resulting in more than 250,000 deaths. In the United States, prostate cancer is the most frequently diagnosed cancer in men with more than 200,000 new cases each year and the second most deadly, killing roughly 30,000 men annually (Jemal et al., 2010). Nearly one in six men will develop prostate cancer at some point in their life, with the majority of incidences occurring after the age of 50. The risks for cancer development include both genetic and dietary factors, but the cause of developing prostate cancer still remain
Figure 1: The prostate gland. The prostate is a walnut-sized gland that is located anterior to the rectum and inferior to the bladder. It has many roles, including storing and secreting an alkaline fluid that aids in the ejaculatory function and protects sperm as they exit the seminal vesicles. (http://medicalcenter.osu.edu/greystone/images/ei_0327.jpg).
unknown. Despite decades of research spent looking into the mechanisms that cause prostate cancer cells to become malignant and metastatic, very little is understood. Thus, new treatment options need to be developed and this begins with uncovering the mechanisms underlying this disease.

**Role of Androgen in Prostate Cancer Progression**

Both normal prostate and prostate carcinoma depend on the presence of androgen for growth. The development of the prostate is dependent on a functional androgen receptor (AR) (Heinlein and Chang, 2004). The primary circulating androgen in men is testosterone, with 95% being produced in the Leydig cells of the testis and the other 5% by the adrenal gland (Debes and Tindall, 2002). Once inside the prostate gland, testosterone is converted into 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase where it can bind to the AR and induce growth of the male urogenital structures. Men lacking a functional 5α-reductase gene have only a small partial prostate or the prostate is completely undetectable (Heinlein and Chang, 2004). Thus, indicating that a high level of DHT is necessary for complete prostate development.

Prostate cancer is also dependent on the actions of androgens and functional AR expression. AR is expressed in both androgen-dependent (AD) and -independent (AI) prostate cancers and is sustained throughout progression of the disease to hormone refractory prostate cancer (Knudsen and Penning, 2010; Yuan and Balk, 2009). Prostate cancer therapy is focused upon blocking androgen activity and androgen ablation therapy causes atrophy of the prostate epithelium. Treatment of patients with metastatic
prostate cancer includes therapy in combination with anti-androgens, such as bicalutamide (casodex). When androgen ablation therapies fail, advanced prostate cancer ultimately progresses to an AI late stage that is refractory to current therapies, also known as castrate resistant prostate cancer (CRPC), and this recurrence results from a reactivation of AR activity.

**Androgen Independent/Castrate Resistant Prostate Cancer**

Decreasing levels of AR protein expression reduces both AD prostate cancer and CRPC growth. While anti-androgens and other forms of treatment for non-organ-defined disease are initially successful in most patients (~80%) resulting in tumor regression and AR suppression, these therapies eventually fail and the cancer progresses to a stage where it is unresponsive to blockage of androgen and growth becomes androgen independent. Several mechanisms have been proposed to play a role in the reactivation of AR including, deregulation, causing over-expression of AR, mutation of AR (gain of function), alternative splicing (causing AR to be constitutively active), co-activator gain of function or loss of co-repressor function, and intracrine androgen synthesis (Figure 2) (Knudsen and Penning, 2010). This therapeutic failure causes adaptations resulting in restored AR activity, which in turn cause prostate specific antigen (PSA) levels to rise and incurable androgen independent tumor development. Therefore, AR signaling pathways must play critical roles in both AD and CRPC. Unlike AD signaling that depends on actions of androgens to bind AR and activate it, AI pathways do not require androgens, but can be activated by growth factors acting through kinase pathways such as
Figure 2: Development of CRPC. When hormone therapy fails, tumor regression occurs and various different adaptation events occur that restore AR signaling. These events include deregulation leading to amplification or overexpression of AR, mutations leading to gain of function, aberrant modifications in growth factor or cytokine pathways, alternative splicing causing AR to be constitutively active, gain of co-activators or loss of co-repressors, and intracrine androgen synthesis all leading to castrate resistant prostate cancer (CRPC) development and thus failure of therapy (Knudsen and Penning, 2010).
the mitogen-activated protein kinase (MAPK) pathway or the phosphatidylinositol 3-kinase (PI3K) pathway, which phosphorylate and activate AR in the absence of androgens (Lattouf et al., 2006) (Figure 3). Thus new improved therapies must be uncovered to determine the mechanisms of by which AR activity is restored and how CRPC can be maintained.

**Androgen signaling and AR**

The AR is a zinc finger transcription factor that is a member of the nuclear receptor superfamily. The AR gene encodes three functional domains: the N-terminal domain (NTD), the DNA-binding domain (DBD), and the androgen-binding domain or ligand binding domain (LBD) (Galani et al., 2008) (Figure 4). The AR gene codes for a 110 kDa phosphoprotein that functions as a steroid-hormone activated transcription factor. Androgen signaling occurs mainly via ligand binding to the AR and the complex binding gene promoters of androgen responsive genes. After binding to hormone, the androgen receptor dissociates from accessory proteins (such as heat shock proteins, HSP), translocates into the nucleus, dimerizes, and then stimulates transcription of target genes (Figure 5). Once in the nucleus, ligand-bound AR binds to chromatin at specific sequences termed androgen response elements (AREs) of target genes and recruits coactivators and chromatin remodeling complexes (Heinlein and Chang, 2004; Wang et al., 2007; Kaarbø et al., 2007) as well as RNA polymerase II, initiating the transcription of androgen responsive genes (Figure 5).
Figure 3: AD and AI signaling pathways. Androgen dependent prostate cancer results from classical androgen signaling which takes place through DHT stimulation of the AR. Androgen independent or castrate resistant prostate cancer cells survive through stimulation of multiple signaling pathways, such as the MAPK or PI3K pathways, which then activate AR leading to AR signaling in the absence of hormone (Lattouf et al., 2006).
Figure 4: Structure of the human androgen receptor. The gene of the androgen receptor is located on the long arm of the X-chromosome (Xq11-12) and contains 8 exons. The protein contains several functional domains including the NH2 domain (NTD), the DNA binding domain (DBD), and the ligand binding domain (LBD) (Galani et al., 2008).
Figure 5: **Classical AR signaling pathway.** In the absence of hormone, AR is bound to heat shock proteins (HSPs) in the cytoplasm of the cell. Once hormone ligand enters the cell and binds to the receptor, AR dissociates from HSPs and enters the nucleus of the cell where it binds to androgen response elements (ARE) sites in the regulatory regions of androgen responsive genes (Galani et al., 2008).
There are many genes that are known to be regulated by androgen, but the most characterized and studied is PSA, a serine protease that is a member of the kalikrein family of genes (Riegman et al., 1989). It is the most widely used screening tool for prostate cancer, as an increase in PSA levels correlates with prostate cancer progression. In the normal prostate, PSA levels are a million fold higher than in the serum. During prostate cancer (PrCa) progression, serum PSA levels are elevated due to the fact that PSA enters the serum through aberrations in the prostate ductal structure of neoplastic epithelial cells, allowing PSA to be actively secreted into the extracellular matrix and enter the circulation (Heinlein and Chang, 2004). AR is known to be the major regulator of PSA. It induces PSA expression through three AREs located in the proximal PSA promoter and distal enhancer regions (Shang et al., 2002; Cleutjens et al., 1997).

**AR cofactors (activators and repressors) and transcription factor partners**

Several coregulators or co-factors are needed for AR transcriptional regulation of androgen responsive genes. Coregulators are either coactivators or corepressors. Coactivators are proteins which interact with nuclear receptors such as AR to enhance transcription of androgen responsive genes, while corepressors are proteins that interact with nuclear receptors to reduce transcription. Type I coregulators facilitate DNA occupancy, chromatin remodeling, and recruitment of the general transcription factors that are associated with the RNA polymerase II holocomplex. Type II coregulators modulate AR folding and ligand binding enabling AR to direct target gene expression
Type I coregulators Creb binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) are both coactivators of AR that have histone acetyltranferase (HAT) activity allowing them to interact with not only AR, but also basic transcriptional machinery as well (Figure 5). Type II coregulators, ARA70 and filamen, stabilize ligand-bound androgen receptor (ARA70) and aid in the translocation of AR to the nucleus (filamen).

Transcription factors (TFs) are also known to interact with AR and may potentially act as co-factors in the regulation of androgen responsive genes. These include zinc finger transcription factors (ZFTFs), such as Sp1 and Egr1. The SP1-AR transcription factor complex has been shown to induce transcription of the Cdk inhibitor p21 gene in LNCaP prostate cancer cells (Lu et al., 2000). Not only do these factors bind to their consensus binding sites in the p21 promoter, but they also functionally interact as demonstrated by both co-immunoprecipitation and mammalian one-hybrid assays. Egr1 and AR also form a complex (Yang and Abdulkadir, 2003) to co-regulate PSA. Egr1 binds to AR in prostate cancer cells and an Egr1-AR complex can be detected bound to the enhancer region of the AR responsive PSA gene. Knock-down of Egr1 by siRNA inhibited the AR signaling pathway activity and suppressed PSA expression. Taken together these findings show that multiple co-factors and transcription factors are involved in hormone regulation of androgen responsive genes. However, delineating AR and ZFTF interactions has been hampered by the difficulty in identifying functional ARE in androgen responsive genes.
Evolutionary conservation as a tool for identification of potential ARE binding sites in target genes

Identifying androgen responsive gene targets has proven difficult and the recent analyses of the androgen transcriptome reveals a network much larger and more complex than expected (Wang et al., 2007; Bolton et al., 2007; Massie et al., 2007). However, delineating regulatory networks and uncovering fundamental mechanisms of gene interaction in prostate cancer are crucial to understanding how AR regulates not only AD PrCa, but also AI/CR PrCa. Global gene expression analyses have led to a better understanding of growth control of prostate cancer cells (Massie et al., 2007; Brown et al., 2006; Eisermann et al., 2008). Previous studies have identified more than 200 genes predominantly expressed in prostate cancer epithelial cells (Brown et al., 2006) and included genes likely to influence growth of prostate cancer cells, such as growth factors, growth factor receptors and TFs (as identified by Gene Ontology and KEGG pathway analyses). To determine whether these genes are included within the androgen signaling network is problematic but approaches that involve computational analyses have been utilized by us and others (Eisermann et al., 2009).

To assess the level of evolutionary conservation of regulatory sequences, transcription factor binding sites (TFBS) can be identified by tools such as MatInspector (Cartharius et al., 2005). Putative TFBS can be computationally predicted; however, the identification of functional TFBS is a challenge and requires an alternative approach. Since functionally important elements are likely to experience purifying selection pressure (Thomas and Touchman, 2002; Dubchak et al., 2000; Stojanovic et al., 1999;
Wang et al., 2006; Johnson et al., 2004), we utilized the degree of evolutionary conservation to identify TFBS that are likely to be functional. Availability of complete genomic sequence from multiple species allowed identification of evolutionary conserved elements, e.g. cis-regulatory elements. Our approach was to identify regions (and TFBS) evolutionary conserved across multiple mammalian genomes, including those separated by 170 million years (human and opossum) (Hedges and Kumar, 2003). Presence of putative ARE sites overlapping with other ZFTF sites suggested the potential for competition for binding between TFs, while adjacent sites suggested either competitive or cooperative binding. The latter was consistent with our model for ZFTF interactions with AR at non-canonical binding sites.

Alternative approaches to scan the genome for potential ARE binding sites have involved DNA binding assays that have also revealed that a majority of androgen responsive AR target genes lack classical dimeric ARE (shown in the sequence logo in Figure 6) (Wang et al., 2007; Massie et al., 2007). These unexpected transcriptional targets have been identified in part, by the use of high-throughput approaches such as chromatin immunoprecipitation (ChIP) microarray (CHIP) screening of hormone treated cells. Some AR binding regions identified by these methods appeared to identify other (non- ARE) TF binding sites that likely function via “tethering” AR to other TF binding sites. In these cases AR is not directly binding chromatin, but could bind to ZFTFs themselves bound at GC sites, and not at ARE sites. However, DNA binding assays also identified non-canonical 6 bp ARE binding sites that include only half the dimeric 15mer (Figure 6) (Wang et al., 2007; Bolton et al., 2007; Massie et al., 2007). Although many
Figure 6: Consensus ARE binding sequence. Sequence logo of a classical dimeric ARE binding site (Bolton et al., 2007) often described as 5'-GGA/TACANNNTGTCT-3'.
of these novel sites may be located > 10 kb from the androgen responsive gene promoter, nearly 40% of those identified in a recent study of genes on chromosome 21 and 22 were located within 500 bp of the transcriptional start site (Wang et al., 2007). While fundamental functional mechanisms of monomeric ARE half-sites remain unclear, it is known that AR interacts with other TFs, and ARE sites are often located within regions containing other ZFTF binding sites. Once potential ARE and ZFTF binding sites are identified it remains a challenge to assess functional relationships, but it is critical to determine mechanisms of regulation of androgen responsive genes important in PrCa.

**VEGF**

Growth of prostate cancer and any solid tumor depends on new blood vessel growth (neoangiogenesis) as solid cancers cannot grow beyond a small size without an adequate blood supply. Vascular Endothelial Growth Factor (VEGF) is the most prominent gene involved in the formation of new blood vessels or angiogenesis. Patients with metastatic prostate cancer have greater VEGF plasma levels than those with localized disease, as over-expression of VEGF contributes to tumor growth and metastasis (Delongchamps et al., 2006). Thus, VEGF assists tumor cell entry into the circulation and aids in the development of distant metastases (Figure 7).

There are five isoforms of VEGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF-A is the most important form in tumor tissues and is known to specifically bind both VEGF tyrosine kinase receptors VEGFR-1 (flt-1) and VEGR-2 (KDR/flk-1), but not VEGFR-3 (flt-4) (Shalaby et al., 1995). Both VEGF and its
Figure 7: The role of VEGF in cancer. VEGF is involved in tumor angiogenesis. When there is an increase in VEGF production, blood vessels are able to grow resulting in an increase of tumor size (Srinivas, 2007).
receptors are highly expressed in bladder cancer (Droller, 1998), breast cancer (Kurebayashi et al., 1999), colon cancer (Shaheen et al., 1999), hepatoma (Yoshiji et al., 1999), and prostate cancer (Balbay P.C. et al., 1999). In mouse xenograft models of prostate cancer, the VEGF receptor flk is overexpressed in LNCaP tumors when compared to normal prostate epithelium (Balbay P.C. et al., 1999). Also, flk-1 is up-regulated on the endothelial cell membrane of highly metastatic LNCaP-LN3 xenograft tumors when compared with LNCaP, demonstrating that VEGF auto-stimulation of its own receptors increases as tumor progresses.

**Regulation of VEGF expression**

Several factors are known to regulate VEGF expression including growth factors, cytokines, hypoxia, etc. Specifically, EGF, TGF-beta, keratinocyte growth factor, IGF-1, FGF, and PDGF are all known to up-regulate VEGF mRNA expression (Ferrara et al., 2003). It has also been shown that the presence of various stimuli including TNF-α (Ryuto et al., 1996), retinoic acid (Akiyama et al., 2002), PDGF (Finkenzeller et al., 1997), HGF (Reisinger et al., 2003), TGF-β1 (Benckert et al., 2003), interferon α (Von Marschall et al., 2003), and estrogen (Stoner et al., 2004) enhance regulation of the VEGF proximal promoter depending on the organ and/or cell type. Previous studies (Levine et al., 1998; Joseph et al., 1997; Li et al., 2005, Stewart et al., 2001) have shown that androgen increases VEGF levels, but the mechanism involved is unknown. The inflammatory cytokines IL-1 and IL-6 are also known to induce VEGF expression in a
number of cell lines. The mechanisms by which these factors up-regulate VEGF are not all known, but in some cases involve actions of TFs.

The VEGF promoter lacks a TATA box and is GC rich, like “housekeeping genes”, but unlike “housekeeping genes”, it is induced by multiple stimuli. Several transcription factors, such as Sp1, Sp3, AP-2, and Egr-1 are known to regulate VEGF (Pagès and Pouysségur, 2005). Sp1 binding sites (see below) located in the core promoter are also known to play a role in VEGF transcriptional regulation in a variety of cell lines including NIH3T3 cells (Finkenzeller et al., 1997), ZR-75 breast cancer cells (Stoner et al., 2004), Y79 retinoblastoma cells (Akiyama et al., 2002), NCI-H322 bronchioloalveolar cells (Maeno et al., 2002), and PANC-1 pancreatic cells (Shi et al., 2001). TNF-α regulates VEGF through a cluster of Sp1 sites in the proximal promoter in human glioma cells (Ryuto et al., 1996). IL-1β induces VEGF transcription through MAP kinases and Sp1 sites in cardiac myocytes (Tanaka et al., 2000). This same group also demonstrated that retinoic acid induces VEGF gene expression in retinoblastoma Y79 cells (Akiyama et al., 2002). Also, TGF-β1 has been shown to stimulate VEGF transcription in cholangiocellular carcinoma cells via Sp1 binding sites in the VEGF core promoter that are essential for TGF-β1 responsiveness (Benckert et al., 2003). In Panc-1 pancreatic cells, the regulation of VEGF by SP1 has been extensively documented (Shi et al., 2001; Abdelrahim et al., 2004). Both constitutive Sp1 activity and a 109bp core promoter region containing SP1 sites are essential for VEGF expression (Shi et al., 2001). Mutation analysis of Sp1 binding sites in this region confirmed that this region is responsible for Sp1 activation of the VEGF promoter. Both SP1 and Sp3 were shown by
RNA interference to affect VEGF expression in Panc-1 pancreatic cells, but other factors also appeared to be involved (Abdelrahim et al., 2004). Taken together, these studies determined that Sp1 is a key regulator of VEGF expression in pancreatic cells. Additional transcription factors such as AP-2, HIF1-α, Egr1, and WT1 are also known to regulate the VEGF promoter in other cell lines (Pagès and Pouységur, 2005), thus indicating that VEGF can be regulated in a multitude of different ways and by a variety of different factors. Overall, the transcriptional regulation of VEGF is cell specific involving different stimuli and factors, but SP1 plays a prominent role.

**Regulation of VEGF by hormones**

In breast cancer cells, estrogen regulation of VEGF expression is thought to act through ER-α/Sp1 and ER-α/Sp3 interactions with GC-rich motifs (Stoner et al., 2004). In ZR-75 breast cancer cells treated with estradiol, VEGF mRNA levels were induced greater than fourfold. Additionally, the GC-rich region of the VEGF core promoter (-66 to -47) was required for E2 activation of VEGF, despite a lack of classical ER binding sites. Both Sp1 and Sp3 were demonstrated to bind the VEGF promoter *in vitro* by EMSA and *in vivo* by ChIP in these cells further supporting their functional relevance in E2 regulation of VEGF.

In contrast, androgens are also thought to regulate VEGF expression via an unknown mechanism. Previous studies have demonstrated that VEGF mRNA levels are elevated by androgen treatment of both human fetal prostatic fibroblasts and LNCaP
prostate cancer cells (Levine et al., 1998; Li et al., 2005, Stewart, et al., 2001). Also, VEGF protein levels are increased after treatment with hormone as demonstrated by ELISA assays (Joseph et al., 1997) and flutamide, an anti-androgen, has been shown to block this up-regulation (Mabjeesh et al., 2003). Taken together, both the mRNA and protein of VEGF are induced by androgens and estrogen, but the mechanism of androgen activation is unknown. Thus, the focus of this dissertation is to determine how androgen is regulating VEGF in prostate cancer cells.

**Transcriptional regulation of VEGF by ZFTFs**

*Importance of G rich binding sites: SP1*

Specificity protein 1 (Sp1) is a ubiquitous transcription factor that belongs to the Sp/KLF family of transcription factors and has a role in the expression of many genes (Black et al., 2001). It was the first mammalian transcription factor to be cloned (Kadonaga et al., 1987) and contains a zinc finger protein motif, by which it binds directly to DNA and enhances gene transcription (Figure 8). The zinc fingers of Sp1 are of the Cys2/His2 type and bind the consensus sequence 5’- (G/T)GGGCGG(G/A)(C/T)-3’ (GC box element). It is thought that Sp1 is responsible for recruiting the TATA binding protein (TBP) to TATAA-less promoters and fixing the start site of transcription (Pugh and Tjian, 1991; Blake et al., 1990; Jolliff et al., 1991; Kollmar et al., 1994).

However, Sp1 binding sites have also been demonstrated to be involved in tissue specific gene expression and regulation of transcription of genes in response to a number
Figure 8: Structure of the Sp1 protein. Sp1 is a zinc finger transcription factor known to be involved in gene transcription. It contains three zinc fingers in the C terminus that are of the Cys2/His2 type and bind the consensus sequence 5’-(G/T)GGCGG(G/A)(G/A)(C/T)-3’ (GC box element).
of different stimuli. This indicates that these sites are important and binding by Sp1 may have the potential to be highly regulated depending on the cell type and stimuli involved. The regulation of VEGF is very cell specific, as described above, and in the majority of cases requires the Sp1 binding sites in the VEGF core promoter region. These sites are very GC-rich and overlap other transcription factor binding sites, such as binding sites for the zinc finger transcription factors Egr1 and WT1.

**Other potential VEGF regulators: EGR1/WT1 family**

*Early Growth Response 1, Egr1*

Egr1 (Early growth response 1) is an immediate early gene known to be involved in growth, development, and differentiation that is a member of the Egr family of zinc finger transcription factors (Liu et al., 1996; Sukhatme, 1990). It contains three zinc finger motifs at the C terminus that are known to bind DNA at GC-rich elements (5’-GCGGGGGCG-3’) on the promoters of genes that it is known to regulate (reviewed in Liu et al., 1996). Additionally, Egr1 is known to interact with overlapping Sp1 and WT1 binding sites in a variety of gene promoters, including PDGF (Khachigian et al., 1995), CSF (Harrington et al., 1993), MDR1(Cornwell and Smith, 1993), TNF (Krämer et al., 1994), EGFR (Englert et al., 1995), and Egr1 (Cao et al., 1993) itself. This overlap of transcription factor binding sites results in competition for binding at these sites and different regulation patterns in different cell types or tissues. Previously Egr1 was thought to be an activator whereas a related TF, WT1 was thought to be a repressor of
gene expression, but these simplistic roles no longer fit as cell and gene specific
differences have been observed.

Wilms tumor 1, WT1

The Wilms tumor 1 (WT1) gene is a tumor suppressor and has been proven to
play a central role in the development of Wilms’ tumor, a childhood tumor of the kidney
(Rivera and Haber, 2005). The Wilms’ tumor suppressor gene functions as one of the
essential regulators of normal development of the kidneys and the urogenital system.
Mutations in this gene have been shown to result in developmental abnormalities, such as
Denys-Drash syndrome and Frasier syndrome (Barbaux et al., 1997).

WT1 belongs to the EGR1 family of zinc finger transcription factors (Figure 9).
It has four zinc fingers and many isoforms and is known to bind the consensus sequence,
5'-GNGNGGGNG-3' which includes the Egr1 consensus sequence (Madden et al., 1991).
Two of the more prominent isoforms vary by the inclusion or deletion of a three amino
acid peptide located between exons nine and 10 are +KTS and –KTS (Haber et al., 1991).
The +KTS isoform has a role in RNA splicing and RNA editing, while the –KTS isoform
regulates transcription of different genes and is thus a transcription factor. The WT1
transcription factor has been shown to activate or repress different genes depending on
the cell type and target gene with which it interacts. WT1 activates expression of VEGF
in prostate cancer cells, but represses VEGF in 293 cells (Hanson et al., 2007), thus
showing that its function is cell-type specific and suggesting that WT1 must interact with
other gene regulatory proteins to have these differing effects on the different genes.
Figure 9: Structure of the WT1 protein. WT1 is a transcription factor that contains four zinc fingers in the C terminus of the protein. The DNA binding domain is also located in the C terminus as well as 9 base pairs which make up three amino acids, KTS that are known to be form two of the important isoforms (+KTS and –KTS) of WT1 (Morrison et al., 2008).
**WT1 and prostate cancer**

In prostate tumor tissues, elevated WT1 mRNA and protein levels have been observed in advanced stage tumors (Devillard et al., 2006; Gregg et al., 2010). Previously in our lab, WT1 protein was identified by immunohistochemistry in tissue microarrays containing moderate and high grade prostate cancer (Gregg et al., 2010). In contrast, WT1 protein was absent in non-neoplastic adjacent tissue and BPH (benign prostatic hyperplasia). Similarly, in paired tumor and non-neoplastic adjacent tissue samples, WT1 expression was elevated in more than 70% of high grade tumor samples examined. As such, WT1 has been investigated as a potential prognostic marker. Clearly the identification of VEGF as a potential WT1 target gene would suggest a possible role for WT1 in prostate tumor progression.

Our previous studies have suggested that WT1 transcriptionally regulates VEGF and that this regulation may be cell type specific (Hanson et al., 2007). It was demonstrated by luciferase reporter assays that over-expression of WT1 activated the VEGF proximal promoter in LNCaP cells, whereas, in both MOLT-4 leukemia and HEK-293 embryonic kidney cells, WT1 over-expression repressed the VEGF proximal promoter (Hanson et al., 2007; Moazam et al., 2010). This ability to either activate or repress target genes in different cell systems supports the observation that WT1 has both oncogenic and tumor suppressor capabilities. However the role of SP1 and EGR1/WT1 in mediating androgen regulation of VEGF is unknown and this has been examined in this study.
**In vitro prostate cancer models**

Several prostate cancer cell lines are available for the study of the mechanisms of androgen signaling and prostate cancer tumor progression. Among them include the most widely studied LNCaP cell line and its derivative LNCaP C4-2, and another mouse tumor xenograft CWR22Rv1, the AR deficient PC3 and DU145 cells and a line derived from normal prostate epithelium, RWPE-1 (van Bokhoven *et al.*, 2003; Bello *et al.*, 1997; Sramkoski *et al.*, 1999). These cell lines are different with respect to androgen sensitivity and have different characteristics associated with them as highlighted in Table 1. LNCaP prostate cancer cells are from a lymph node metastases and are androgen responsive requiring androgen to grow. They are the most commonly used cell line when studying androgen effects on prostate cancer as they are androgen dependent and contain both AR mRNA and protein. The LNCaP C4-2 cell line was derived from the parental LNCaP by xenograft coinoculations with the bone stromal cell line, MS; they are highly tumorigenic and metastatic and can grow in castrated mice, thus are AI (Thalmann *et al.*, 2000).

Other cell lines that are also AI include CWR22Rv1, PC3, and DU145. PC3 and DU145 cells are from bone and brain metastases, respectively, and both lack AR mRNA and protein as well as PSA (van Bokhoven *et al.*, 2003). CWR22Rv1 cells were developed from a relapsed androgen independent xenograft from castrated mice (Sramkoski *et al.*, 1999). Although these cells were developed from an androgen independent xenograft, similar to C4-2 cells, they still express AR mRNA and protein and show a growth response in the presence of androgens. RWPE-1 cells were derived from normal prostate epithelial cells immortalized by HPV-infection (Bello *et al.*, 1997).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>AR</th>
<th>Androgen sensitivity</th>
<th>PSA</th>
<th>Year</th>
<th>Site of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>+</td>
<td>Sensitive</td>
<td>+</td>
<td>1980</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LNCaP C4-2</td>
<td>+</td>
<td>Hypersensitive</td>
<td>+</td>
<td>2000</td>
<td>xenograft</td>
</tr>
<tr>
<td>CWR22Rv1</td>
<td>+</td>
<td>Hypersensitive</td>
<td>+</td>
<td>1999</td>
<td>xenograft</td>
</tr>
<tr>
<td>PC3</td>
<td>-</td>
<td>Insensitive</td>
<td>-</td>
<td>1978</td>
<td>bone</td>
</tr>
<tr>
<td>DU145</td>
<td>-</td>
<td>Insensitive</td>
<td>-</td>
<td>1977</td>
<td>brain</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>+</td>
<td>Sensitive</td>
<td>+</td>
<td>1997</td>
<td>primary</td>
</tr>
</tbody>
</table>
Models of Hormone Regulation of VEGF

Since the mechanism of androgen regulation of VEGF in prostate cancer cells is not known, three different models of regulation were proposed that differ primarily in the manner that AR binds the VEGF promoter (Figure 10). The first model proposes that AR binds to AREs as a dimer (Figure 10, i). This is the classical way that AR binds to androgen responsive genes, such as PSA. However, recently there have been reports that non-canonical ARE half-sites are extremely important and may contain monomeric half-sites (Wang et al., 2007; Bolton et al., 2007; Massie et al., 2007). Thus, the second proposed model (Figure 10, ii) shows AR binding to a monomeric ARE half-site and bridged to other ZFTF binding sites, such as Sp1, Egr1, or WT1 by co-factors (marked as ?), such as CBP or SRC-1. Since AR is known to interact with Sp1, EGR1, and potentially WT1, the third and final model (Figure 10, iii) proposes that AR is not bound to an ARE binding site, but is tethered via a ZFTF, which is bound to the VEGF promoter.
Figure 10: Proposed models of androgen regulation of VEGF in prostate cancer. Three potential ways that androgen is proposed to regulate VEGF in prostate cancer by: (i) binding to androgen response elements (AREs) as a dimer, (ii) binding ARE and bridged to other ZFTF binding sites (ZFTFBS), or (iii) tethering to ZFTF, but not bound to ARE.
OVERALL GOAL OF RESEARCH

Our first goal was to identify functional ARE and interacting ZFTF binding sites based on the degree of evolutionary conservation. Presence of putative overlapping ZFTF sites adjacent to ARE sites suggested the potential for cooperative binding, consistent with our model for ZFTF interactions with AR at non-canonical binding sites. One gene lacking classical ARE but regulated by androgen is vascular endothelial growth factor (VEGF) (Levine et al., 1998; Joseph et al., 1997; Sordello et al., 1998), an important signaling protein involved in angiogenesis and tumor progression. Since the mechanism(s) of androgen induction of VEGF are not well understood, our second goal was to determine how androgen induces VEGF expression.
SPECIFIC AIMS

AIM 1: Determine if candidate binding elements in genes coordinately expressed with WT1 in prostate cancer epithelial cells are conserved across multiple mammalian genomes (Eisermann et al., 2008).

Identifying regulatory networks and uncovering fundamental mechanisms of gene interaction in prostate cancer are crucial to understanding how AR regulates not only AD PrCa, but also AI PrCa. Global gene expression analyses have led to a better understanding of growth control of prostate cancer cells (Wang et al., 2007; Bolton et al., 2007; Massie et al., 2007). Earlier studies identified more than 200 genes predominantly expressed in prostate cancer epithelial cells (Brown et al., 2006) and included genes likely to influence growth of prostate cancer cells, such as growth factors, growth factor receptors and TFs (as identified by Gene Ontology and KEGG pathway analyses). We focused on 24 genes known as prostate cancer growth regulatory genes. To assess the level of evolutionary conservation of regulatory sequences, TFBS for six different ZFTFs were surveyed. Presence of overlapping sites suggests the potential for competition for binding between TFs, while adjacent sites may participate in competition as well as cooperative binding. To go beyond the candidate genes approach and identify novel gene targets coordinately expressed with WT1 in tumor epithelial cells, a more systematic and unbiased high-throughput computational approach was used. These in silico analyses were based on 24 genes expressed in prostate cancer epithelium that were likely to influence growth of prostate cancer cells. Putative TFBS were computationally predicted; however, the identification of functional TFBS is a challenge and requires an alternative
approach. Availability of complete genomic sequence from multiple species allows identification of evolutionary conserved elements, e.g. cis-regulatory elements. Functionally important elements are likely to experience purifying selection pressure (Thomas and Touchman, 2002; Dubchak et al., 2000; Stojanovic et al., 1999; Wang et al., 2006; Johnson et al., 2004), thus, we can utilize the degree of evolutionary conservation to identify TFBS that are likely to be functional. Our approach was to identify regions (and TFBS) evolutionary conserved across multiple mammalian genomes, including those separated by 170 million years (human and opossum) (Hedges and Kumar, 2003). Overall, this targeted approach identified important candidate binding elements in genes coordinately expressed with WT1 in prostate cancer epithelial cells.

**AIM 2: Determine if androgen up-regulates VEGF expression by binding at non-classical ARE sites**

The mechanism of hormone regulation of VEGF is unknown, therefore it was hypothesized that androgen acts through classical androgen signaling and up-regulates VEGF by binding the proximal promoter. If the induction of VEGF is AR mediated, then treatment with the androgen antagonist bicalutamide (casodex) should block the effect of hormone by disrupting binding of AR to R1881. LNCaP cells were treated with R1881 along with casodex to block androgen signaling, showing change in expression is indeed due to androgen. After treatment with casodex, real-time PCR was performed to see if the increase in VEGF transcript was shown to decrease. Since no reports have shown the presence of AR binding sites in the VEGF promoter, in silico analysis was performed to
determine if there were any ARE sites located within this regulatory region and chromatin immunoprecipitation was performed to determine if hormone increased binding of AR to the promoter of VEGF. Mutations were made in these predicted monomeric ARE binding sites and luciferase reporter assays were performed to examine if these sites were responsible for VEGF activation by androgen.

**AIM 3: Determine whether WT1 and AR interact and transcriptionally regulate VEGF**

In LNCaP cells, over-expression of WT1 has been demonstrated to increase VEGF promoter activity, thus to extend this idea, we examined other hormone responsive prostate cancer cells lines, CWR22Rv1 and LNCaP C4-2, to determine if they show a similar pattern of regulation as LNCaP cells. A functional WT1 binding site in the VEGF proximal promoter was mutated and luciferase reporter assays were performed to access the effect on VEGF up-regulation by WT1. siRNA specific for WT1 was used to knockdown WT1 gene expression and determine the effect on VEGF mRNA levels and see if WT1 was necessary for VEGF regulation. Co-immunoprecipitation was performed to determine if WT1 and AR functionally interact in prostate cancer cells. Once shown to interact, luciferase assays were performed to see if the two transcription factors were acting synergistically to regulate the VEGF promoter.
AIM 4: Determine how androgen activates the VEGF core promoter despite lack of ARE binding sites

*In silico* analysis determined that the VEGF core promoter does not contain any ARE binding sites for AR to bind to and up-regulate transcription of VEGF. Thus, other factors must be involved in the hormone activation of this region. Since there are four potential Sp1 sites in this region, it was hypothesized that these sites are involved in androgen regulation of this region. Mutations were made in the Sp1 binding sites and luciferase assays were performed to test the effect on hormone up-regulation.
CHAPTER II

METHODS

Promoter sequence compilation

For each of the 24 prostate cancer growth regulatory genes differentially expressed, the complete or draft genomes of eight different mammalian species were downloaded from the Ensembl Genome Browser (http://uswest.ensembl.org/index.html) (Hubbard et al., 2007). The following genome assemblies were used: the NCBI 36 assembly of human (Homo sapiens) genome, the NCBI m36 assembly of mouse (Mus musculus) genome, the Pan Tro 2.1 assembly of chimp (Pan troglodytes) genome, a whole genome shotgun (WGS) preliminary assembly Btau_3.1 of cow (Bos Taurus) genome, a WGS assembly Can Fam2.0 of dog (Canis familiaris) genome, a WGS preliminary assembly Mmul_1 of rhesus monkey (Macaca mulatta) genome, the Mon Dom5 assembly of opossum (Monodelphis domestica) genome, and the RGSC3.4 assembly of rat (Rattus norvegicus) genome. Since major regulatory elements are located within several hundred base pairs of transcription start sites (Dieterich et al., 2002), 1.5 kb of human nucleotide sequence 5' of the translational start site (that is, 5' of the first exon as defined in Ensembl (Hubbard et al., 2007)) was collected. Orthologous sequences from other mammalian genomes were obtained from respective genome assemblies. In the case of the EGR1 promoter this extended beyond 1.5 kb, so was
assigned a negative number. The genome viewer and annotation program Artemis was used to ensure the correct context of genomic sequences (Rutherford et al., 2000). In each sequence, the nucleotide positions were numbered sequentially, with the targeted promoter region occupying positions 1 through 1500 (5' to 3' direction) of the forward strand, and ATG start codon located at positions 1501–1503 of the genomic sequence analyzed. AR and VEGF promoter sequences containing the functional WT1 TFBS for the human AR and VEGF promoters were obtained from Ensembl (ENSG00000169083 and ENSG00000112715, respectively). For alignment analyses of known functional sites (Hanson et al., 2007; Shimamura et al., 1997), an orthologous promoter region (3 kb) was then collected from eight mammalian genomes as described above.

**TFBS predictions, evolutionary conservation and multiple sequence alignments**

TFBS of WT1, EGR1, SP1, SP2, AP2 and GATA1 were predicted for each gene by the program MatInspector (http://www.genomatix.de/) (Cartharius et al., 2005) that utilizes the TRANSFAC libraries of TF binding motifs (Cartharius et al., 2005). The default parameters of similarity thresholds were used for all examined genes, and they were as follows: core similarity > 0.75 and optimized matrix similarity thresholds (i.e., those that minimize false positives for each individual matrix as available in the library) (Cartharius et al., 2005). In MatInspector, core similarity is one of the built-in program parameters that determines whether the observed sequence match will be analyzed further. It refers to the four most conserved consecutive nucleotides of the matrix, usually the most critical sites for protein binding, and reaches 1.0 only when there is a perfect
match (Cartharius et al., 2005; Werner, 2000). Sequence matches with low core similarity (less than 0.75) are not, by default, reported to the user. Vertebrate matrices of the Matrix Family Library Version 6.2 (October 2006) that included 464 matrices were used (Cartharius et al., 2005). Multiple sequence alignments of the promoter sequences were reconstructed with the program blastZ using MultiPipMaker (Schwartz, 2003), and predicted human TFBS were mapped onto the alignments. Regions that are conserved in multiple genomes are often found to correspond to functionally important ones (Frazer et al., 2004). However, because of the species-specific differences in gene regulation due to underlying differences in morphogenesis and development, such as those between different segments of human and rodent prostate (Berquin et al., 2005), it can be expected that some functionally important regions will be conserved only in a limited set of genomes where they play a critical role. Thus, we used a flexible definition of "evolutionary conservation" to accommodate such potential differences between genes and/or TFBS: here a TFBS was considered evolutionary conserved if it was predicted as a respective TFBS in orthologous position in at least three of eight surveyed genomes. In other words, the same genomic region was predicted to function as a candidate binding site for a particular TF in at least 3 surveyed genomes. Further, because differences in presence/absence of particular TFB sites between genomes may also be attributed to differences in the role of respective genes in each of the organisms, we examined evolutionary conserved sites at different levels of resolution: Human-Primates, Human-Rodents, and Human-Opossum, thereby, allowing us to identify genes and TFB sites that are functionally relevant to each of these comparisons.
**ARE binding site predictions**

MatInspector (http://www.genomatix.de/) was used to predict transcription factor binding sites in the 3kb region of the VEGF and PSA promoters. Here we focused on androgen receptor elements (ARE’s) as well as adjacent GC rich zinc finger transcription regulators Sp1, Egr1, and WT1.

**Cell culture and hormone treatment**

LNCaP (ATCC CRL-1740), LNCaP C4-2 (from Dr. Alex Almasan, CCF, Cleveland, OH), and CWR22Rv1 (ATCC CRL-2505) prostate cancer cells were cultured in RPMI media, while human embryonic kidney 293 cells (ATCC CRL-1573) were cultured in DEM/F12 (HyClone Laboratories, Utah) media. All cells were grown in media supplemented with 10% FCS and 1% penicillin/streptomycin in a 37°C incubator with 5% CO2. For hormone treatment, cells were grown to 60-80% confluency and then serum starved in either serum-free media or media supplemented with 5% charcoal-dextran stripped FBS RPMI for 18-24 hours. The synthetic androgen methyltrienolone (R1881) (Perkin Elmer, Boston, MA) was then added to the charcoal-dextran stripped FBS RPMI media and cells were treated with 5nM R1881 for 24 hours unless otherwise noted in figure legends. For inhibition of AR, bicalutamide (LKT Labs, St. Paul, MN) was used and added 2 hours prior to treatment with R1881.
**Plasmid transfections and Epifluorescence**

The cytomegalovirus (CMV) promoter-driven pGFP-WT1 (A) expression construct encoding the murine Wt1 gene (lacking both KTS insertion and exon 5) fused to GFP coding region was obtained from Dr. A. Ward (Dutton *et al.*, 2006). The pGL3-VEGF luciferase reporter constructs (V88, V411, and V2274) were obtained from Dr. K. Xie (Shi *et al.*, 2001). All DNA was purified by the Qiagen plasmid Maxi Kit (Qiagen, Valencia, CA). Restriction digest analyses were performed on plasmid preps using restriction enzymes appropriate for the plasmid backbone. DNA quantity and quality was determined using a Nanodrop spectrophotometer. Transfections were performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum and antibiotic-free media as described (Hanson *et al.*, 2007). For over-expression studies, the amount of DNA used was adjusted by adding the empty CMV expression vector, pCMV4 (Promega) for WT1. Transfection media was replaced with complete full serum media (10% FBS RPMI or DEM/F12) 4-6 hours after transfection. Green fluorescing cells were visualized by epifluorescence microscopy (Olympic) at 100–400× magnification at 24 and 48 hrs after transfection to verify effective transfection of pGFP-WT1.

**Chromatin immunoprecipitation**

The Farnham ChIP protocol (Weinmann and Farnham, 2002) was used with some modifications. Two million cells were treated with formaldehyde to crosslink proteins to DNA and lysed in PBS-PI as recommended for the EZ ChIP Assay (Upstate Biotechnology Inc.). Lysates were centrifuged and DNA sheared by sonication (Biosonik
III, Bronwill Scientific, Rochester, NY) to fragments of 200–1,000 bp in length. The supernatant was pre-cleared by incubation with Protein G Agarose and incubated overnight at 4°C with AR, WT1 (a mixture of C19 and N18 polyclonal Abs) (Santa Cruz Biotechnology), Sp1, pol II, or non-immune IgG (Upstate Biotechnology Inc.). The antibody/protein/DNA complex was collected by incubation with Protein G Agarose and washed in increasing salt buffers, then rinsed in TE as recommended (Upstate Biotechnology Inc.). The complexes were recovered from agarose beads with an elution buffer, crosslinks were reversed and DNA was purified using G-50 spin columns. Four percent of both immunoprecipitated and input chromatin were amplified by PCR using Taq polymerase (Applied Biosystems by Roche Molecular System, Inc) and the appropriate primers (see Table 2). Following an initial 10 min denaturation at 95°C, DNA was amplified by 32-35 cycles of: 1) 20 sec denaturation at 95°C, 2) 30 sec annealing at either 52°C (for ARE III primers), 53°C (for ARE II primers), 58°C (for ARE I and VEGF primers) or 59°C (for AR and PSA primers) and 3) 30 sec extension at 72°C; amplification was completed with a 2 min final extension at 72°C. PCR products were fractionated on 1% agarose gel, and ethidium bromide stained DNA was visualized by a gel doc system (BIORAD, CA). Amphiregulin (AREG) primers were used as a specificity control as WT1 is known to bind the AREG promoter (Kim et al., 2007).

ChIP quantification was performed using SYBR Green qRT-PCR (see below) and results are presented as percentage of input chromatin. The starting input fraction was 1%, so a dilution factor of 100 or 6.444 cycles (log2 of 100) was subtracted from the CT value of the diluted input fraction. The average CT values of the immunoprecipitations
were then subtracted from the adjusted input CT and the percent input was determined using the following formula: 100*2^(Adjusted input - CT (IP). Image J analysis was performed to determine binding affinity differences in immunoprecipitations.

**RNA isolation**

RNA was isolated from subconfluent cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO) as per manufacturer’s recommendation. Briefly, cells were lysed using a lysis solution containing 2-mercaptoethanol (2-ME) and then filtered through a GenElute Filtration Column to remove cellular debris and shear the DNA. Ethanol was added to the filtered lysate and this mixture was loaded into a GenElute Binding Column. The RNA was then washed 3 times and eluted with elution solution. Concentration of the purified RNA was determined using a Nanodrop spectrophotometer.

**Quantitative real-time PCR**

RNA (1μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed using either Taqman Universal Master Mix with pre-designed Taqman Gene Expression Assay probe sets for VEGF, PSA, and 18S or SYBR Green Master Mix with primers specific for VEGF, GAPDH, and Beta-actin (Table 4). SYBR Green is a fluorescent dye that binds to all double stranded DNA. Fluorescence is measured as the dye binds more amplified DNA in the PCR reaction. When SYBR Green binds to double stranded DNA,
the intensity of the fluorescent emission increases. No probe is required, but since it binds to all double stranded DNA, false positives can be generated, however dissociation or melting curves generate specific melting temperature peaks for each DNA product. The comparative Ct method (Livak and Schmittgen, 2001) was used to analyze gene expression differences between control (untreated) cells and cells treated with R1881 alone or with bicalutamide.

**siRNA Transfection**

LNCaP cells were seeded into 10mm dishes using antibiotic-free complete medium (10% FBS RPMI). Transfection of siRNA oligos was accomplished using DharmaFECT transfection reagent (Dharmacon RNAi Technologies) as described by the manufacturer. Briefly, WT1 siRNA (25nM final concentration) and Dharmafect were added to serum-free medium and then combined for 20 minutes at room temperature. The WT1 oligo/Dharmafect mixture was then added to LNCaP cells containing complete medium without antibiotics for 24-48 hours, then RNA was isolated as described above. WT1 mRNA levels were measured by SYBR Green qRT-PCR to confirm WT1 knockdown. WT1 exon 1-2 primers were used and expression was normalized to GAPDH (to determine dCT). Percent suppression was determined by comparison to random siRNA oligos (RISC-free), which was used as a negative control (to determine ddCT). VEGF levels were measured using primers specific for VEGF and using SYBR Green qRT-PCR as described above. Percent knockdown was determined using GAPDH
as a normalizer (to determine dCT) and comparing to the negative control RISC-free siRNA (to determine ddCT). Calculations were as follows:

\[ 2^{-\text{ddCT}} \times 100\% = \% \text{ remaining}, \]
\[ 100\% - \% \text{ remaining} = \% \text{ knockdown (KD)}. \]

E-cadherin (ECAD) primers were used as a positive control, as it is known to be regulated by WT1 and strongly affected by siWT1 RNA oligonucleotides.

**Western blot**

Subconfluent monolayers from LNCaP and CWR22Rv1 cells were washed in PBS and extracted using RIPA lysis buffer (1% NP40, 0.5% Na Deoxycholate, 0.1% SDS, and 150 mM NaCl) containing protease inhibitors. Bicinchoninic acid (BCA) assays (Pierce, Thermo Fisher Scientific, Rockford, IL) were performed to quantify the amount of proteins present in each lysate and protein concentrations were determined by using a BSA standard curve. Proteins (25-50ug) were diluted in 6x laemmli sample buffer containing DTT, boiled for 5 minutes, and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a Polyvinylidene Fluoride (PVDF) membrane and blocking with 5% casein (to prevent non-specific binding), blots were probed with primary antibodies specific for VEGF, AR (Santa Cruz), and beta-actin (GenScript) overnight at 4°C with rocking. Membranes were washed twice with PBST and incubated in the appropriate HRP-conjugated secondary antibody (anti – mouse, Santa Cruz; anti – rabbit, GenScript) for 1 hour. Proteins were visualized by incubating the membrane in a luminol ECL solution followed by chemiluminescent
detection using a Fuji LAS 3000 detection system. Bands were quantified using ImageJ analysis and normalizing to actin levels.

For comparison of nuclear and cytoplasmic enriched proteins, the cytoplasmic proteins were first extracted (@6,000 rpm) using lysis buffer containing 10mM HEPES, pH 7.9, 20mM KCl, 3mM MgCl₂, 0.5% NP-40, 5% glycerol, 1mM PMSF, and protease inhibitors, while for nuclear lysates, proteins were extracted (@14,000 rpm) using lysis buffer containing 20mM HEPES, pH 7.9, 3mM MgCl₂, 0.225M NaCl, 1mM EDTA, 0.5% NP-40, 10% glycerol, 1mM PMSF, and protease inhibitors.

**Co-Immunoprecipitation**

Nuclear extracts from either LNCaP or CWR22Rv1 cells were obtained using Active Motifs Universal Magnetic Co-IP kit (Carlsbad, CA) by first resuspending cell pellets in Complete Hypotonic Buffer (10X Hypotonic Buffer, phosphatase inhibitors, deacetylase inhibitor, protease cocktail inhibitors, and 100mM PMSF), incubating on ice for 15 minutes and then adding 5% detergent. This suspension was then centrifuged at 14,000 x g and the supernatant was discarded leaving the nuclear fraction. Complete Digestion Buffer (Digestion Buffer, phosphatase inhibitors, deacetylase inhibitor, protease cocktail inhibitors, and 100mM PMSF) was then added to nuclear fractions followed by addition of enzymatic shearing cocktail. This mixture was incubated for 10 minutes at 37°C before addition of EDTA to stop the reaction. Nuclear extracts (150-200µg) were then combined with 5µg of either AR (C-19, Santa Cruz), Sp1 (Upstate), WT1 (C-19, Santa Cruz), or negative control IgG antibodies in Complete Co-IP/Wash
buffer (Co-IP Wash buffer, Phosphatase inhibitors, deacetylase inhibitor, protease cocktail inhibitors, and 100mM PMSF) for two hours at 4°C with rotation. Following antibody incubation, Protein G magnetic beads were added for one hour to pull down the antibody-protein complexes. These complexes were then washed four times with Complete Co-IP/Wash buffer and resuspended in 2X SDS loading buffer, followed by SDS-PAGE Western blot analysis (as described above).

**Plasmid Transfection and Luciferase Assay**

LNCaP, LNCaP-C42, and CWR22RV1 cells were plated in 12-well plates using RPMI with 10% FCS and 1% penicillin/streptomycin. After reaching ~70-90% confluency, cells were serum-starved for 18-24 hours with serum-free RPMI and then transfected with VEGF reporter constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). VEGF luciferase reporter constructs (V88, V411, or V2274) were obtained from Dr. Xie (Shi et al., 2001) and purified by the Qiagen plasmid Maxi prep kit (Qiagen, Valencia, CA). For co-transfection, cells were co-transfected with WT1-GFP expression construct or CMV empty vector as described above and one of the VEGF reporter constructs again using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 4-6 hours, transfection media was replaced with appropriate growth media. For hormone induction, 5-10% charcoal-dextran stripped FBS was used as described above with the addition of 5nM R1881. After 48 hours cells were lysed and luciferase activity was measured using a Promega luciferase assay kit (Promega, Sunnyvale, CA) and a Turner luminometer (Promega, Sunnyvale, CA) following manufacturer’s recommendations.
The luciferase activity was normalized using a micro BCA protein assay, and absorbance was measured at 600nm on a Dynex Technologies (Chantilly, VA) MRX Revelation plate reader. All experiments were done in triplicate and repeated at least three times. Standard errors of the mean were determined using GraphPad InStat software (San Diego, CA). Significance was determined by Student’s t-test.

**Site directed mutagenesis**

Predicted binding sites in the VEGF promoter construct were mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA). Primers containing the desired mutation (Table 3) were designed according to the manufacturer’s suggestions using the QuikChange Primer Design Program (https://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15). PCR was performed using the V88, the V411, or the V2274 reporter construct and the appropriate mutant primers. Parental strands were digested for 1 hour at 37°C using the enzyme DpnI. Mutant DNA was then transformed into XL1-Blue super competent cells for 45 seconds at 42°C. Following heat shock, reactions were placed on ice for 2 minutes and then incubated in LB broth at 37°C for 1 hour with shaking at 225–250 rpm. Transformations were then spread onto LB plates containing ampicillin and incubated overnight at 37°C. Individual colonies were grown and plasmids were purified (Qiagen, Valencia, CA). Mutant constructs were sequenced to verify that the correct base pairs were changed (Cleveland Clinic Genomics Core, Lerner
Research Institute, Cleveland, OH). Luciferase assays were performed using mutant constructs as described above.
### Table 2: ChIP primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (FOR)</td>
<td>5’-TTCCTAGCAAAGAGGGAACG-3’</td>
</tr>
<tr>
<td>VEGF (REV)</td>
<td>5’-ACCAAGGTTTCACAGCCTGAA-3’</td>
</tr>
<tr>
<td>AR (FOR)</td>
<td>5’-TATCTGCTGGCTTGGTCATGGCTTG-3’</td>
</tr>
<tr>
<td>AR (REV)</td>
<td>5’-CTGCTTCCTGAATAGCTCCTGCTT-3’</td>
</tr>
<tr>
<td>PSA (FOR)</td>
<td>5’-TCTGCCTTTGTCCCCCTAGAT-3’</td>
</tr>
<tr>
<td>PSA (REV)</td>
<td>5’-AACCTTCATTCCCCAGGACT-3’</td>
</tr>
<tr>
<td>AREG positive (FOR)</td>
<td>5’-GTTTTCGGGTAGCACCTTCTGG-3’</td>
</tr>
<tr>
<td>AREG positive (REV)</td>
<td>5’-AGGTGTGCGAACGTCTGTAGG-3’</td>
</tr>
<tr>
<td>AREG negative (FOR)</td>
<td>5’-CTGACCTCAGGTGATCCACC-3’</td>
</tr>
<tr>
<td>AREG negative (REV)</td>
<td>5’-TAGTGCTCTAGACTGTGCCTC-3’</td>
</tr>
<tr>
<td>ARE I (FOR)</td>
<td>5’-TTCGAGAGTGAAGCCTGTA-3’</td>
</tr>
<tr>
<td>ARE I (REV)</td>
<td>5’-AGGGAGCAGAAGGAGGT-3’</td>
</tr>
<tr>
<td>ARE II (FOR)</td>
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<tr>
<td>ARE III (REV)</td>
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</table>
Table 3: Site-directed mutagenesis primer sequences

WT1mV411 (FOR): 5’-GTGTCTCTGGACAGAGTTTCCGAATACGGATGGGTAATTTCAGGCTG-3’

WT1mV411 (REV): 5’-CAGCCTGAAAAATTACCCATCCGTATTCGGAAACTCTGTCCAGAGACAC-3’

ARE I (FOR): 5’-CTCTATCGATAGGTACCGTGTCAGCTCTCCCCACCCGTCCCTGTC-3’

ARE I (REV): 5’-GACAGGGACGGGTGGGAGAGCTGACACGGTACCTATCGATAGAG-3’

ARE II (FOR): 5’-GGAACCACACAGCTTCCCACCTGAGCTCAGCTCCACAAACTTGG-3’

ARE II (REV): 5’-CCAAGTTTTGTGAGCTGACAGTGGGAAGCTGTGTTGGTCC-3’

ARE III (FOR): 5’-GCCCAAGATGTCTACAGCTTACGGTCCTGGGGGTGC-3’

ARE III (REV): 5’-GCACCCCAGGACCGTAAGCTGTAGACATCTTGGGGGC-3’

Sp1.2/Sp1.3 (FOR): 5’-GCCCCCGGTTGCGGGCCGTTCCGGGTCCC-3’

Sp1.2/Sp1.3 (REV): 5’-GGGACCCCGAACCAGGGCCGAGACCGGGGGGC-3’

Sp1.4 (FOR): 5’-GGGGTCCCGGGGCGGTTCCGAGCCATGCG-3’

Sp1.4 (REV): 5’-CGCATGGCTCCGAAACCAGCGGACCC-3’
Table 4: Real-time PCR primer sequences

**SYBR Green primers**

*VEGF* (FOR): 5’-CGAAACCATGAACCTTTCTGC-3’

*VEGF* (REV): 5’-CCTCAGTGGGACACACTCC-3’

*WT1* exon 1-2 (FOR): 5’-GAGAGCCAGCCGCTATTC-3’

*WT1* exon 1-2 (REV): 5’-CATGGGATCCTCATGCTTG-3’

*GAPDH* (FOR): 5’-CCATCACCATCTTCCAGGAG-3’

*GAPDH* (REV): 5’-GGATGATGTCTGGAGAGCC-3’

*Beta-actin* (FOR): 5’-GTGGGGCGCCCCCAGGCACCA-3’

*Beta-actin* (REV): 5’-GTCCCTTAATGTCACGCACGATTTC-3’

**Taqman Gene Expression Assays primers**

*VEGFA*  Hs00900057_m1

*PSA*  Hs03063374_m1

*18S*  Hs99999901_s1
CHAPTER III

RESULTS

PART I: IDENTIFICATION OF CONSERVED AND FUNCTIONAL BINDING SITES IN GENES IMPORTANT IN PROSTATE CANCER PROGRESSION

*Evolutionary conservation analysis: TFBS conserved in prostate cancer growth genes*

Genomic sequences of proximal promoter regions of 24 genes (Eisermann *et al.*, 2008) expressed in prostate cancer epithelial cells were analyzed to determine the degree of evolutionary conservation and to identify potentially important regulatory regions. Binding sites for six TFs (WT1, EGR1, SP1, SP2, AP2, and GATA1) (*Table 5*) were investigated for evolutionary conservation over a range of eight different mammalian species (human, chimpanzee, macaque, cow, dog, mouse, rat and opossum). *Tables 6* and *7* highlight 11 of these genes whose promoter sequences could be aligned in at least five mammalian species (human, chimpanzee, macaque, rat and mouse) and were found to have at least one evolutionary conserved TFBS.

Among the TFBS investigated, WT1, EGR1 and SP1 sites showed the highest frequency of evolutionary conservation in the gene promoters surveyed. For example, the promoters of EGR1, GATA2 and WT1 were found to have multiple WT1, EGR1 and SP1 candidate binding sites that were conserved through multiple species (*Table 7*). In the EGR1 promoter, 50% of WT1 sites are conserved between human and primates.
Table 5 - Transcription factors potentially involved in coordinate gene expression in prostate cancer epithelial cells

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Expression in prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>Wilms tumor 1</td>
<td>(Devilard et al., 2006)</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>(Ogishima et al., 2005; Eid et al., 1998)</td>
</tr>
<tr>
<td>SP1</td>
<td>Specificity protein 1</td>
<td>(Sroka et al., 2007; Husbeck et al., 2006)</td>
</tr>
<tr>
<td>SP2</td>
<td>Specificity protein 2</td>
<td>(Phan et al., 2004; Ho et al., 2006)</td>
</tr>
<tr>
<td>AP2</td>
<td>Transcription factor AP-2</td>
<td>(Ruiz et al., 2004; Zhang et al., 2007b)</td>
</tr>
<tr>
<td>GATA1</td>
<td>GATA binding protein 1</td>
<td>NR(^a)</td>
</tr>
</tbody>
</table>

\(^a\)None reported.
Table 6 – Genes co-expressed with WT1 in prostate cancer epithelium

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Synonyms</th>
<th>Ensembl gene ID</th>
<th>Entrez gene ID</th>
<th>Summary of function</th>
<th>Regulation</th>
<th>Binding</th>
<th>Expression in prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDCA</td>
<td>cadherin 1, type 1, E-cadherin (epithelial)</td>
<td>CDH1</td>
<td>ENSG000000390</td>
<td>599</td>
<td>signaling</td>
<td>+</td>
<td>+</td>
<td>(Rhodes et al., 2003)</td>
</tr>
<tr>
<td>EGR1</td>
<td>early growth response 1</td>
<td>TIS8, GOS30, AT225</td>
<td>ENSG000001207</td>
<td>1958</td>
<td>TF</td>
<td>+, ++</td>
<td>+</td>
<td>(Ogishima et al., 2005; Eid et al., 1998)</td>
</tr>
<tr>
<td>GATA2</td>
<td>GATA binding protein 2</td>
<td>NFE1B</td>
<td>ENSG000001793</td>
<td>2624</td>
<td>TF</td>
<td>+, ++</td>
<td>+</td>
<td>(Perez-Stable et al., 2000)</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>insulin-like growth factor binding protein-2</td>
<td>IBP2</td>
<td>ENSG000001154</td>
<td>3485</td>
<td>signaling</td>
<td>+</td>
<td>++</td>
<td>(Mehriyan-Shai et al., 2007; Chatterjee et al., 2004; Le et al., 2006)</td>
</tr>
<tr>
<td>KLK3</td>
<td>kallikrein 3, (prostate specific antigen)</td>
<td>PSA</td>
<td>ENSG000001425</td>
<td>354</td>
<td>enzyme, signaling</td>
<td>++</td>
<td></td>
<td>(Yin et al., 2006; Borghino and Diamandis, 2004; Nagpal et al., 2006)</td>
</tr>
<tr>
<td>NDRG1</td>
<td>N-myc downstream regulated gene 1</td>
<td>DRG1, RTP, TDD5, NDR1, PYY4</td>
<td>ENSG000001044</td>
<td>10397</td>
<td>enzyme</td>
<td>++</td>
<td></td>
<td>(Caruso et al., 2004; Tu et al., 2007)</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
<td></td>
<td>ENSG000001225</td>
<td>4852</td>
<td>signaling</td>
<td>+</td>
<td></td>
<td>(Ruscia et al., 2006; Rasiah et al., 2006)</td>
</tr>
<tr>
<td>SOX4</td>
<td>SRY (sex determining region Y)-box-4</td>
<td></td>
<td>ENSG000001247</td>
<td>6659</td>
<td>TF</td>
<td>+, ++</td>
<td>+</td>
<td>(Liu et al., 2006; Vanaja et al., 2006)</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY (sex determining region Y)-box-9</td>
<td>CMD1, CMPD1, SRA1</td>
<td>ENSG000001253</td>
<td>6662</td>
<td>TF</td>
<td>+, ++</td>
<td>+</td>
<td>(Wang et al., 2007; Drivdahl et al., 2004)</td>
</tr>
<tr>
<td>TFAP2C</td>
<td>transcription factor AP-2 gamma</td>
<td>ERF1, TFAP2G</td>
<td>ENSG000000875</td>
<td>7022</td>
<td>TF</td>
<td>+, ++</td>
<td>+</td>
<td>(Zhang et al., 2007b)</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumor 1</td>
<td>WAGR, WIT-2, GUD</td>
<td>ENSG000001849</td>
<td>7490</td>
<td>TF</td>
<td>+, ++</td>
<td>+</td>
<td>(Devillard et al., 2006; Hanson et al., 2006)</td>
</tr>
</tbody>
</table>
*Function as defined in the respective SwissProt annotation.

b Broad functional categories are based on Gene Ontology (Ashburner *et al.*, 2000) functional annotation by DAVID (Dennis *et al.*, 2003).


d+ designates GOTERM_MF_ALL: binding, ++ designates GOTERM_MF_ALL: protein binding.
Table 7 - TFBS in promoters of genes expressed in prostate cancer are conserved between Human and Primates or Rodents

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT1</th>
<th>EGR1</th>
<th>SP1</th>
<th>SP2</th>
<th>AP2</th>
<th>GATA1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conserved between(^a)</td>
<td>#Cons. sites(^b)</td>
<td>Total #(^c)</td>
<td>#Cons. sites(^b)</td>
<td>Total #(^c)</td>
<td>#Cons. sites(^b)</td>
</tr>
<tr>
<td>ECAD</td>
<td>H-Pr</td>
<td>1/PA</td>
<td>2</td>
<td>1/0</td>
<td>1</td>
<td>3/0</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>0/0</td>
<td>PA/PA</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>EGR1</td>
<td>H-Pr</td>
<td>4/4</td>
<td>8</td>
<td>7/8</td>
<td>10</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>2/2</td>
<td>3/4</td>
<td>2/2</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>GATA2</td>
<td>H-Pr</td>
<td>7/8</td>
<td>8</td>
<td>1/1</td>
<td>1</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>1/2</td>
<td>PA/PA</td>
<td>1/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>H-Pr</td>
<td>3/2</td>
<td>3</td>
<td>6/6</td>
<td>6</td>
<td>6/3</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>0/0</td>
<td>3/1</td>
<td>1/1</td>
<td>0/0</td>
<td>NP/NP</td>
</tr>
<tr>
<td>KLK3</td>
<td>H-Pr</td>
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<td>3</td>
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<td>1/1</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>NSA/</td>
<td>NSA/</td>
<td>NSA/</td>
<td>NSA/</td>
<td>NP/NP</td>
</tr>
<tr>
<td>NDRG1</td>
<td>H-Pr</td>
<td>1/1</td>
<td>1</td>
<td>NP/NP</td>
<td>NP</td>
<td>NP/NP</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>0/0</td>
<td>NP/NP</td>
<td>NP/NP</td>
<td>NP/NP</td>
<td>NP/NP</td>
</tr>
<tr>
<td>NPY</td>
<td>H-Pr</td>
<td>9/7</td>
<td>9</td>
<td>2/1</td>
<td>2</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>1/1</td>
<td>0/0</td>
<td>1/1</td>
<td>0/0</td>
<td>1/1</td>
</tr>
<tr>
<td>SOX4</td>
<td>H-Pr</td>
<td>2/1</td>
<td>2</td>
<td>2/2</td>
<td>2</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>1/1</td>
<td>0/0</td>
<td>2/2</td>
<td>0/0</td>
<td>NP/NP</td>
</tr>
<tr>
<td>SOX9</td>
<td>H-Pr</td>
<td>3/2</td>
<td>4</td>
<td>1/1</td>
<td>1</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>0/1</td>
<td>0/PA</td>
<td>1/NA</td>
<td>NP/NP</td>
<td>NP/NP</td>
</tr>
<tr>
<td>TFAP2C</td>
<td>H-Pr</td>
<td>NP/NP</td>
<td>NP</td>
<td>4/4</td>
<td>4</td>
<td>5/4</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>NP/NP</td>
<td>3/3</td>
<td>2/2</td>
<td>NP/NP</td>
<td>NP/NP</td>
</tr>
<tr>
<td>WTI</td>
<td>H-Pr</td>
<td>4/2</td>
<td>7</td>
<td>3/3</td>
<td>3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>H-Ro</td>
<td>1/1</td>
<td>0/0</td>
<td>2/2</td>
<td>0/0</td>
<td>1/0</td>
</tr>
</tbody>
</table>

\(^a\)H-Pr = TFBS conserved between human and other primates (chimpanzee / macaque), H-Ro = TFBS conserved between human and rodents (mouse / rat).

\(^b\)Number of conserved sites: PA = only partial alignment of promoters as constructed by MultiPipMaker (Schwartz, 2003), NP = no TFBS in human promoters as predicted by the MatIspector (Cartharius et al., 2005), 0 = TFBS not conserved, and NSA = no orthologous sequence is available in Ensembl.

\(^c\)Total number of predicted sites is based on TFBS in human promoters.
Additionally, in the GATA2 gene promoter, 94% of WT1 sites, 70% of SP1 sites, and 100% of EGR1 sites are conserved between human and other primates. Similarly, in the WT1 gene promoter 50% of SP1, 43% of WT1 and 100% of EGR1 sites are conserved between human and other primates. WT1, EGR1, and SP1 TFBS within the promoters of IGFBP2, KLK3, NPY, SOX4, SOX9, and TFAP2C are also conserved between human and other primates.

Importantly, for the WT1 and EGR1 gene promoters this conservation extended into the marsupials (Table 8). The EGR1 gene promoter is relatively conserved between human and opossum with 20% of predicted EGR1, 12% of predicted WT1 and 14% of predicted SP1 sites conserved between human and opossum. Similarly, the WT1 gene promoter exhibited conservation between human and opossum, with 33% of predicted SP1 and 14% of predicted WT1 sites shared between human and opossum. In the GATA2 promoter only 12% of predicted WT1 sites are shared between human and opossum (Table 8). Overall, TFBS for the three TF (WT1, EGR1, and SP1) were evolutionary conserved between human and the distantly related species, opossum, in seven different promoters (WT1, EGR1, GATA2, IGFBP2, SOX4, SOX9, and TFAP2C).

Tables 7 and 8 show that there were fewer SP2, AP2 and GATA1 than WT1, EGR1 and SP1 TFBS in the 11 gene promoters analyzed. While evolutionary conservation between primates was similar for all six TFBS, conservation between human and rodents diminished for SP2 and AP2 TFBS. AP2 sites in the promoters of the GATA2, WT1, and NPY genes showed 25% to 100% conservation between human and other primates. Conservation of AP2 sites was the strongest in the NPY gene promoter as...
Table 8 - TFBS in promoters of genes expressed in prostate cancer are conserved between Human and Opossum

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT1</th>
<th>EGR1</th>
<th>SP1</th>
<th>AP2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conserved between(^a) &amp; #Cons. sites(^b) &amp; Total(^c) &amp; #Cons. sites(^b) &amp; Total(^c) &amp; #Cons. sites(^b) &amp; Total(^c) &amp; #Cons. sites(^b) &amp; Total(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGR1</td>
<td>H-Op</td>
<td>1</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>GATA2</td>
<td>H-Op</td>
<td>1</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>H-Op</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>NPY</td>
<td>H-Op</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>SOX4</td>
<td>H-Op</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TFAP2C</td>
<td>H-Op</td>
<td>NP</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>WT1</td>
<td>H-Op</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) H-Op = Conservation between human and opossum.
\(^b\) Number of conserved sites: PA = only partial alignment of promoters as constructed by MultiPipMaker (Schwartz, 2003), NP = no TFBS in human promoters as predicted by the MatIspector (Cartharius et al., 2005), and 0 = TFBS not conserved.
\(^c\) Total number of predicted sites is based on sites in human promoters.
these sites are also conserved between human and opossum (Table 8). In addition to
conservation of GC-rich TFBS, the AT-rich GATA1 binding sites were shown to be
highly conserved in several gene promoters including SOX4, EGR1, IGFBP2 and NPY
(Table 7). All of the GATA1 sites in these four promoters are conserved between human
and chimpanzee, and for the SOX4 gene promoter this strong conservation extends to
rodents as well.

The overall evolutionary conservation of predicted TFBS of these 11 different
genes expressed in prostate cancer cells was analyzed. As would be expected,
conservation of TFBS decreased as species became more evolutionarily divergent (Table
9). TFBS were found to be the most conserved among primates, followed by rodents, and
the least amount of conservation was found between human and opossum. Of the 47
predicted WT1 sites in the 11 genes analyzed, primates had 68% of these sites conserved
between human and primate genomes, while rodent genomes had only 15% of these sites
being conserved, and opossum only 6% of these sites conserved, clearly showing a
drastic drop in conservation as species diverge. This same pattern was shown for the
other TFBS that were analyzed, including EGR1 and SP1. In particular, 85% of the
EGR1 sites were conserved between human and other primates, 26% between human and
rodents, and 19% between human and opossum. Similarly, there were 50 predicted SP1
binding sites, of which 62%, 22% and 12% were conserved between human and primates,
rodents, and opossum genomes, respectively, therefore, exhibiting decreasing
conservation of these sites with evolutionary divergence. Thus, with this approach of
Table 9 - Summary of evolutionary conserved sites shared between genomes of human and other species

<table>
<thead>
<tr>
<th></th>
<th>WT1</th>
<th>EGR1</th>
<th>SP1</th>
<th>SP2</th>
<th>AP2</th>
<th>GATA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # of TFBS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47</td>
<td>31</td>
<td>50</td>
<td>12</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td><strong>Primates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>36 (77%)</td>
<td>28 (90%)</td>
<td>35 (70%)</td>
<td>11 (92%)</td>
<td>11 (69%)</td>
<td>13 (72%)</td>
</tr>
<tr>
<td>Macaque</td>
<td>28 (60%)</td>
<td>25 (81%)</td>
<td>27 (54%)</td>
<td>6 (50%)</td>
<td>9 (56%)</td>
<td>8 (44%)</td>
</tr>
<tr>
<td>Primate % conserved&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68%</td>
<td>85%</td>
<td>62%</td>
<td>71%</td>
<td>63%</td>
<td>58%</td>
</tr>
<tr>
<td><strong>Rodents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>6 (13%)</td>
<td>9 (29%)</td>
<td>12 (24%)</td>
<td>1 (8%)</td>
<td>2 (13%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>Rat</td>
<td>8 (17%)</td>
<td>8 (26%)</td>
<td>10 (20%)</td>
<td>0 (0%)</td>
<td>1 (6%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Rodent % conserved&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15%</td>
<td>26%</td>
<td>22%</td>
<td>4%</td>
<td>9%</td>
<td>14%</td>
</tr>
<tr>
<td><strong>Marsupials</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opossum</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>% conserved</td>
<td>6%</td>
<td>19%</td>
<td>12%</td>
<td>0%</td>
<td>6%</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total # of TFBS = The total number of TFBS predicted by MatInspector (Cartharius et al., 2005) based on TFBS in human promoters of 11 genes. Numbers of evolutionary conserved TFBS shared between human and each species are shown below (percent of sites conserved shown in parenthesis).

<sup>b</sup>Primate % conserved = Average number of chimpanzee and macaque evolutionary conserved TFBS divided by the total number of TFBS predicted for that particular TF.

<sup>c</sup>Rodent % conserved = Average number of mouse and rat evolutionary conserved TFBS divided by the total number of TFBS for that particular TF.
identifying evolutionary conserved sequences we were able to pinpoint specific candidate binding sites that could be tested for functional relevance.

**Conservation of overlapping WT1, EGR1, and SP1 TFBS**

Several of the genes investigated have multiple overlapping WT1, EGR1, and SP1 binding sites in their proximal promoter regions. For example, the promoter of the human *EGR1* gene has evolutionary conserved overlapping WT1/SP1 binding sites (one of which is shown in Figure 11A). Both the overlapping WT1 (human 565–581) and SP1 (human 563–577) sites are conserved between seven of eight species compared, and the SP1 site is also conserved between human and opossum. A second WT1 site (human 614–630) located 33 bp downstream overlaps an EGR1 site (human 608–624) and both sites are conserved among all eight species, including opossum (Figure 11A). The promoter of the *GATA2* gene also contained overlapping SP1 and WT1 TFBS (located in human positions 1125–1139 and 1127–1145, respectively) that are conserved among several mammalian genomes (Figure 11B). The *WT1* gene promoter also has overlapping WT1/SP1 binding sites and when aligned with multiple species, one 3’ WT1 site (human 1444–1468) was conserved between all primates, rodents, and opossum, thus, depicting millions of years of conservation of this particular site (Figure 11C). The SP1 site (human 1420–1434) is conserved between all primates and rodents tested, and overlaps with a WT1 site (human 1409–1425) that is conserved between human and chimpanzee (Figure 11C). Interestingly, the sequence similarity is so great between human and chimpanzee for this *WT1* promoter region that no insertions or deletions were observed in
Figure 11: Alignment of TFBS in *EGRI*, *GATA2*, and *WT1* promoters reveals overlapping SP1, *EGR1* and *WT1* sites. Dots indicate nucleotides identical to human, while gaps are shown with dashes. Predicted TFBS are based on human sequences and are marked by boxes: *EGR1*, dashed; SP1, dash-dotted; WT1, solid. (A) Two separate WT1 sites in the *EGR1* promoter are conserved between multiple species and both overlap an *EGR1* site, and one also overlaps an SP1 site. WT1 site (human 614–630) overlaps EGR1 site (human 608–624) and both sites are conserved between all eight species surveyed. The WT1 site (human 565–581) overlaps both an EGR1 site (human 563–575) and an SP1 site (human 563–577). The SP1 site is conserved between all eight species, the WT1 site is conserved between all but opossum and the EGR1 site is conserved between primates. Negative numbers in the chimpanzee *EGR1* promoter sequence indicate that the orthologous region was located 1,668 base pairs from the ATG site (further upstream than 1.5 kb analyzed for other species). (B) Two overlapping WT1 sites (human 1127–1143 and human 1129–1145) overlap an SP1 site (human 1125–1139) in the *GATA2* gene promoter region. The WT1 sites are conserved between human, chimpanzee, and macaque, while the SP1 site is conserved between human, chimpanzee, macaque, and cow. (C) Two WT1 and an SP1 TFBS in the *WT1* promoter are conserved. The WT1 site (human 1444–1468) is conserved between human, chimpanzee, macaque, mouse, rat, and opossum. The WT1 site (human 1409–1425) that overlaps an SP1 site is conserved between human and chimpanzee only, while the SP1 site (human 1420–1434) is conserved between human, chimpanzee, macaque, mouse, and rat.
either genomic sequence; thus, these TFBS were located in exactly the same positions relative to the start ATG codon.

Identification of overlapping TFBS in the gene promoters indicated that WT1 and EGR1 may compete for binding. Analyses of the promoter regions of 11 genes expressed in prostate cancer epithelial cells showed that WT1 TFBS overlapped SP1 and EGR1 TFBS, either separately or together. Overall, it was found that there were 25 overlapping sites in the promoter regions of these genes. There were 12 WT1/SP1, seven SP1/EGR1, three WT1/EGR1, and three WT1/SP1/EGR1 overlapping sites (Table 10). These overlapping sites were found in 10 of the 11 gene promoters analyzed. Seven overlapping sites were identified in the promoter region of the EGR1 gene, and three of these seven overlapping sites are conserved between human and other species. Three other gene promoters, GATA2, IGFBP2, and TFAP2C, have three overlapping sites each, with one SP1/EGR1 site conserved between human and opossum for both the TFAP2C and IGFBP2 promoters. The WT1 and KLK3 promoters have overlapping WT1/SP1 and SP1/EGR1 sites, respectively. All of these overlapping TFBS are excellent candidates for functional testing to determine whether competition for TF binding at these sites results in activation or suppression of the genes they are regulating.

**Sequence conservation of TFBS indicates a potentially functional WT1 binding site in the KLK3 (PSA) promoter**

One of the 24 genes differentially expressed in prostate cancer epithelial cells was KLK3 (PSA), an important diagnostic marker. Sequence alignment of the KLK3 promoter
Table 10 - Conservation of overlapping TFBS between human and other mammals

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT1/EGR1</th>
<th>SP1/EGR1</th>
<th>WT1/SP1</th>
<th>WT1/SP1/EGR1</th>
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</thead>
<tbody>
<tr>
<td>ECAD</td>
<td>0</td>
<td>0</td>
<td>1233-1249: C</td>
<td>0</td>
</tr>
<tr>
<td>EGR1</td>
<td>608-630: Pr, Ro, Op</td>
<td>703-727: Pr</td>
<td>1340-1360: M</td>
<td>563-581: Pr</td>
</tr>
<tr>
<td></td>
<td>1029-1047: C</td>
<td></td>
<td>1466-1486: M</td>
<td>1466-1490: M</td>
</tr>
<tr>
<td>GATA2</td>
<td>0</td>
<td>0</td>
<td>282-300: M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>892-910: Pr</td>
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<td>1125-1145: Pr</td>
<td></td>
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<td>IGFBP2</td>
<td>0</td>
<td>1327-1359: Pr, Ro, Op</td>
<td>716-744: C</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1445-1463: C</td>
<td></td>
</tr>
<tr>
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<td>1384-1403: Pr, Ro, Op</td>
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<td></td>
<td></td>
<td></td>
<td>1409-1434: C</td>
<td></td>
</tr>
</tbody>
</table>

a Position numbers are based on predicted TFBS in the human sequences. Pr = Both primates (chimpanzee and macaque), C = chimpanzee, M = macaque, Ro = Both rodents (mouse and rat), Op = opossum. 0 = No overlapping WT1/EGR1, SP1/EGR1, WT1/SP1, or WT1/SP1/EGR1 TFBS for that particular gene.
revealed three WT1 sites and two SP1 sites, with two-thirds of the WT1 and one-half of the SP1 sites conserved between human and other primates (Table 7). Given the premise that evolutionary conserved sites are more likely to be functionally relevant, we tested these conserved sites for their ability to bind TF in vivo. PCR primers were designed to flank the region where adjacent conserved WT1 (human 1332–1352) and the SP1 sites (human 1404–1418) were identified (Figure 12A). Both of these binding sites in the PSA promoter were tested by chromatin immunoprecipitation (ChIP) in hormone responsive LNCaP prostate cancer cells (Figure 12B). Since LNCaP cells express little WT1 (Fraizer et al., 2004), they were transfected with a green fluorescent protein (GFP)-tagged WT1 expression construct 48 hours prior to the ChIP assay. After crosslinking, the chromatin and TF complexes were immunoprecipitated by both WT1 and SP1 antibodies, as demonstrated by PCR amplification of the promoter region. WT1 and SP1 may bind at adjacent sites within the PSA promoter or at overlapping sites, since the SP1 site overlaps the EGR1 site, to which WT1 may also bind (Liu et al., 1996; Madden et al., 1991; Dey et al., 1994). The importance of these WT1 and SP1 TFBS as candidate binding sites was confirmed by the in vivo ChIP assay.

**Functional WT1 and SP1 binding sites in the VEGF promoter are conserved between human and other primates**

Having tested the significance of identified evolutionary conserved sites, we then asked whether TFBS known to mediate transcriptional regulation would also be
Figure 12: Conservation of the KLK3 (PSA) promoter and ChIP verification of WT1 and SP1 binding. (A) Alignment of predicted TFBS (based on human sequences) in the KLK3 gene promoter of multiple genomes shows the conservation of two overlapping WT1 binding sites (solid box), an EGR1 site (dashed box), an SP1 site (dash-dotted box), and an SP2 site (double dash-dotted box). WT1 sites (human 1332–1348 and 1336–1352) are conserved between human, chimpanzee, macaque, and cow and they overlap an SP2 site (human 1347–1361) conserved between human, chimpanzee, and cow. An EGR1 site (human 1400–1416) overlaps an SP1 site (human 1404–1418) and both are conserved between human, chimpanzee, macaque, and dog. (B) The binding of WT1 and SP1 TFs to native chromatin obtained from WT1-transfected LNCaP cells was confirmed by ChIP. Lane 1 shows the no DNA PCR control and lane 2 shows PCR amplified input DNA. Lanes 3, 4, and 5 show PCR amplified DNA immunoprecipitated by IgG (no antibody control), SP1 or WT1 antibodies, respectively.
conserved. Two genes that regulate prostate cancer progression by enhancing growth and blood supply, AR and VEGF, have multiple WT1 and SP1 binding sites in their proximal promoter regions (Hanson et al., 2007; Shimamura et al., 1997; Husbeck et al., 2006; Kohler et al., 2007; Pore et al., 2006). We have previously identified an EGR1 site in the VEGF promoter that binds both WT1 and SP1 protein in vitro (Hanson et al., 2007), and here demonstrate by ChIP assay that this promoter region binds WT1 and SP1 in vivo (Figure 13). Chromatin from both embryonic kidney 293 cells and LNCaP cells expressing a GFP-tagged WT1 expression construct was immunoprecipitated by WT1 and SP1 antibodies and amplified by PCR. Using primers specific for the VEGF proximal promoter region, products ~140 bp in size were amplified from chromatin of both 293 and LNCaP cells (Figure 13A and 13B). These ChIP assays also demonstrated selective WT1 binding, since an adjacent site 190 nucleotides downstream failed to bind WT1 in the same assay (data not shown). These sites were validated as being transcriptionally regulated in several different assays, including luciferase reporter assays (Hanson et al., 2007), so we asked whether they were evolutionary conserved in different species. In silico analyses predicted that an overlapping EGR1 (human 1717–1733) and SP1 (human 1721–1735) site and a WT1 site (human 1755–1771) were conserved between primates and dogs, but not in rodents (Figure 13C). Furthermore, as seen with the PSA promoter region, WT1 and SP1 may bind at adjacent sites or potentially at overlapping sites since WT1 also binds at EGR1 sites (Liu et al., 1996; Madden et al., 1991; Dey et al., 1994). Both PSA and VEGF promoter regions contain evolutionary conserved WT1 sites.
Figure 13: ChIP verification of WT1 and SP1 binding to endogenous VEGF promoter and sequence conservation. Functional WT1 and SP1 TFBS in the VEGF promoter region were previously identified by EMSA and luciferase reporter assays (Hanson et al., 2007). (A) ChIP analysis of chromatin from WT1 transfected 293 kidney cells verified that these TFBS were functional. Lanes 1 and 7 show the 1 Kb ladder, lane 2 shows the No DNA PCR control, and lane 3 shows PCR amplified input DNA. Lanes 4, 5, and 6 show PCR amplified DNA immunoprecipitated by IgG (no antibody control), WT1 or SP1 antibodies, respectively. (B) ChIP analysis of chromatin from WT1 transfected LNCaP cells verified these TFBS were functional in prostate cancer cells as well. Lanes as described in section (A). (C) Predicted TFBS are based on human sequences and marked by boxes as described in Figure 10. These functional WT1 (human 1755–1771), EGR1 (human 1717–1733) and SP1 (human 1721–1735) sites were conserved between primates (human, chimpanzee, and macaque) and dogs, but not in rodents; and the SP1 site overlapped with the EGR1 site.
adjacent to overlapping EGR1/SP1 TFBS, to which WT1 is also likely to bind, thus facilitating either cooperation or competition between TFs.

**Functional WT1 binding sites in the Androgen Receptor (AR) promoter are conserved between human and other primates**

Similarly, WT1 binding sites previously identified in the AR proximal promoter region by EMSA analysis and verified to mediate transcriptional regulation in luciferase reporter assays (Shimamura et al., 1997; Kohler et al., 2007) were confirmed by ChIP using PCR primers flanking the WT1 and SP1 TFBS (**Figure 14A**). Since these binding sites were tested *in vivo*, evidence of sequence conservation was sought, as described. As shown in **Figure 14B**, both a WT1 site (human 1434–1450) and an EGR1 site (human 1524–1537) were identified within the region amplified by ChIP. This less common pyrimidine-rich EGR1 TFBS, consisting of TCC repeats, has been shown to bind both WT1 and SP1 (Liu et al., 1996; Englert et al., 1995; Shimamura et al., 1997), thus all three zinc finger TFs could compete for binding at this site. Evidence for evolutionary conservation between human and other primates was limited by the lack of genomic sequence information available for chimpanzee (and lack of conservation between human and macaque).
Figure 14: ChIP verification of WT1 and SP1 binding to endogenous AR promoter and sequence analysis. Functional WT1 TFBS in AR promoter region were previously identified by EMSA and reporter assays (Shimamura et al., 1997; Kohler et al., 2007). (A) ChIP analysis of chromatin from WT1 transfected LNCaP prostate cancer cells verified these TFBS were functional. Lane 1 shows the 1 Kb ladder, lane 2 shows the No DNA PCR control, and lane 3 shows PCR amplified input DNA. Lanes 4, 5, and 6 show PCR amplified DNA immunoprecipitated by IgG (no antibody control), WT1 or SP1 antibodies, respectively. (B) Predicted TFBS are based on human sequences and marked by boxes as described in Figure 11. Evidence for conservation of the functional WT1 (human 1434–1450) TFBS was limited by lack of sequence information available for chimpanzee (and lack of conservation with macaque). Surprisingly the TCC rich EGR1 site (human 1524–1537), previously shown to bind WT1 in vitro (Shimamura et al., 1997), also showed no evolutionary conservation.
PART II: ANDROGEN MEDIATED REGULATION OF VEGF

Vascular Endothelial Growth Factor is up-regulated by androgens in PrCa cells

To determine whether VEGF expression was activated by androgen in prostate cancer cells, LNCaP cells were treated with the androgen R1881. Cells were serum starved overnight and then treated with 1nM and 10nM R1881 for 24 hours. Figure 15A shows a dose-dependent response of VEGF mRNA expression to androgen by real-time PCR. As the concentration of R1881 was increased, activation of VEGF also increased from 3.7 fold (1nM R1881) to 9.8 fold (10nM R1881). Given that mRNA increased, VEGF protein expression was examined in LNCaP cells treated with androgen. As shown in Figure 15B, VEGF protein expression increased after 1 hour of treatment with 1nM R1881 and maximal expression was seen after 48 hours, which was similar to mRNA expression. After determining that VEGF expression was hormone responsive in LNCaP cells, another androgen responsive prostate cancer cell line, CWR22Rv1 (22Rv1), was investigated. 22Rv1 cells were serum starved overnight and treated with 5nM R1881 for 48 hours. VEGF mRNA levels increased more than two-fold indicating that VEGF is also hormone responsive in this cell line (Figure 15C).

Casodex treatment attenuates androgen activation of VEGF

To confirm that androgen induction of VEGF required hormone-AR interaction, the effect of anti-androgen treatment was then examined using bicalutamide (casodex). LNCaP and 22Rv1 cells were treated with 5nM R1881 for 24 hours (LNCaP) and 48 hours (22Rv1) with and without pre-treatment with 10µM casodex (Figures 16A and
Figure 15: Androgens regulate VEGF expression in LNCaP and 22Rv1 prostate cancer cells treated with R1881. (A) VEGF mRNA expression in LNCaP cells treated with 1nM and 10nM R1881 measured using real-time PCR. GAPDH was used to normalize VEGF expression. Values represent fold change relative to no hormone treatment. A student t-test was performed and significance was determined (*p < 0.05). (B) VEGF protein expression in LNCaP cells treated for 0-48 hours with 1nM R1881. Beta-actin was used as a loading control. (C) VEGF mRNA expression in CWR22Rv1 cells treated with 5nM R1881. Beta-actin was used to normalize VEGF expression (***p value < 0.01).
Figure 16: Expression of VEGF in LNCaP prostate cancer cells treated with R1881 and the anti-androgen casodex. VEGF mRNA expression of LNCaP (A) and CWR22Rv1 (B) cells was measured by real-time PCR using Taqman VEGF primers. Cells were serum starved overnight with phenol and serum-free RPMI and then treated with 5nM R1881 or DMSO as a vehicle control. For inhibition of androgen activity, cells were pre-treated with 10µM casodex for 2 hrs and then induced with 5nM R1881 for 24 hrs (LNCaP) and 48 hrs (22Rv1). Values represent fold change relative to no hormone treatment. A student t-test was performed and significance was determined * (p < 0.05), ** (p < 0.01). (C, D) Nuclear AR and cytoplasmic VEGF protein expression was measured by western blot of LNCaP cells treated as per (A). AR was used as a control to determine that AR activity was indeed inhibited.
Casodex treatment significantly reduced the hormone activation of VEGF mRNA indicating that classical signaling requiring AR-androgen interaction was occurring. To determine whether casodex blocked a hormone mediated increase in nuclear AR protein levels, nuclear extracts were examined (Figure 16C). Western blot analysis was used to measure AR protein levels in nuclear lysates prepared from LNCaP cells treated with and without hormone (1nM R1881) and with and without casodex (10µM). Figure 16C shows that casodex reduced levels of nuclear AR induced by hormone treatment (from 7 fold to 4 fold compared to 0nM R1881). Cytoplasmic VEGF protein levels were decreased more than 70% by casodex treatment that blocked hormone mediated up-regulation of VEGF (Figure 16D).

**Promoter analysis reveals potential functional ARE sites in the VEGF promoter**

In order to determine if the VEGF promoter contained potential ARE binding sites, TFBS prediction analysis was done using MatInspector software. Analysis of the VEGF promoter sequence revealed numerous transcription factor binding sites including Sp1, WT1, and Egr1 sites as well as three potential ARE binding sites within 2kb of the transcription start site in the VEGF promoter (Figure 17). Since these ARE sites were non-classical monomeric sites, it was important that they be tested for functional binding using chromatin immunoprecipitation (ChIP) (Figure 18A). LNCaP cells were serum starved overnight and then treated with and without 5nM R1881 for 24 hours. As shown in Figure 18B, chromatin of hormone treated LNCaP cells was immunoprecipitated with anti-AR antibody. Immunoprecipitated chromatin was amplified by primers flanking the
gaattctgtg ccctcactcc cctggatccc tgggcaaagc cccagaggga aacacaaaca
5274
ggttgttgta acacaccttg ctgggtac cca ccatggagga cagttggctt atg
121
cgggcctg gggccacgga gtgactggtg atggctatcc ctccttggaa cccctcaca
2274
cgacccctag catgagggcc gctgttgag cccttccata tcccgttca
321
tggggtgcta gaggcccaaa aggaggaaag ttaaggtgctt ccccttcata tccccgttca
421
cagccttggag gggggtgg gaggcgcaca aggaggaaag ttaaggtgctt ccccttcata tcccgttca
521
ttaaactgagc ccatggagga cagttggctt atg
tggggtgcta gaggcccaaa aggaggaaag ttaaggtgctt ccccttcata tcccgttca
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821
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tggggtgcta gaggcccaaa aggaggaaag ttaaggtgctt ccccttcata tcccgttca
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3421
ttaaactgagc ccatggagga cagttggctt atg
tggggtgcta gaggcccaaa aggaggaaag ttaaggtgctt ccccttcata tcccgttca

Figure 17: Promoter analysis of VEGF. The VEGF promoter (VEGFA accession number AB021221) was downloaded from Ensembl (www.ensembl.org). Binding sites were predicted by MatInspector and located on the VEGF promoter sequence. Potential ARE and zinc finger transcription factor binding sites thought to play a role in VEGF regulation are highlighted: | = transcription start site, Yellow = SP1 (2300, 2284, 2273, 2262, 1993, 1257), red = EGR1 (2314, 2297, 2281, 2265, 2116, 1308, 1260, 451), blue = WT1 (2154, 1864, 1716), HIF1 (2046, 1847, 1382), green = ARE (ARE I: 1945, ARE II: 1443, ARE III: 342).
Figure 18: AR protein binds to the VEGF promoter specifically in hormone treated LNCaP cells. (A) Schematic diagram of the VEGF promoter showing predicted ARE binding sites and primers used to amplify the specific regions of the promoter. (B) ChIP assays were performed with chromatin prepared from LNCaP cells treated for 24 hours with either 0nM R1881 or 5nM R1881 after overnight serum starvation using primers specific for the ARE I region of the VEGF promoter. Lane 1 contains input chromatin that was not immunoprecipitated with antibody, lane 2 is chromatin immunoprecipitated with anti-pol II antibody (Upstate), lanes 3 and 4 contain chromatin treated with and without 5nM R1881 and immunoprecipitated with anti-AR C-19 (Santa Cruz) antibody and lane 6 is the negative control immunoprecipitated with anti-IgG antibody (Upstate). (C) Quantification of immunoprecipitation was performed by SYBR Green real-time PCR on chromatin immunoprecipitated with anti-AR antibody from LNCaP cells treated with and without 5nM R1881. Values shown represent % of input immunoprecipitated by the two preps. (D) PCR was performed using chromatin as described in (A) and primers specific for ARE II. Lane 1 is the no DNA control, lane 2 is input diluted 1:10, lane 3 is input, lane 4 is untreated chromatin immunoprecipitated with anti-AR antibody, lane 5 is chromatin treated with 5nM R1881 and immunoprecipitated with anti-AR antibody, and lane 6 is the anti-IgG antibody negative control immunoprecipitation. (E) same as (D) only using primers specific for ARE III. Image J analysis was performed and IP’s are shown as a ratio of Input chromatin.
-286 to -521 region containing the putative ARE I binding site (Figure 18A). Although little amplified PCR product was observed, AR antibody did pull down more chromatin in treated than in untreated cells as measured by SYBR Green quantitative real-time PCR (Figure 18B and C). Results are presented as a percentage of input chromatin and show that the AR immunoprecipitation of hormone treated chromatin increased greater than 2 fold as compared to the AR immunoprecipitation of untreated chromatin (Figure 18C). This suggested that ARE I may be functional and warranted examining the other two potential ARE binding sites. Using the same chromatin preparations, PCR was performed using primers designed to flank ARE II and ARE III (as shown in Figure 18A). Figure 18D shows that AR antibody immunoprecipitates the region of the VEGF promoter containing the ARE II binding site. Comparing chromatin of hormone treated cells to that of untreated, there is more than a 5 fold increase of chromatin immunoprecipitated by AR antibody in cells treated with 5nM R1881. This same pattern was also seen at the region containing the ARE III binding site (Figure 18E) however with only a 2 fold difference between treated and untreated. These results led us to believe that all three binding sites are functional and may be important in the hormone regulation of VEGF.

*The VEGF promoter is hormone responsive*

To determine whether AR binding regions identified by ChIP were also transcriptionally regulated by hormone, VEGF promoter deletion constructs were obtained from Dr. Xie (Shi et al., 2001) ranging in length from 88 bp (V88) to 2274 bp (V2274). Figure 19A shows the location of predicted transcription factor binding sites.
Figure 19: Hormone activates the VEGF promoter in two different cell lines, LNCaP and CWR22Rv1. (A) Schematic diagram of the VEGF promoter showing where predicted ARE binding sites are in relation to V411 and V2274. (B) LNCaP cells were transfected with a 411 bp VEGF promoter construct (V411) in the presence or absence of hormone (as described) and with and without casodex for 48hrs. Luciferase assays were performed and fold activation was determined. (C) CWR22Rv1 cells were treated as in (B). (D) Dose response of hormone on V411 in CWR22Rv1 cells. (E) Effect of casodex on the hormone activation of the 2274 bp VEGF promoter construct V2274 in CWR22Rv1 cells. Ratios are expressed relative to average normalized luciferase activity in the absence of hormone. Experiments were repeated three times in triplicate. Values represent normalized relative luciferase activity. Significance was determined by Students t-test (*p<0.05, ** p<0.01, ***p<0.001).
These constructs were tested in luciferase assays to determine where within the VEGF promoter the hormone responsive element(s) were located. LNCaP and 22Rv1 cells were transfected with a 411 bp (V411) construct containing only the ARE I site and treated with either 5nM R1881, 5nM R1881 with 10µM casodex, or DMSO (control) (Figure 19B and C). After 48 hours, cells were lysed and luciferase assays were performed. LNCaP cells treated with 5nM R1881 showed a greater than a 2.5 fold increase in VEGF promoter activity when compared to cells treated with the DMSO vehicle control (Figure 19B). Confirming the requirement for AR-hormone interaction, casodex treatment inhibited this androgen response in LNCaP cells (Figure 19B). Similarly, in 22Rv1 cells an almost 2 fold increase in VEGF promoter activity was seen in cells treated with 5nM R1881 and again casodex blocked this activation (Figure 19C). Additionally, 22Rv1 cells were shown to be highly sensitive to androgen as even 0.5nM R1881 increased VEGF promoter activity more than 2 fold (Figure 19D).

Since ARE II and III lie outside of the V411 region, a larger promoter construct (V2274) was examined in 22Rv1 cells to determine whether hormone activation of VEGF was greater in the 2kb reporter construct containing all three ARE binding sites. As shown in Figure 19E, hormone activation of V2274 was increased (3.5 fold) in this larger construct, greater than the response shown in the smaller 411 bp reporter construct containing only ARE I. This suggested that all three ARE sites may contribute to androgen activation of the VEGF promoter, although not synergistically.
**Mutations of ARE binding sites attenuates androgen activation of VEGF**

To determine whether all three ARE sites might be involved in the androgen mediated up-regulation of the VEGF promoter, the sequences of all three ARE binding sites were mutated to prevent AR binding and mutations were confirmed by sequence analysis. Mutations were made in the larger V2274 reporter construct which contains all three ARE binding sites (Figure 20A). Site-directed mutagenesis was performed using PCR primers designed to contain base substitutions in both the ARE II and ARE III sites in the V2274 construct (Figure 20B). Transfections were then performed in 22Rv1 cells followed by hormone treatment and luciferase assays. Mutations of either ARE sites were shown to decrease the androgen mediated activation of the V2274 construct from roughly 3 fold activation of wild type to 2 fold activation of mutant ARE II and III constructs (Figures 20C and 20D, respectively). Double mutation of both ARE II and III in V2274 showed similar retention of residual hormone activation when compared to wild type (data not shown).

To determine the contribution of ARE I, the V411 reporter construct (Figure 21A) containing ARE I was mutated by site-directed mutagenesis using PCR primers designed to mutate the core bases of this binding site (Figure 21B). The effect of eliminating AR binding at the ARE site was tested by luciferase reporter assays performed in both 22Rv1 and LNCaP cells. Figure 21C shows that in 22Rv1 cells, the wild type V411 is up-regulated by 5nM R1881 more than 3 fold and this is attenuated in the mutant ARE I V411 construct to less than 2 fold. This similar effect was also seen in LNCaP cells where the wild type V411 construct activity was significantly increased by
Figure 20: Mutation of ARE sites attenuate hormone activation of the VEGF promoter. (A) Schematic diagram of a 2kb region of the VEGF promoter showing the ARE binding sites. Red boxes indicate sites that were mutated. (B) Shows the wild type and mutant sequence of the ARE II and ARE III binding sites. (C) Site-directed mutagenesis was performed using primers containing the mutated bases in (B) for ARE III followed by transfection into CWR22Rv1 cells and treatment with and without 5nM R1881 for 48 hrs. Luciferase assays were performed as previously described. (D) ARE II was mutated as in (C) using primers containing mutated bases for ARE II. Experiments were repeated three times in triplicate. Significance was determined by student’s t-test (**p<0.01).
Figure 21: Mutation of predicted ARE I binding site decreases VEGF promoter activation. (A) Schematic diagram of the VEGF promoter showing the location of ARE I (red box) binding site which was mutated. (B) Shows the wild type and mutant sequence of the ARE I binding site. (C) Site-directed mutagenesis was performed on the VEGF promoter construct V411 and the predicted ARE I binding site was mutated. Mutant constructs were transfected into LNCaP cells serum starved for 24 hrs and treated with and without 5nM R1881 for 48 hours. Luciferase assays were performed as described. (D) This same mutated V411 construct was transfected into 22Rv1 cells and treated the same as LNCaP. Experiments were repeated three times in triplicate. Significance was determined by student’s t-test (**p<0.01).
hormone treatment by almost 3 fold, while the hormone response of the mutant ARE I V411 construct was only increased 1.5 fold (Figure 21D). Thus the response to hormone was reduced 2x in both 22Rv1 and LNCaP cells.

Since complete loss of hormone response was not achieved by double mutation, we reasoned that one possibility was that all three sites were redundant. Another possibility was that other TFs were involved in the hormone response. Thus we examined the involvement of other ZFTFs in the regulation or co-regulation of VEGF transcription.

PART III: INTERACTIONS OF ANDROGEN AND ZFTFs IN THE REGULATION OF VEGF

Although we had demonstrated that ARE sites were necessary for a full hormone response, residual activity remained in the mutant ARE constructs. Thus, we wondered whether other transcription factors might interact with AR to enhance hormone response. We hypothesized that if AR-ZFTF interactions were important mediators of androgen response, then cognate binding sites should be located within the promoter regions of hormone responsive genes expressed in prostate cancer. To determine whether AR binding sites were found adjacent to previously described ZFTF binding sites (PART I) in promoter regions of genes expressed in prostate cancer, in silico analysis was once again performed on eleven of the promoter regions examined in PART I. Non-classical AR half-sites were identified adjacent to WT1/EGR1/Sp1 sites in 8 of 11 promoters analyzed (Table 11). This discovery and our earlier transcriptional regulation studies
TABLE 11: EVOLUTIONARY CONSERVED ZFTF (WT1/EGR1/SP1) SITES ARE FOUND ADJACENT TO NON-CLASSICAL AR HALF-SITES IN GENE PROMOTERS


<table>
<thead>
<tr>
<th>Genes</th>
<th>AR half-sites</th>
<th>WT1 sites</th>
<th>EGRI sites</th>
<th>SP1 sites</th>
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<td>1</td>
<td>1</td>
<td>3</td>
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<td>11</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>FGFR3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>NRDG1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NKX3.1</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>4</td>
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<td>6</td>
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<tr>
<td>VEGF</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

\[^a\text{Genes and zinc finger transcription factors (ZFTF) expressed in prostate cancer cells are listed. Note that in 8 of 11 promoters analyzed non-canonical AR half-sites were identified adjacent to potential ZFTF sites. KLK3, kallikrein 3 (prostate specific antigen); FGFR3, Fibroblast growth factor receptor3; IGFBP2, insulin-like growth factor Binding protein2; NRDG1 N-myc downstream regulated gene1.}\]
support our hypothesis that androgen signaling is mediated in part by interaction of AR with specific ZFTFs at non-classical ARE binding sites. Binding at one of the three predicted non-classical ARE half-sites (ARE I) in the VEGF promoter region was tested by ChIP analysis of hormone treated WT1 transfected LNCaP cells (Figure 22). Endogenous AR and Sp1 proteins, along with exogenous WT1, were immunoprecipitated from native chromatin of these hormone treated cells. This indicates that the predicted WT1, Sp1 and AR sites in the VEGF proximal promoter region were functional and suggests the three ZFTFs may bind individually or as a complex. Thus, the effect of these ZFTFs on hormone mediated activation of VEGF expression was further investigated. Our hypothesis was that these ZFTFs might enhance the activation of VEGF promoter by androgen.

**WT1 binds to and activates the VEGF promoter in PrCa cells**

Although previous studies had demonstrated that WT1 regulates the VEGF promoter (Hanson *et al.*, 2007) in LNCaP prostate cancer cells, we asked whether this same pattern of regulation was occurring in other hormone responsive prostate cancer cell lines. Using two other hormone responsive prostate cancer cell lines, CWR22Rv1 and C4-2, it was found that WT1 regulated the VEGF proximal promoter (Figure 23A) similarly in all three cell lines (Figure 23C-E). The V411 proximal promoter construct was co-transfected with a GFP-WT1 expression construct and fluorescence was observed, indicating efficient transfection of WT1 into these cells (Figure 23B).
Figure 22: Predicted WT1, Sp1 and AR binding sites in VEGF proximal promoter are functional in vivo. Top Panel: Schematic diagram of the VEGF proximal promoter showing predicted binding sites of WT1, AR, Sp1, and Egr1. The red box indicates the region that the primers flanked in the ChIP assay. Bottom Panel: The binding functions of the predicted ARE I, WT1 and Sp1 binding sites in the VEGF proximal promoter were tested by ChIP using hormone induced WT1-transfected LNCaP cells. PCR negative control (lane 2) and unprecipitated positive control input DNA (1% and 5% of total chromatin) (lanes 3 and 4) are shown along with negative IgG Ab control (lane 5). Remaining lanes show PCR amplified DNA immunoprecipitated by: WT1 Ab (cocktail of C19/N20, Santa Cruz) lane 6, SP1 Ab (Upstate) lane 7, or AR Ab (N20, Santa Cruz), lane 8.
Figure 23: WT1 activates the VEGF promoter in prostate cancer cells. (A) Shows the VEGF promoter with WT1 binding sites as predicted by MatInspector. (B) Expression of GFP tagged WT1 expression construct was observed by epifluorescence microscopy 24 hours after transfection of LNCaP cells. (C-E) Transcriptional activation of the VEGF promoter construct V411 by WT1. LNCaP (C), CWR22Rv1 (D), and C42 (E) prostate cancer cells were co-transfected with 250ng of V411 and 500ng of CMV or WT1 expression construct and harvested after 48 hrs. Luciferase activity was measured and normalized to protein concentration. The data represents the relative fold activation by WT1 normalized to CMV vector control. Each experiment was repeated at least twice in triplicate. Statistical significance was determined by student's t-test (p< 0.01 **, p< 0.001 ***).
Luciferase assays were performed and it was determined that WT1 activated the proximal VEGF promoter more than two fold in LNCaP and CWR22Rv1 cells (Figure 23C-D) and slightly less, but still significantly in LNCaP C-42 cells (Figure 23E). Since there are a number of potential WT1 binding sites in the VEGF promoter, functional studies were done to see if WT1 was binding to this region. We previously verified binding at one predicted WT1/Egr1 binding site using ChIP in LNCaP cells (PART I, Figure 12). Chromatin binding of WT1 to this site in the VEGF promoter, suggested it might play a role in the regulation of VEGF expression. We hypothesized that mutating this WT1 binding site would reduce VEGF activation by WT1. Using site-directed mutagenesis, primers containing base pair substitutions specific for this WT1 binding site in the VEGF promoter construct V411 (Figure 24A) were used to PCR amplify and create this mutant WT1mV411 construct. This construct was then used in luciferase reporter assays to determine if this site was necessary for WT1 mediated transcriptional activation of V411 proximal VEGF promoter. Co-transfection of WT1 expression construct and mutant reporter into LNCaP cells revealed that mutation of this WT1 binding site significantly decreased the ability of WT1 to up-regulate the proximal VEGF promoter (Figure 24B). Taken together this data showed that WT1 bound and activated the VEGF proximal promoter and this binding site was necessary for WT1 mediated activation of the VEGF promoter. Given the location of the WT1 site within 200 bp of the ARE site, we then asked whether WT1 would modulate the hormone response of the proximal VEGF promoter.
Figure 24: VEGF promoter activity is decreased when specific predicted WT1 site is mutated. Site-directed mutagenesis was performed on the VEGF promoter construct V411. (A) A predicted WT1 binding site shown with a red box was mutated. (B) For the WT1mV411 reporter construct, cells were transfected with and without WT1 (500ng) along with the mutant construct using the empty CMV expression construct as comparison. Luciferase assays were performed using mutant and wild-type V411 reporter constructs. Values represent normalized luciferase activity relative to CMV empty vector. Experiments were repeated three times in triplicate. Student’s t-test was performed and significance was determined, *** (P < 0.001).
To investigate whether WT1 was playing a role in the hormone activation of VEGF, co-transfections of WT1 and the proximal promoter V411 construct were performed in hormone treated LNCaP cells. Cells were serum starved and then after transfection treated with 5nM R1881 or vehicle control (DMSO). Luciferase assays confirmed that either hormone or WT1 alone increased VEGF transcription 3-4 fold compared to CMV empty vector, vehicle control. However, the combination of WT1 and 5nM R1881 activated this reporter construct more than 12-fold (Figure 25), suggesting that their interaction strongly enhances hormone response. This synergistic up-regulation led to the hypothesis that WT1 and AR may form a complex in the nucleus and, therefore, both cytoplasmic and nuclear lysates were isolated from different prostate cancer cell lines and examined for the presence of these two proteins (Figure 26). WT1 protein was found only in the nuclear fraction of LNCaP, LNCaP C-42, and PC3 cells. In hormone responsive LNCaP and C42 cells grown in full serum (containing endogenous hormone), AR was present in both the cytoplasmic and nuclear preps. However, there was more AR in the nucleus than in the cytoplasm, as would be expected. Co-immunoprecipitation of WT1 and AR revealed that these two proteins may form a complex in the presence of androgens (Figure 27). Antibodies specific for WT1 were used to immunoprecipitate WT1-GFP protein from nuclear extracts of transfected LNCaP cells grown in full serum. Using Western blot analysis, AR antibody was used to probe the membrane demonstrating that AR was also immunoprecipitated with WT1. Conversely, AR immunoprecipitated complexes contained low levels of WT1 protein.
Figure 25: WT1 and R1881 synergistically activate the VEGF proximal promoter. LNCaP cells were serum starved and co-transfected with V411 and either pcDNA3.1 vector control DNA or GFP-WT1 (250ng) and cultured in media containing 10% charcoal stripped FCS with 0 or 5nM R1881. Normalized luciferase values are shown as relative activation by GFP-WT1 and compared to pCMV4 vector control in the absence of hormone treatment (white). Each experiment was performed in triplicate and repeated twice. Student’s t-test was performed, p < 0.001.
Figure 26: Nuclear localization of WT1 and AR in different prostate cancer cell lines. Prostate cancer cell lines were investigated for the presence of nuclear AR and WT1 proteins. Nuclear and cytoplasmic lysates were prepared from LNCaP, LNCaP-C42, and PC3 prostate cancer cells. After electrophoresis, membranes were probed with anti-WT1 rabbit monoclonal Ab (Epitomics) or anti-AR polyclonal Ab (N-20, Santa Cruz) followed by incubation with HRP-labeled secondary antibody and visualized with chemiluminescence using a Fuji LAS3000 developer.
Figure 27: Co-Immunoprecipitation of AR and WT1 reveals an interaction between the two proteins in LNCaP cells. The interaction of AR and WT1 in hormone treated LNCaP cells was demonstrated by co-immunoprecipitation. Nuclear extracts were incubated with AR Ab (C19, Santa Cruz), WT1 Ab (Epitomics), or serum IgG and the complexes were collected by sepharose magnetic beads (Active Motif, CA). Proteins eluted from the beads, were denatured, electrophoresed, and probed with either AR (top) or WT1 (bottom) antibodies. Left lanes show nuclear lysates, middle lanes show proteins immunoprecipitated by IgG Ab, and right lanes show proteins immunoprecipitated by WT1 Ab.
(data not shown). Taken together, these results suggest that WT1 could be having a role in the androgen activation of VEGF.

Enhanced expression of VEGF mRNA in WT1 transfected LNCaP cells confirmed the in vitro promoter activation studies. VEGF mRNA was also up-regulated by over-expression of WT1. Using real-time PCR, it was shown that WT1 increased VEGF mRNA levels almost 3-fold (data not shown). Although over-expression of WT1 increased VEGF mRNA levels, the converse was not true. Knockdown of WT1 expression using siRNA did not significantly effect VEGF mRNA levels, as shown in Figure 28. LNCaP cells were transfected with siWT1 (Dharmacon) and 48 hours later harvested for RNA. Although WT1 levels were reduced almost 80% (compared to siRISC-free control) by transfection with siRNA oligonucleotides specific for WT1, VEGF mRNA levels only decreased 25%, when compared to control siRISC-free (shown as fold change decreased compared to RISC-free control). Taken together, these results indicate that WT1 is sufficient to regulate VEGF, but not necessary. This suggests that other transcription factors must be playing a role in the androgen activation of VEGF.

**Sp1 binding sites in the VEGF core promoter are vital for hormone responsiveness**

Given that potential SP1 binding sites were also identified in the VEGF promoter region and SP1 was observed to bind the VEGF promoter in chromatin of LNCaP cells, we asked whether SP1 binding might contribute to androgen activation of the VEGF promoter. Although the V88 core promoter region is a very G-rich region, containing multiple potential SP1 binding sites (Figure 29A), it was not expected to respond to
Figure 28: WT1 knockdown has no significant effect on VEGF mRNA levels. WT1 gene expression in LNCaP prostate cancer cells was reduced nearly 80% (compared to siRISC-free control) using WT1 siRNA. LNCaP cells were transfected with siWT1 oligonucleotides (Dharmacon) and 48 hours later cells were harvested for RNA. Real-time PCR was performed to determine whether WT1 was knocked down and then to determine the effects of WT1 suppression on VEGF mRNA levels. Values shown are shown as fold change decreased compared to RISC-free as determined by dCT using GAPDH as a normalizer. Student’s t-test was used to determine significance (p = 0.296).
Figure 29: Hormone activates a *VEGF* promoter region lacking ARE sites. (A) Schematic diagram of an 88bp region of the *VEGF* promoter showing predicted Sp1 and Egr1 binding sites. (B) CWR22Rv1 cells were serum starved overnight and transfected the next day with V88 followed by treatment with 0nM or 5nM R1881 for 48 hours. (C) same as (B) except cells were pre-treated with 10µM casodex for 2 hours. Luciferase assay was performed as described. Experiments were repeated three times in triplicate. Significance was determined by student’s t-test (*p<0.05, ** p<0.01).
hormone induction as it does not contain any ARE binding sites. Since androgen had been demonstrated to induce AR binding to three sites within the VEGF promoter in chromatin of hormone treated cells and mutation of these sites attenuated androgen activation of VEGF, it was expected that the hormone responsive region of the VEGF promoter would be limited to the ARE containing region. Initially, to test the hormone response of the G-rich core promoter region, 22Rv1 cells were transfected with the V88 reporter construct followed by treatment with or without 5nM R1881 for 48 hours. Luciferase assays were performed and surprisingly androgen activated this VEGF core promoter region 2-fold (Figure 29B). To confirm this hormone activation was mediated by AR/androgen interaction, V88 transfected 22Rv1 cells were treated with casodex. Figure 29C shows that casodex treatment completely blocked the androgen activation of V88, implicating classical androgen signaling at the G-rich core promoter. This unexpected result led us to examine AR interaction with SP1, another potential activator of VEGF transcription. Since Sp1 has been shown to regulate VEGF transcription in other cell lines (Akiyama et al., 2002; Finkenzeller et al., 1997; Benckert et al., 2003) and Sp1 has been identified as a co-activator of other steroid receptors for other target genes (M et al., 2004; Lu et al., 2000b), we hypothesized that Sp1 binding sites were required for androgen mediated up-regulation of VEGF. Site-directed mutagenesis was used to mutate three potential Sp1 binding sites within the core promoter. Primers were designed incorporating mutations for both the Sp1.2 and Sp1.3 binding sites as shown (Figure 30A). Hormone treatment significantly activated the core promoter with mutant Sp1.2/.3 binding sites 2.5 fold as demonstrated by luciferase assays of R1881 treated
Figure 30: Mutation of the Sp1.4 binding site attenuates hormone activation of the VEGF core promoter. (A) Illustration of the potential zinc finger transcription factor binding sites within the human VEGF promoter sequence -88 to -1. Yellow highlights the Sp1 sites and red text delineates the Egr1 sites. (B) Mutations were introduced into the Sp1.2 and Sp1.3 binding sites in the V88 reporter construct. 22Rv1 cells were serum starved overnight and transfected with WT and mutant (mSp1.2/3) constructs followed by treatment with and without 5nM R1881 for 48 hours. (C) The Sp1.4 binding site was mutated in V88 reporter construct. 22Rv1 cells were serum starved overnight and transfected with WT and mSp1.4 followed by treatment with and without 5nM R1881. Luciferase assays were performed as described. Experiments were repeated three times in triplicate. Significance was determined by student’s t-test (*** p<0.001).
22Rv1 cells (Figure 30B). This suggested that these two potential Sp1 binding sites were not essential for hormone response, so another Sp1 binding site (Sp1.4) closer to the transcriptional start site was examined. Mutation of this site suppressed activation by R1881 (Figure 30C). That is, wild-type core promoter was activated by hormone treatment 3 fold, whereas the mSp1.4 mutant core promoter was not significantly activated (1.3 fold increase). This reduction of hormone response suggests that the Sp1.4 site is required for a full-strength response of the core promoter to R1881. Since the basal activity of mSp1.4 was less than wild-type V88, this mSp1.4 construct was then compared with the pGL3 empty vector to determine whether the mSp1.4 mutation eliminated all promoter activity. Figure 31A shows that was not the case, as significant levels of VEGF promoter activity remained (compared to a promoter-less construct, PGL3-Basic) despite mutation of the Sp1.4 site. In contrast to the Sp1.2/.3 or Sp1.4 mutations, all four potential Sp1 binding sites in the VEGF core promoter region were mutated and this eliminated promoter activity (Figure 31B).

**Sp1 interacts and forms a complex with AR**

Although AR and Sp1 have previously been demonstrated to interact in LNCaP cells (Lu et al., 2000), it was unclear whether these two zinc finger transcription factors would form a complex in CWR22Rv1 cells. Nuclear lysates from CWR22Rv1 cells were immunoprecipitated using AR C-19 (Santa Cruz) and Sp1 (Upstate) antibodies as well as a negative control IgG antibody. Using Western blot analysis, the immunoprecipitated
Figure 31: Mutating all Sp1 sites in the V88 core promoter completely diminishes basal activity of VEGF. (A) mSp1.4 was transfected into 22Rv1 cells and compared to the empty vector pGL3 to determine if basal activity remained. (B) Site-directed mutagenesis was used to mutate all Sp1 binding sites in the V88 core promoter. Primers were designed with mutated bases and PCR was performed. Mutant and WT reporter constructs were transfected into 22Rv1 cells followed by treatment with and without 5nM R1881 for 48 hours. Luciferase assays were performed as described. Experiments were repeated three times in triplicate. Significance was determined by student’s t-test (** p<0.01, *** p<0.001).
proteins were then probed with AR antibody, and indeed AR was immunoprecipitated by Sp1 Abs (Figure 32). This interaction was hormone specific occurring only in cells that were treated with 5nM R1881. Thus it is an androgen dependent interaction, indicating that in the presence of hormone, AR and Sp1 are bound to each other and most likely bind the VEGF core promoter activating VEGF expression.
Figure 32: AR forms a complex with Sp1 in CWR22Rv1 cells in the presence of R1881. The interaction of AR and Sp1 in hormone treated CWR22Rv1 cells was demonstrated by co-immunoprecipitation of cells treated with 5nM R1881 for 48 hours prior to harvest. Nuclear extracts were incubated with either AR Ab (C19, Santa Cruz), Sp1 Ab (Upstate), or serum IgG and the complexes were collected by sepharose magnetic beads (Active Motif, CA). Proteins were eluted from the beads, denatured, electrophoresed, and probed with AR Ab. Left panel shows proteins from immunoprecipitations of cells treated with DMSO vehicle control. Right panel shows proteins from immunoprecipitations of cells treated with 5nM R1881.
CHAPTER IV

DISCUSSION

Analysis of conserved binding sites in promoters of genes expressed in prostate cancer revealed novel monomeric AR binding sites adjacent to other ZFTFs in the promoter of VEGF and other androgen responsive genes. To further extend the investigation of VEGF regulation in prostate cancer cells, the effects of hormone on VEGF expression were examined. Androgens were found to increase both VEGF mRNA and protein and the anti-androgen casodex attenuated this VEGF activation. Mutations of three functional ARE half-sites attenuated, but did not eliminate activation of VEGF by androgen. Both WT1 and Sp1 interacted with AR and bound the VEGF promoter in vivo. AR was co-immunoprecipitated with both Sp1 and WT1. Importantly, the Sp1.4 binding site located 50 bp downstream of the transcription start site of VEGF was necessary for androgen activation of the VEGF core promoter. Taken together, these novel results demonstrate for the first time a mechanistic explanation for how androgen regulates VEGF in prostate cancer cells.
PART I: IDENTIFICATION OF CONSERVED AND FUNCTIONAL BINDING SITES IN GENES IMPORTANT IN PROSTATE CANCER PROGRESSION

Identification of evolutionary conserved sequences derived from comparisons of multiple genomes (so-called "phylogenetic footprints") has been successful in identifying functionally important regions, including those regions that regulate gene expression (Wang et al., 2006; Tagle et al., 1988; Elnitski et al., 1997; Hardison et al., 1997; Vuillaumeier et al., 1997; Loots et al., 2000; Cliften et al., 2003; Kellis et al., 2003; Xie et al., 2005). However, some regulatory genomic sequences do not appear to be conserved or the level of evolutionary conservation varies between different genomic comparisons (Hughes et al., 2005; King et al., 2005). Importantly, some functional regions have been reported to experience a relatively fast rate of turnover, where the functional significance of the element is retained despite changes at the nucleotide sequence level (e.g., transcription start sites, (Frith et al., 2006)). Thus, it is likely that gene expression in mammalian genomes is controlled by both types of regulatory elements, i.e., those elements that exhibit evolutionary and functional conservation and those that exhibit functional conservation only. Moreover, while numerous algorithms are available to computationally predict potential regulatory elements, it is often challenging to narrow down the list of those that are likely to be functional, particularly for relatively short elements such as TFBS. One of the approaches that utilizes evolutionary conservation as a predictor of TFBS functionality is the rVISTA tool that uses pairwise sequence alignments to identify the most highly conserved TFBS between the pair of genomic sequences (Loots and Ovcharenko, 2004). Another set of tools, the Mulan, takes advantage of evolutionary conservation information obtained from multi-sequence
alignments of several genomes (Ovcharenko et al., 2005). However, the latter requires the TFBS to be shared among all genomes present in the alignment (Ovcharenko et al., 2005) and may potentially miss the lineage-specific regulatory elements that are absent from some subsets of genomes. Therefore, in this work we used TFBS elements shared between some but not necessarily all of the available genomes.

We used evolutionary sequence conservation, as determined by both the multi-species sequence alignments and the in silico TFBS predictions, to identify those sites most likely to regulate expression of target genes that influence growth of prostate cancer cells. Regulatory regions with functional importance can be expected to exhibit sequence conservation due to selection. Thus, predicted TFBS that are located in the orthologous positions in multiple genomes are likely to be truly functional. Our identification of evolutionary conserved WT1 and SP1 binding sites in the PSA promoter indeed supports this notion (Figure 12). As expected, conservation of TFBS decreased as species became more evolutionarily divergent (Dermitzakis and Clark, 2002), so those TFBS that were conserved between multiple species including opossum are more likely to be functionally important in the regulation of gene expression.

The abundance of overlapping zinc finger TFBS also supported the functional importance of these regulatory regions. Thus, we identified many TFBS in potential target genes that were co-expressed with WT1 in prostate cancer epithelial cells. Evolutionary conserved WT1 and SP1 sites in the PSA promoter were confirmed by ChIP to bind both WT1 and SP1 in LNCaP prostate cancer cells chromatin. Although it is a novel discovery that both SP1 and WT1 bind the PSA promoter and may play a role in its
regulation, reporter assays are needed to confirm their contribution to transcription. In addition, a WT1 binding site known to transcriptionally regulate the VEGF promoter (Hanson et al., 2007) was confirmed by ChIP and found to be in an evolutionary conserved region. Interestingly, transcriptionally active WT1 and EGR1 binding sites in the AR promoter (Eid et al., 1998) were not conserved between human and macaque, although adjacent genomic regions could be aligned between multiple species (Figure 14). This suggests that the AR promoter may have experienced faster turn-over than the VEGF promoter, yet remained functionally conserved despite sequence changes at the nucleotide level.

Many of the genes expressed in prostate cancer epithelial cells have previously been reported to interact and regulate each other, suggesting multiple potential targets for altered pathways that may lead to prostate cancer progression. We and others have identified gene interactions (Madden et al., 1991; Hanson et al., 2007; Shimamura et al., 1997; Kohler et al., 2007; Hewitt et al., 1995; Mayo et al., 1999; Werner et al., 1993; Werner et al., 1994; Drummond et al., 1992; Hosono et al., 2000; Hewitt et al., 1996) that are consistent with WT1 regulating the progression and/or growth of tumors in the prostate. However, PSA was a candidate gene target identified by our in silico evolutionary conservation approach and confirmed by in vivo chromatin binding assays. PSA is a member of the kallikrein family of serine proteases and is a marker of epithelial differentiation in the prostate (Yin et al., 2006). It is up regulated in prostate cancer cells when compared to normal adjacent tissue (Borgono and Diamandis, 2004) and its expression is regulated by the ligand bound androgen receptor (AR) (Yin et al., 2006).
Since WT1 activates the AR promoter in prostate cancer cells (Kohler et al., 2007), this suggests that WT1 may directly or indirectly regulate PSA gene expression.

In addition to PSA, genes that were co-expressed with WT1 in prostate cancer epithelial cells and that could potentially interact with, or be regulated by, WT1 included GATA2, ECAD, EGR1, and NDRG1 (Brown, 2006). GATA binding proteins are zinc finger transcription factors that bind the WGATAR consensus motif and are expressed in multiple tissues, including endocrine glands (Merika and Orkin, 1993; Tremblay and Viger, 2003; Viger et al., 2008). Interestingly, GATA TFs regulate WT1 expression, as multiple GATA TFBS are found within the WT1 promoter and enhancer regions (Fraizer et al., 1994; Wu et al., 1995; Zhang et al., 1997). GATA binding protein 2 (GATA2) has been shown to be one of the main GATA family members expressed in the prostate of human and mouse (Perez-Stable et al., 2000). It has been suggested that GATA2 plays a role in androgen mediated regulation of PSA expression, possibly through interaction with AR, as GATA sites are adjacent to AR TFBS in the PSA promoter (Perez-Stable et al., 2000). WT1 could contribute to GATA2 mediated regulation of target genes in prostate cancer cells, if WT1 also physically interacts with GATA2. This notion is consistent with the observation that WT1 interacts with GATA4 to regulate SRY gene expression (Miyamoto et al., 2005). This complex pattern of zinc finger-protein interaction between WT1 and GATA, along with regulation of WT1 expression by GATA TF, suggests a potential for WT1 feedback control of GATA activity.

The WT1 promoter is itself a target of auto-regulation by WT1 (Hewitt et al., 1996). WT1 is a multifunctional transcription factor; its four major isoforms are formed
by alternative splicing at two sites resulting in the inclusion or exclusion of (1) exon V and/or (2) a tripeptide (KTS) in exon 9 that alters the zinc finger DNA binding structure (Rauscher et al., 1990). While the functions of the various isoforms of WT1 are still being discovered, the -KTS isoform is a transcriptional regulator with G-rich recognition sequence. The +KTS isoform is also likely to be present in prostate cancer cells but would contribute to gene regulation via splicing and posttranscriptional gene regulation (Larsson et al., 1995; Ladomery et al., 2003). Here we have identified potential target genes with well-described DNA binding sites recognized by the -KTS isoform and have not assessed the less well understood RNA binding sites recognized by the +KTS isoform (Ladomery et al., 2003).

The early growth response 1 gene (EGR1) is a homolog of WT1 (Liu et al., 1996). Although it has only three zinc-fingers, it shares some TFBS with WT1. EGR1 has been implicated as a cancer suppressor gene and activates genes required for differentiation. In human prostate cancer, EGR1 is overexpressed (Ogishima et al., 2005; Eid et al., 1998) and in a mouse model of prostate cancer, EGR1 regulates genes essential for progression of tumor growth (Abdulkadir et al., 2001). Since WT1 regulates the EGR1 promoter in vitro (Madden et al., 1991) it may indirectly regulate other EGR1 target genes, such as the N-myc downstream regulated gene 1 (NDRG1), a α/β hydrolase. In many cancer cell lines it has been shown to be up-regulated by both hypoxia and hormone treatment, suggesting that it could be linked to androgen induced differentiation and signaling in the prostate (Caruso et al., 2004; Ellen et al., 2007). Since EGR1 regulates NDRG1, WT1 could either directly or indirectly regulate NDRG1.
While analyzing the homologous sequences of the different gene promoters, numerous overlapping TFBS were found, suggesting competition for binding and differential regulation of these gene promoters. Several studies have shown that EGR1 and SP1 TFBS often overlap (Liu et al., 1996; Rong et al., 2006; Zhang et al., 2007a). When EGR1 binds to a site also bound by SP1, it displaces the SP1 "activator" from the binding site and represses transcription of these genes (Liu et al., 1996). For example, the promoter of NDRG1 was shown to be regulated by an overlapping EGR1/SP1 binding site (Zhang et al., 2007a) (located outside of the surveyed region of our study). It was shown that this evolutionary conserved site was vital in positively regulating expression of NDRG1. Similarly, our results showed evolutionary conserved overlapping EGR1/SP1 sites in several other gene promoters, including VEGF and PSA. In the latter, overlapping EGR1/SP1 sites were found to be conserved between human and two other primate species (chimpanzee and macaque).

Additionally, WT1 and EGR1 compete for binding at shared TFBS. WT1 recognizes and binds to EGR1 sites on the promoters of many different genes (Liu et al., 1996; Harrington et al., 1993; Dey et al., 1994; Minc et al., 1999; Wang et al., 1992). WT1 generally functions as a transcriptional repressor when bound to EGR1 TFBS in the transforming growth factor beta 1 (TGFβ1) and EGR1 promoters, while EGR1 functions as an activator (Madden et al., 1991; Dey et al., 1994). Many gene promoters with overlapping WT1, EGR1, and SP1 binding sites have been identified (reviewed in (Liu et al., 1996)). For example, three-way competition occurs between EGR1, SP1 and WT1 for binding and regulation of superoxide dismutase expression (Minc et al., 1999). However,
the mechanisms of gene regulation at overlapping sites, including TF competition, are not well understood.

Combinations of adjacent and overlapping EGR1, WT1 and SP1 TFBS conserved between multiple species were found in multiple gene promoters. Adjacent sites were found in the PSA promoter where an overlapping EGR1/SP1 site is 50 base pairs downstream of a WT1 site and in the VEGF promoter where an EGR1/SP1 overlapping site is 20 base pairs away from a WT1 site. Such sites can facilitate synergistic interactions or may be required for inducible expression, as described for AR and GATA2 interactions in the PSA promoter (Perez-Stable et al., 2000). Additionally, in the VEGF promoter an SP1 site adjacent to a non-canonical estrogen receptor (ER) TFBS contributes to hormone induction of VEGF expression (M et al., 2004). Similarly, WT1 appears to interact with ER at neighboring sites in the insulin like growth factor 1 receptor (IGF1R) promoter (Reizner et al., 2005). These complex arrangements of EGR1, WT1, and SP1 TFBS could facilitate cooperative or competitive binding by these TFs and would have pleiotropic effects on the regulation of these genes. Genes with evolutionary conserved overlapping TFBS could be part of a prostate epithelial cell transcriptome regulated by WT1.

PART II: ANDROGEN MEDIATED REGULATION OF VEGF

Androgens are known to regulate a multitude of genes, with the most well studied androgen regulated gene being PSA. There are three known dimeric ARE binding sites in the regulatory region of PSA, but regulation of PSA by androgens is mainly through an
ARE binding site located 4kb upstream of the transcription start site in the enhancer region (Cleutjens et al., 1997). Cleutjens et al. demonstrated that all three ARE binding sites were involved in androgen regulation and that although mutation of the two AREs in the proximal promoter of PSA significantly decreased activation by R1881, mutation of the ARE in the distal enhancer completely blocked androgen activation of PSA. Similar to the PSA promoter, three ARE binding sites were identified in the VEGF promoter and all three ARE binding sites were involved in the hormone regulation of VEGF by R1881. ChIP analysis demonstrated that these sites were bound by AR specifically in the presence of androgen. However, mutation of these sites did not totally diminish activation by R1881. In contrast to PSA, the ARE sites in the VEGF promoter are monomeric sites that contain only half of the canonical dimeric ARE sequence. Thus, binding at these sites may not be as strong as in the PSA promoter although other factors might enhance binding of AR causing increased VEGF expression.

Previous studies of the effect of androgen on VEGF determined that in both normal and malignant prostatic tissue, VEGF levels correlated with androgens (Joseph et al., 1997) and DHT increased VEGF mRNA levels in primary cultures of human prostate fetal fibroblasts (Levine et al., 1998). Here we show that VEGF mRNA is transcriptionally up-regulated by R1881 in both LNCaP and CWR22Rv1 prostate cancer cell lines. Our results are similar to those of Li (Li et al., 2005) who showed R1881 increased VEGF mRNA levels in LNCaP cell lines, but did not determine a mechanism. Conversely, anti-androgens decreased VEGF protein levels for six days following treatment with flutamide, another anti-androgen that competes with DHT for binding to
androgen receptors (Mabjeesh et al., 2003). Similarly we observed that treatment with the anti-androgen casodex suppressed androgen activation of VEGF two days after treatment. Thus, our studies revealed that VEGF was transcriptionally regulated by the androgen R1881 and led to our identification of novel ARE sites and cooperating ZFTF sites.

To determine how androgen was acting on the VEGF promoter, *in silico* analysis was performed using the transcription factor binding site tool MatInspector. Since there are no known classical dimeric ARE binding sites in the VEGF promoter, it was not expected that any would be identified within the region analyzed. However, surprisingly three non-classical monomeric ARE half-sites were predicted and AR binding was observed by ChIP at all three sites. These non-canonical ARE half-sites are extremely important to identify as up to 78% of potential AR regulatory regions identified by ChIP-chip technology contain these monomeric half-sites (Wang et al., 2007; Bolton et al., 2007; Massie et al., 2007). The identification of these sites and demonstration of binding at them are novel findings important to the characterization of the VEGF promoter.

Based on these findings, it was then hypothesized that the VEGF distal promoter construct, containing all three ARE half-sites, would be up-regulated by treatment with R1881. However, although mutation of these sites diminished hormone activation, it did not totally eliminate the up-regulation by androgen. This implies that although the three ARE sites contribute to the androgen mediated activation of the VEGF promoter, they are not required for hormone induction. Overall these results suggested that classical dimeric binding of AR to ARE(s) was not occurring and an alternative model of androgen regulation was hypothesized. Model ii in Figure 10 suggests that AR is bound to both
monomeric ARE half-sites and to other zinc finger transcription factors also bound to the VEGF promoter. This region is highly GC-rich and therefore we investigated the role of other zinc finger transcription factors known to bind GC-rich promoter regions, such as WT1 and Sp1. Our characterization of the VEGF promoter region resembles that of the NRIP (nuclear receptor interaction protein) promoter in that that mutation of two ARE binding sites decreased activation by androgen, but did not completely eliminate it (Chen et al., 2008). Sp1 binding sites were also examined for their involvement in the androgen mediated up-regulation of the NRIP promoter and it was found that AR and Sp1 associate on the NRIP promoter and cooperatively regulate NRIP promoter activity. Similarly, we hypothesized that WT1 and/or Sp1 worked together with AR to cooperatively activate VEGF promoter activity.

PART III: INTERACTIONS OF ANDROGEN AND ZFTFs IN THE REGULATION OF VEGF

Androgens have been known to act in concert with other zinc finger transcription factors to regulate androgen responsive genes (Perez-Stable et al., 2000; Lu et al., 2000; Chen et al., 2008). Adjacent WT1 and AR binding sites have been identified in the VEGF proximal promoter and immunoprecipitations of these factors were observed binding chromatin of hormone treated LNCaP cells in this region (Eisermann et al., 2009). Since both WT1 and androgen independently regulate VEGF, we hypothesized that WT1 would enhance hormone activation of VEGF in androgen responsive cell lines. Initially transfection of WT1 was tested in three androgen responsive cell lines and over-expression of WT1 significantly increased the VEGF proximal promoter construct. In
contrast, no effect was shown in the AR negative PC3 cell line (data not shown). A functional WT1 binding site in the VEGF proximal promoter (Eisermann et al., 2008; Hanson et al., 2007) was then mutated to determine its necessity for up-regulation of VEGF by WT1.

Once a WT1 binding site in the VEGF proximal promoter was identified and its activity characterized in prostate cancer cells (Eisermann et al., 2008; Eisermann et al., 2009), its role in hormone induction of VEGF was then tested. Over-expression of WT1 in hormone treated LNCaP cells did indeed enhance hormone up-regulation of the VEGF proximal promoter. Additionally, co-immunoprecipitation of AR and WT1 proteins confirmed that they form a complex, thus indicating that they interact within the nucleus of the cell. Taken together, WT1 and AR are acting as co-regulators of VEGF, increasing transcription by interacting at the proximal promoter. Although WT1 and AR interact and WT1 enhances androgen activation of VEGF, WT1 is not required for VEGF expression, as WT1 specific siRNA knockdown had no significant effect on VEGF mRNA levels. Since a number of different factors are involved in the regulation of VEGF, the fact that WT1 is sufficient but not necessary to up-regulate VEGF is not surprising. One possible interpretation of this finding is that hormone activation is so important that redundancy is built in, so that the hormone regulation of VEGF remains intact even after eliminating a specific factor.

Another ZFTF well known to regulate VEGF is Sp1 and Sp1 binding sites in the core promoter region are known to control VEGF transcriptional regulation in a number of different cell lines. Sp1 mediated regulation of VEGF involves the presence of specific
stimuli, such as stress (Schafer et al., 2003), estrogen (M et al., 2004), retinoic acid (Akiyama et al., 2002), TGF-β1 (Benckert et al., 2003), PDGF (Finkenzeller et al., 1997), etc. depending on the cell type. Previously, Sp1 sites have been shown to be involved in androgen induction of the p21 gene and co-IP demonstrated that AR interacts with Sp1 to regulate p21 expression (Lu et al., 2000). Thus we hypothesized that Sp1 may have a role in the androgen responsiveness of the VEGF promoter.

Since mutation of the three ARE sites in the distal and proximal VEGF promoter did not fully eliminate the hormone response of the VEGF promoter, this suggested that the remaining core promoter might be involved in androgen mediated regulation, despite the lack of ARE sites. Many Sp1 binding sites were predicted by MatInspector in the VEGF promoter, but the core promoter sequence contained four Sp1 sites, some previously described to regulate VEGF in other systems. Surprisingly, we discovered that androgen did in fact up-regulate the VEGF core promoter region. Since no ARE binding sites are in this promoter region, this suggested that other cis-regulatory elements such as Sp1, EGR1/WT1 binding sites maybe contributing to androgen mediated up-regulation of VEGF expression.

Three different Sp1 binding sites predicted by MatInspector were mutated to determine if they were involved in the androgen activation of this region. While mutating two of these sites in a single construct did not have a significant effect on hormone regulation, mutation of a single Sp1 binding site, Sp1.4, significantly diminished the up-regulation by androgen in the core promoter. Previously electrophoretic mobility shift assays had demonstrated that purified Sp1 protein bound an oligonucleotide sequence
containing this site (Hanson, 2006). This novel finding is the first report of an Sp1 binding site necessary for androgen up-regulation of the VEGF core promoter in prostate cancer cells. Also, co-immunoprecipitation of Sp1 with AR revealed that these two proteins form a nuclear complex and likely bind together at the VEGF core promoter. These results further strengthen the case for Sp1 involvement in the hormone activation of VEGF.

The VEGF promoter is similar to the NRIP promoter (Chen et al., 2008) described earlier, they are both TATA-less GC rich promoters that are induced by androgen in prostate cancer cells. The NRIP promoter also contains three Sp1 sites and two hormone responsive elements (ARE and GRE). Additionally, mutation of these sites did not eliminate hormone response, they demonstrated that Sp1 and AR cooperatively interact by several methods including sequential chromatin immunoprecipitation (or re-ChIP) and co-IP. Overall a comparison of our findings and those of others demonstrate the complexity of hormone activation of VEGF and the importance of protein interaction, especially Sp1 and AR, in its regulation.
OVERALL CONCLUSIONS

PART I

Genes coordinately expressed in prostate cancer epithelial cells have conserved regulatory elements and an abundance of overlapping zinc finger TFBS. Potential WT1 gene targets were identified based on TFBS sequence conservation, and the significance of the WT1 TFBS in the PSA promoter was verified in vivo by ChIP assays. Similarly, a transcriptionally active WT1 binding site in the VEGF promoter was confirmed by ChIP and found to be in a region conserved amongst primates. Thus, these genes could be part of a novel network of regulatory pathways initiated by WT1 and have important implications in the progression of prostate cancer.

PART II

Androgen is necessary for VEGF activation as the anti-androgen casodex inhibits androgen actions. MatInspector predicted three non-classical ARE monomeric half-sites in the VEGF promoter. Androgen increased AR binding to predicted ARE binding sites in the VEGF promoter ~2-6x. Mutation of the three predicted ARE sites in the VEGF promoter reduced, but did not eliminate hormone response. Thus, these ARE sites appear to be redundant. Since there were no predicted ARE dimeric binding sites in the VEGF promoter other factors must be involved in facilitating the hormone activation observed.
PART III

WT1 over-expression increased VEGF mRNA. WT1 binds and up-regulates the VEGF proximal promoter in androgen responsive PrCa cell lines. AR and WT1 both bind chromatin of LNCaP cells and functionally interact to increase VEGF transcription. WT1 is sufficient to up-regulate VEGF, but is not required for VEGF expression. AR and Sp1 co-immunoprecipitate together forming a complex that may bind to the VEGF promoter and activate transcription. The Sp1.4 binding site is necessary for androgen activation of the VEGF core promoter.

SIGNIFICANCE (IMPACT OF WORK)

Regulation of VEGF and, thereby, angiogenesis can be affected by ZFTFs and their interactions may therefore play a positive role in promoting prostate cancer progression. The elevated expression of these ZFTFs may indicate a worse prognosis. Therapy targeting disruption of AR-ZFTF complexes might help maintain the indolent form of prostate cancer by dampening VEGF and angiogenesis and, thereby, extend lives.
FUTURE DIRECTIONS

- Determine if AR and SP1 bind chromatin of hormone treated cells at the VEGF core promoter

- Determine if Sp1 siRNA decreases VEGF expression

- Determine if Mithramycin A, which prevents Sp1 from binding GC rich promoter regions, decreases VEGF transcription
APPENDIX A

ABBREVIATIONS

AD- androgen-dependent
AI- androgen independent
AP2- Transcription factor AP-2
AR- androgen receptor
ARE- androgen response element
AREG- Amphiregulin
BCA- Bicinchoninic acid
BPH- benign prostatic hyperplasia
CBP- creb binding protein
ChIP- chromatin immunoprecipitation
CMV- cytomegalovirus
CRPC- castrate resistant prostate cancer
DBD- DNA-binding domain
DHT- 5α-dihydrotestosterone
ECAD- E-cadherin
EGR1- Early growth response 1
FGFR3- Fibroblast growth factor receptor 3
GATA1- GATA binding protein 1
GATA2- GATA binding protein 2
HAT- histone acetyltranferase
HSP- heat shock proteins
IGFBP2- insulin-like growth factor binding protein 2
IGF1R- insulin-like growth factor 1 receptor
KLK3- prostate specific antigen (PSA)
LBD- ligand binding domain
MAPK- mitogen-activated protein kinase
NDRG1- N-myc downstream regulated gene 1
NRIP- nuclear receptor interaction protein
NPY- neuropeptide Y
NTD- N-terminal domain
PI3K- phosphatidylinositol 3-kinase
PrCa- prostate cancer
PSA- prostate specific antigen
PVDF- Polyvinylidene Fluoride
SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOX4- SRY (sex determining region Y)-box-4
SOX9- SRY (sex determining region Y)-box-9
SP1- Specificity protein 1
SP2- Specificity protein 2
SRC-1- steroid receptor coactivator-1
TBP- TATA binding protein
TF- transcription factor
TFAP2C- transcription factor AP-2 gamma
TFBS- transcription factor binding site
TGFβ1- transforming growth factor beta 1
2-ME- 2-mercaptoethanol
VEGF- Vascular Endothelial Growth Factor
WT1- Wilms tumor 1
ZFTF- zinc finger transcription factors
REFERENCES


sequences. Proc. by identification, subclassification, and functional assessment of multispecies conserved


Liu, C., Calogero, A., Ragona, G., Adamson, E. and Mercola, D. (1996). EGR-1, the reluctant suppression factor: EGR-1 is known to function in the regulation of growth, differentiation, and also has significant tumor suppressor activity and a mechanism involving the induction of TGF-beta1 is postulated to account for this suppressor activity. Crit Rev Oncog. 7, 101-25.


Shaheen, R.M., Davis, D.W., Liu, W., Zebrowski, B.K., Wilson, M.R., Bucana, C.D., McConkey, D.J., McMahon, G. and Ellis, L.M. (1999). Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the
growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. Cancer Res. 21, 5412-6.


R) gene by the tumor suppressor WT1 involves binding to sequences both upstream and downstream of the IGF-I-R gene transcription start site. J. Biol. Chem. 17, 12577-12582.


