

Chapter 14

Functional Role of WT1 in Prostate Cancer

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Abstract

Although initial discoveries of Wilms tumor 1 (WT1) expression in extrarenal disease generated controversy, we and others have examined WT1 expression in non-Wilms cancers and have demonstrated that the WT1(A) isoform, lacking the lysine-threonine-serine tripeptide (KTS) insertion, transcriptionally regulates the expression of growth control genes in other cancer types. Here, we review our evidence that WT1 is expressed in prostate cancer (PC) epithelial cells and regulates PC critical genes. That WT1 may promote metastatic disease is consistent with previous findings that WT1 suppressed E-cadherin and enhanced motility of PC cells with low migratory and metastatic potential. Recent findings led us to ask

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whether WT1 acts as an angiogenic switch in PC. Although vascular endothelial growth factor (VEGF) is regulated at several levels and by a number of different factors, a mechanistic understanding of WT1-mediated transcriptional regulation in PC cells was previously lacking. Here, we discuss the evidence of WT1- and androgen receptor (AR)-binding sites in the VEGF promoter and show the potential for cooperation between hormone and WT1. These findings revealed that in AR-intact PC cells, WT1 was sufficient to upregulate VEGF transcription, and WT1 expression enhanced the hormone activation of VEGF expression. This notion that WT1 can activate an angiogenic switch in PC cells, to enhance tumor growth and progression to metastatic disease, is consistent with our understanding of the oncogenic nature of WT1 overexpression in inappropriate tissues or at inappropriate times. The potential for WT1 to promote both tumor angiogenesis and PC cell migration suggests that WT1 regulates genes that promote PC progression to lethal metastatic disease. Therapies targeting WT1 in PC may reduce metastatic spread and increase overall survival.

Key words: AR; E-cadherin; Prostate cancer; Transcription; VEGF; WT1

Introduction

The WT1 gene is a member of the early growth response gene I (EGR-1) family of transcription factors containing four Kruppel-like zinc fingers in the carboxyl terminus that bind nucleic acids (both DNA and RNA). The functions of the Wilms tumor 1 (WT1) protein are isoform-specific and reflect its structural domains (1). The four major isoforms of WT1 are formed by alternative splicing at two sites resulting in the inclusion or exclusion of (1) exon V and/or (2) a lysine-threonine-serine tripeptide (KTS) in exon 9 that alters the relative orientation of the 3rd and 4th zinc fingers and affects the DNA-binding structure. The isoform WT1(A), which lack both exon V and the KTS tripeptide, binds DNA and functions as a transcription factor, while isoform WT1(D) contains both elements and can function as a post-transcriptional regulator in certain contexts. Additional, less common isoforms initiate from internal or upstream start sites. Three of the four Cys₂-His₂ zinc fingers (2, 3) of the (-)KTS isoforms are involved in binding a common G-rich DNA consensus sequence, GNGNGGGNG, as well as the related Egr-1 recognition elements (4). The importance of the zinc finger domain for DNA binding is underscored by congenital syndromes associated with naturally occurring WT1 mutations, such as the Denys-Drash syndrome and Frasier syndrome, characterized by urogenital anomalies and elevated risk of Wilms tumor or gonadoblastoma, respectively (5, 6). While controversy exists over the ability of mutant forms of WT1 to bind DNA, it is possible that protein interaction sites remaining within the mutant WT1 protein could play a direct role in these anomalies (7). Indeed, controversy exists over the role of normal cytoplasmic WT1 protein, with some evidence supporting a shuttle function, as WT1 contains cytoplasmic and nuclear localization signals, as well as a nuclear export signal (8). The activity of

the cytoplasmic form may be related to phosphorylation status, as phospho-WT1 is thought to be retained in the cytoplasm (9, 10). Alternatively, as both +/- KTS isoforms have been identified in polysomes and bound to polyA RNPs (11), a post-transcriptional function has been suggested. Interestingly, one example of post-transcriptional regulation of vascular endothelial growth factor (VEGF) by WT1 involved transcriptional regulation of a splicing factor kinase that, in turn, altered VEGF splicing in podocytes (12). Recent evidence indicating the association of WT1 protein with histone and chromatin modifying enzymes also suggests an epigenetic function for WT1 [reviewed in reference (1)], mediated, in part, by WT1 recruitment of DNA methyltransferase DNMT1 and polycomb group protein enhancer of zeste homolog 2 (EZH2) (13) and CREB-binding protein (CBP), a histone acetyltransferase (14). Additionally, the evidence of epigenetic regulation of WT1 expression by lncRNA in acute leukemia (15) suggests that WT1 is intimately involved in both direct transcriptional and indirect epigenetic regulation. Thus, study of WT1 as a regulator of gene expression in key developmental processes, such as hematopoiesis, continues to be relevant.

Developmental expression of WT1

WT1 expression in the developmental processes was initially viewed as growth suppressive and necessary for cell differentiation, consistent with its earliest descriptions as a tumor suppressor gene (TSG). Within the developing kidney and genitourinary system, the timing of WT1 expression is exquisitely controlled, and once kidney development occurs, WT1 expression is tightly restricted to podocytes (16). WT1 is an essential regulator of nephrogenesis (17–19) and is expressed in both normal podocytes and in some Wilms tumors (18, 20). In addition to the kidney, WT1 is normally expressed in many other organs (6), including hematopoietic tissues such as the spleen, fetal liver, bone marrow, and lymph nodes, gonads, and peripheral nervous system (3, 21–25). However, its role is ambiguous depending on the organ involved and whether epithelial or mesenchymal differentiation occurs. For instance, in the normal development of the kidneys and the urogenital system, WT1 is needed to induce mesenchymal-epithelial transition (MET) leading to the formation of nephrons (26) and kidneys (16). In MET, the mesenchymal cells undergo multiple morphological changes associated with differentiation into epithelial cells and condensation into structures forming the organ. WT1 expression accompanies the opposite developmental role, epithelial to mesenchymal transition (EMT), in the developing heart where epithelial cells transform into motile mesenchymal cells that contribute to the organ's cellular structure and generate important signals (27). Furthermore, it has been demonstrated that WT1 is required for cardiovascular progenitor cell formation through the upregulation of Snail and downregulation of E-cadherin, two of the major molecules involved in EMT (28). Although WT1 has been proposed to regulate EMT by repressing E-cadherin; more recently, WT1 has been linked to the regulation of epicardial EMT through the β -catenin and retinoic acid signaling pathways (29). Interestingly, it has

been found that WT1 transcriptionally activates Snail with partial maintenance of E-cadherin, and WT1 is associated with epithelial characteristics in kidney cells and in clear cell renal cell carcinoma (31). Thus, in these examples, WT1 induces an epithelial-mesenchymal hybrid transition defined by Snail upregulation with E-cadherin maintenance, a tumor cell differentiation state in which cancer cells retain both mesenchymal and epithelial features that may contribute to tumor cell plasticity and tumor progression (30). Similarly in prostate cancer (PC), a partial EMT with features of both epithelial and mesenchymal cells has been observed (31). The transformation of metanephric mesenchyme to epithelial cells within the condensing glomeruli also is similar to the metastatic process of cancer cells, whereby motile cancer cells, after extravasation, must revert back to their epithelial state to survive at the metastatic site (32). Because WT1 is required for normal MET within the developing kidney, it seems plausible that it may also play a role in the metastatic MET process. Yet, little is known about the requirement for WT1 expression during the metastatic process.

WT1 expression in non-Wilms cancer

CD34+ hematopoietic progenitor cells express WT1, but like metanephric mesenchyme, once hematopoietic progenitors become lineage-committed then expression of WT1 is highly restricted within a small subpopulation of cells [reviewed in reference (33)]. Increased expression appears to persist in cancer cells, and WT1 expression in tumor tissue exceeds that of the normal cell counterparts. This dysregulated expression was regarded as an indicator of a potential growth-promoting effect of WT1 and led to the controversy over whether WT1 was truly a TSG as originally identified in Wilms tumor or whether WT1 was actually an oncogene-driving cancer cell proliferation and blocking differentiation, as observed in leukemia cell lines (25). As evidence accumulates on different tumor types that overexpress WT1 relative to their normal counterparts, it is clear that WT1 has a dichotomous role in cancer, and indeed, WT1 has been referred to as a chameleon [reviewed in references (33) and (25)]. Within the hematopoietic system, it is clear that WT1 can behave as a survival gene, enhancing cell viability, but also can induce quiescence, depending on the differentiation state of the leukemia cells involved [reviewed in reference (33)]. Many studies have shown elevated *WT1* expression in diverse cancer types, including leukemia (34–37), breast (38–40), Ewing sarcoma (41), ovarian (42), mesothelioma, and pulmonary adenocarcinomas (43). Additionally, *WT1* is being investigated as a potential prognostic marker for both leukemia and breast cancer (39, 44).

Expression and potential role of WT1 in PC

WT1 is expressed mainly during development, and it plays an important role in adreno-gonadal development and sex determination [reviewed in reference (45)] via its regulation of SRY (46), so its expression in hormone-responsive tumors such as breast, ovarian, and

prostate was not unexpected. We and others initially identified WT1 mRNA in cultured PC cells (47–51) and then WT1 mRNA and protein in PC tissues (49). Because the prostate is a complex tissue and PC is a heterogeneous disease, we used laser capture microdissection (LCM) to isolate distinct cell-type populations from epithelial and stromal tissues in PC and identified *WT1* among the nearly 500 genes whose expression was significantly different between epithelial and stromal PC cells (49). Results of microarray analysis are posted at NCBI (Geo #GSE 20758). This differential expression of *WT1* in PC epithelial cells was validated by quantitative real-time polymerase chain reaction (PCR) and relevance confirmed by analysis of additional frozen tumor tissue biopsies and tissue microarray (TMA) sections (49). This cell-specific expression suggests a potential role for *WT1* in PC, likely involving the acquisition of characteristics necessary for metastatic growth of PC.

Metastatic disease is associated with a marked increase in the risk of mortality among PC patients. Ninety-nine percent of patients who develop primary PC are expected to live at least 5 years after diagnosis (52). Ninety-eight percent are alive after 10 years, and 94% live for at least 15 years if the disease remains localized. By contrast, patients with metastatic disease at diagnosis have a 5-year survival rate of only 28% (52). The process of metastasis requires that cancer cells acquire characteristics of enhanced motility and invasiveness. That *WT1* may be involved in PC metastasis was suggested by immunohistochemical analysis of PC TMAs, demonstrating that *WT1* protein was more often expressed in high Gleason grade PC epithelial cells than that in low grade, and it was not observed in non-neoplastic prostate tissue (49). Others have also suggested that *WT1* could serve as a marker for PC progression (53). While Devillard et al. (53) demonstrated the expression of *WT1* by microarray analysis in a hormone-refractory LuCaP xenograft PC progression model, our results provide the most complete evidence of elevated *WT1* mRNA and protein in prostate tumors, and our study was the first to identify *WT1* expression in LCM human prostate epithelial tissue (49). We confirmed the relevance of the microarray analysis of LCM-captured tissue RNA by real-time PCR quantifying *WT1* expression in 20 additional sets of paired tumor and non-neoplastic tissues. *WT1* mRNA levels were elevated in 70% of invasive-stage T3 tumors examined when compared to the adjacent non-neoplastic tissue. Similarly, in three of four established PC cell lines, *WT1* expression was also significantly higher than the nontumorigenic, immortalized prostate epithelial cell line RWPE-1 (49). Further analysis of *WT1* protein in formalin-fixed, paraffin-embedded TMAs identified *WT1* expression in 65% of tumor samples (of Gleason grade 6–10) and, importantly, the absence of expression in non-neoplastic and benign prostatic hyperplasia (BPH) samples. *WT1* expression in high-grade PC may indicate that *WT1*-responsive pathways promote the slow progression of latent PC to aggressive, hormone-refractory PC. Two possible mechanisms whereby *WT1* expression in prostate could enhance metastatic tumor growth warrant discussion.

WT1 target genes relevant in PC

The transcriptionally active isoform of the Wilms tumor gene, WT1(A), regulates a large family of genes involved in growth control, sex determination, and genitourinary development [for reviews see references (6, 16, 54)]. We and others have demonstrated that WT1 regulates important PC pathways – both growth-promoting pathways, e.g., insulin-like growth factor axis (55, 56) and androgen signaling via androgen receptor (AR) (46, 50), and growth suppressing/apoptotic pathways via Bcl-2 (57–61). Recently, WT1 has been shown to control differentiation of epicardial cells by repressing E-cadherin expression, thereby inducing mesenchymal transformation (EMT) resulting in vascular endothelial cells, smooth muscle cells, and cardiomyocytes in the heart (28). WT1 could similarly facilitate the metastatic progression of PC cells by inducing EMT, which is marked by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as N-cadherin. WT1 could also enhance metastatic tumor growth by inducing expression of the angiogenic regulatory gene, VEGF. Together, these gene regulatory functions could promote acquisition of the lethal metastatic phenotype of PC.

WT1 suppression of E-cadherin promotes cell motility

Initial studies in NIH-3T3 cells, in which it was demonstrated that E-cadherin is a WT1 target gene (62), and studies in cardiac epithelial cells have established the role of WT1 in E-cadherin regulation (28). E-cadherin is a transmembrane protein that mediates epithelial cell–cell interactions in the adherent junctions of the plasma membrane (63) through homophilic protein–protein interactions (64). Downregulation of E-cadherin results in increased invasiveness of distinct types of cancer, such as gastric (65, 66), breast (67), ovary (68, 69), endometrial (70), thyroid (71), hepatocellular carcinoma (72), oral (73), and pancreatic (74), and has been well documented in prostate adenocarcinoma (75–77). In PC, E-cadherin expression has been shown to be reduced by activation of AKT signaling (78), by high expression of transcription factors such as Snail (79, 80), Slug (81), Twist (82) and WT1 (48), and by hypermethylation of the E-cadherin promoter (83). The loss of this important cell adhesion molecule is a critical early event in invasion and metastasis that leads to the conversion from a stationary to a migratory cell phenotype (84). When cancer cells acquire motility and invasiveness, they exhibit marked morphological changes, lose epithelial features, and acquire a more mesenchymal phenotype (EMT) (85, 86). Interestingly, androgen exposure has been reported to increase levels of Snail, decrease levels of E-cadherin and β -catenin, and induce expression of the mesenchymal marker N-cadherin in PC cells (87). TGF- β also has been implicated in induction of EMT in PC through activation of SMAD3 (88) and promotion of PARP4 nuclear localization with the subsequent increase of Snail, vimentin and N-cadherin, and decrease of E-cadherin (89).

While initial experiments associated growth suppression and characteristics of epithelial differentiation, including upregulation of E-cadherin, with stable expression of WT1 in NIH 3T3 cells

(62), more recent studies in cardiac epithelial cells showed that WT1 transcriptionally repressed E-cadherin expression both directly and indirectly by the upregulation of Snail (28). Furthermore, it has been demonstrated that WT1 expression promotes metastasis and invasion in non-small-cell lung carcinoma patients through the suppression of E-cadherin (90). In the context of PC, we observed that WT1 expression was inversely related to E-cadherin expression in several PC cell lines, and, importantly, WT1 expression correlated with migratory potential (48). Mechanistic studies showed that WT1 could bind to the E-cadherin promoter *in vivo* and decrease the E-cadherin promoter activity through a novel-binding site located at -146 bp upstream from the transcription start site. Additionally, overexpression of WT1 in LNCaP cells decreased E-cadherin mRNA expression (2-fold, $p \leq 0.05$). Although LNCaP cells have low migratory potential as measured in migration chamber assays, forced expression of WT1 not only suppressed E-cadherin but also enhanced LNCaP cell migration 3-fold compared to control vector-transfected cells ($p \leq 0.001$). Moreover, silencing WT1 in PC3 cells, which exhibit higher WT1 expression and greater migratory potential, reduced their motility in migration chambers by 50% compared to scrambled control-transfected cells ($p \leq 0.01$). This strong inhibition of motility was confirmed in wound-healing assays showing a 4.4 \times reduction in the motility of siWT1 RNA-transfected PC3 cells compared to controls ($p \leq 0.001$) (48). Our study, the first to undertake a complete analysis of the effect of WT1 on E-cadherin expression and motility in PC cells, thus demonstrated that WT1 binding decreased activity of the E-cadherin promoter in the presence of WT1 and that repression of E-cadherin expression led to an increase in cell migration (Figure 1). Suppression of E-cadherin expression and enhancement of motility are both associated with EMT.

WT1 may contribute to tumor angiogenesis via regulation of VEGF

We have demonstrated that, in addition to enhancing PC migration by suppressing E-cadherin expression, WT1 also upregulates VEGF, thereby potentially promoting tumor angiogenesis and metastasis. VEGF is a mitogen secreted by tumor cells that is essential for tumor angiogenesis and is necessary for tumor growth beyond 1–3 mm³ in volume (91). VEGF regulation is complex and occurs at both transcriptional and post-transcriptional levels (92, 93). While the VEGF promoter lacks a TATA-binding site, it contains a GC-rich core promoter region and additional distal enhancer sites including hypoxia response elements that bind hypoxia-inducible factor (HIF1)-alpha.

Coexpression of WT1 and VEGF

WT1 was previously shown to play a role in neovascularization in the proliferative response of coronary vasculature to regional ischemia (94). In vascular cells, WT1 expression was associated with an increase in proangiogenic molecules such as VEGF (95). Similarly, both VEGF and WT1 are elevated in some PC cells (96), consistent with its ability to regulate growth control pathways important in PC (46, 50, 55–61). Additionally, WT1

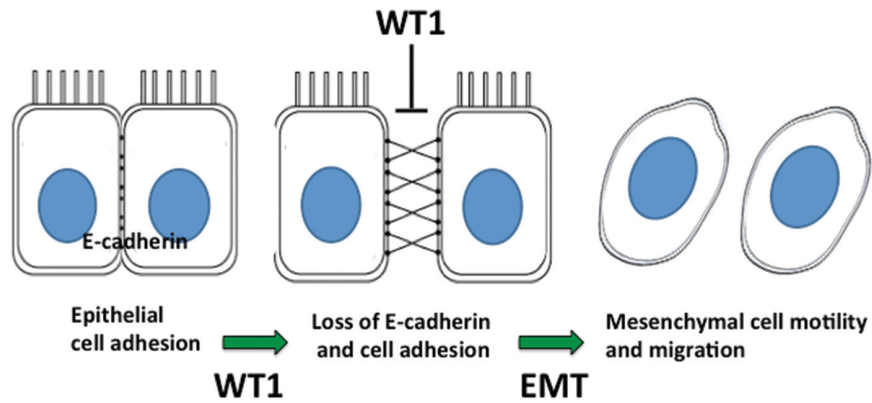


Figure 1. Proposed mechanism for WT1 regulation of motility of prostate cancer cells. WT1 transcriptionally represses E-cadherin, which would lead to loss of cell adhesion and promote prostate cancer cell migration, potentially enhancing the epithelial to mesenchymal transition (EMT) of PC cells expressing WT1.

and VEGF are coexpressed in both normal podocytes and some Wilms tumors (18, 20, 97). These findings of coordinate expression led to suggestions that WT1 plays an important regulatory role in developmental and tumor angiogenesis (20, 51, 98). For all these reasons, it seemed likely that VEGF was a physiologically relevant target of WT1 regulation in the prostate. In Ewing sarcoma cell lines, knockdown of WT1 expression using WT1-specific shRNA downregulated VEGF mRNA expression and decreased angiogenic activity (99). Conversely, overexpression of WT1 upregulated VEGF mRNA and increased angiogenic activity (99). Additionally, WT1 bound to the promoter of VEGF and increased promoter activity in response to hypoxia in Ewing sarcoma cells (100). Together, these results demonstrated that WT1 could directly regulate VEGF expression in Ewing sarcoma cells.

Regulation of VEGF by WT1 in prostate cancer

We assessed the WT1-mediated regulation of VEGF in PC cells. WT1-binding sites predicted by *in silico* analysis of the VEGF proximal promoter (101, 102) were demonstrated functional by reporter assays and protein binding *in vitro* and *in vivo* using electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assays, respectively. The latter result indicated the ability of WT1 protein to bind to native chromatin in LNCaP PC cells (101) and is consistent with results of luciferase reporter assays, showing that WT1 upregulates the VEGF promoter (102) in LNCaP cells. One of the functional binding sites identified initially as an Egr1-binding site was verified to bind WT1 by ChIP analysis in LNCaP cells (101). Site-directed mutagenesis of the proximal VEGF promoter construct V411 (Figure 2A)

was used to determine if this site was necessary for WT1-mediated transcriptional activation of the VEGF promoter. Cotransfection of a green fluorescent protein (GFP)-tagged WT1 expression construct (103) and mutant reporter into LNCaP cells revealed that disruption of this site significantly decreased the ability of WT1 to upregulate the proximal VEGF promoter (Figure 2B). We then asked whether this same pattern of regulation was occurring in other hormone-responsive PC cell lines. Testing two other hormone-responsive PC cell lines, CWR22Rv1 and C4-2, we found that WT1 regulated the VEGF proximal promoter (Figure 2C and 2D) similarly in all three cell lines. Thus, the data showed that WT1 bound and activated the VEGF proximal promoter in several PC cell lines.

The enhanced expression of VEGF mRNA in WT1-transfected LNCaP cells confirmed the *in vitro* promoter activation studies. Although overexpression of WT1 increased VEGF mRNA levels, the converse was not true (data not shown). Knockdown of WT1 expression in LNCaP cells using siRNA did not significantly affect VEGF mRNA levels. Together, these results indicate that WT1 is sufficient to upregulate VEGF expression, but not necessary, suggesting that other transcription factors (possibly SP1) play a role in the androgen activation of VEGF (104). Additionally, WT1-mediated regulation of VEGF appears to be cell specific as transfection of hormone-insensitive PC3 cells did not enhance VEGF promoter activity, and WT1 appears to repress the VEGF promoter in embryonic kidney HEK293 cells (102).

Combined androgen and WT1 activation of VEGF expression in hormone-responsive PC

Although hormone responsive (105–108), the VEGF promoter lacks canonical AR or estrogen receptor (ER)-binding sites. VEGF regulation by estrogen in endometrial and breast cancer cells involves interactions of ER- α and Sp1 (or Sp3) with GC boxes in the core promoter region of VEGF (–66 to –47 bases from start site) (108, 109). VEGF mRNA levels were significantly induced in ZR-75 breast cancer cells treated with estradiol, and the intact GC-rich core VEGF promoter region (–66 to –47) was required for such activation. The relevance of Sp1 and Sp3 in estradiol regulation of VEGF in breast cancer was suggested by binding assays *in vitro* (by EMSA) and *in vivo* (by CHIP). Similarly, multiple groups have shown that androgen treatment of human fetal fibroblasts and LNCaP cells significantly increases VEGF mRNA expression levels (110–112, 102). Additionally, VEGF protein levels have been demonstrated to be upregulated after the treatment of LNCaP cells with hormone (106), and the androgen antagonist flutamide blocked this upregulation (113). The mechanism of androgen-mediated regulation of VEGF expression, however, is less well understood.

In examining the mechanism of androgen-mediated regulation of VEGF expression, we identified AR/GC sites within the VEGF GC-rich core. Based on our earlier *in silico* analyses of the VEGF promoter (101) and the discovery that site-directed mutation of three AR half-sites did not eliminate hormone activation of the VEGF promoter (104), we hypothesized that

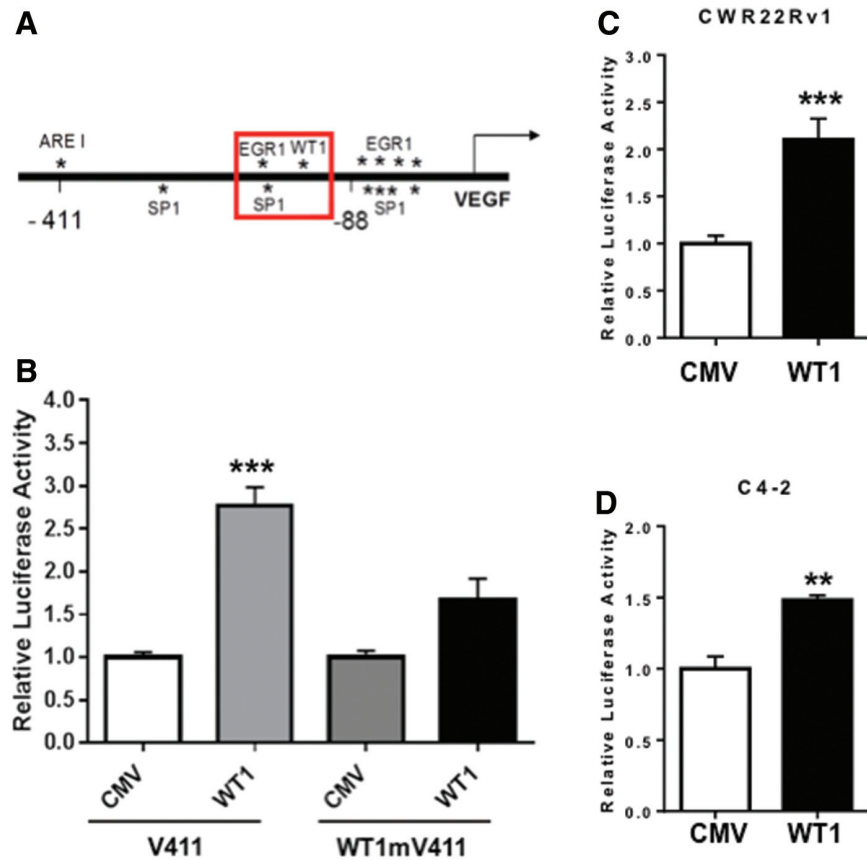


Figure 2. WT1 activates the VEGF promoter in prostate cancer cells via a WT1-binding site within the proximal VEGF promoter. (A) Site-directed mutagenesis of a predicted WT1-binding site (red box) was performed on the VEGF proximal promoter construct V411. (B) LNCaP cells were cotransfected with GFP-WT1 or the empty CMV expression construct, pcDNA3.1, along with 250 ng of either the wild-type V411(left) or the mutant (right) reporter constructs. (C) Cotransfection of CWR22Rv1 and (D) C42 prostate cancer cells with V411 and CMV or WT1 expression construct as described above. Luciferase activity was normalized to protein concentration, and values represent average normalized luciferase activity (+SEM) relative to empty vector control. Experiments were repeated three times in triplicate, and statistical significance was determined by Student’s t-test (**p< 0.01, ***p< 0.001).

WT1 might regulate the hormone-responsive VEGF promoter. Thus, we asked whether AR might bind at other sites via interaction with other zinc finger transcription factors (ZTFs), such as SP1, EGR-1, or WT1. We hypothesized that if AR-ZTF interactions were important mediators of androgen response, then cognate-binding sites should be located within the

promoter regions of hormone-responsive genes expressed in PC (101, 114). As expected, nonclassical AR half-sites were identified adjacent to WT1/EGR1/Sp1 sites in 8 of 11 promoters analyzed including VEGF (114). Binding at one of the three predicted nonclassical androgen receptor element half-sites (ARE-I) in the VEGF promoter region was tested by ChIP analysis of hormone-treated, WT1-transfected LNCaP cells (114). Endogenous AR and Sp1 proteins, along with exogenous WT1, were immunoprecipitated from native chromatin of these hormone-treated cells, indicating that the predicted WT1, Sp1, and AR sites in the VEGF proximal promoter region were functional and suggesting that the three factors may bind individually or as a complex. Based on these *in silico* predictions, we proposed three alternative models for AR-mediated regulation of VEGF promoter activity. The models differ primarily in the manner that AR binds the VEGF promoter (Figure 3). The first model proposes that AR binds to AREs as a dimer (Figure 3, model i), in the classical way that AR binds to many androgen-responsive genes, such as prostate-specific antigen (PSA). However, there have also been reports that noncanonical monomeric ARE half-sites are important (115–117). Thus, the second proposed model (Figure 3, model ii) shows AR monomers binding to an ARE half-site and bridged to WT1 (or other ZFTF, such as Sp1 or Egr1) binding sites by cofactors (marked as?), such as CBP or SRC-1; alternatively, AR dimers may bind to half-site ARE and bridge to WT1-binding site. Because AR is known to interact with Sp1, Egr1, and potentially WT1, the third and final model (Figure 3, model iii) proposes that AR is not bound to an ARE-binding site but is tethered via a ZFTF, which is bound to the G-rich VEGF promoter at either Egr1-/WT1-binding sites or GC boxes (SP1-/Sp3-binding sites).

To test the model for WT1 AR interaction, we examined the WT1 site within 200 bp of the ARE site to determine whether WT1 would modulate the hormone response of the proximal VEGF promoter. Cells were serum-starved to deplete androgens, cotransfected with the VEGF proximal promoter and either WT1 or empty vector control, then treated with 5 nM R1881, an androgen analog, or vehicle control dimethyl sulfoxide (DMSO) (Figure 4A). Luciferase assays confirmed that either hormone or WT1 alone increased VEGF transcription 3- to 4-fold compared to cytomegalovirus (CMV) empty vector, vehicle control. However, the combination of WT1 and 5nM R1881 activated this reporter construct more than 12-fold (Figure 4A), suggesting that their interaction strongly enhanced hormone response. This strong upregulation suggested that WT1 and AR may form a complex in the nucleus and bind the G-rich and the AR half-site (similar to Figure 3, model ii). Nuclear lysates from WT1-transfected LNCaP cells grown in full serum (containing endogenous hormone) were co-immunoprecipitated with WT1 and AR antibodies. Immunoblot analysis revealed that complexes precipitated by antibodies specific for WT1 also contained AR protein (Figure 4B). Conversely, AR-immunoprecipitated complexes contained low levels of WT1 protein (data not shown). Together, these results indicate that WT1 may interact with AR to enhance androgen induction VEGF expression in PC cells.

Surprisingly, the GC-rich VEGF core promoter (-88 to +51), which lacks AR half-sites, but contains multiple EGR-1/WT1/Sp1 overlapping sites, also demonstrated a hormone response. Consequently, the third model we tested (Figure 3, model iii) proposes that AR is not bound to an ARE but is tethered via SP1, which is bound to GC boxes in the VEGF core promoter. Because estrogen regulation of the VEGF core promoter has been shown to require Sp1 sites in breast cancer cells (109), we asked whether androgen might regulate VEGF in a similar fashion in PC cells. Sp1-associated binding of AR to novel-binding sites in the VEGF promoter was demonstrated *in vivo* by ChIP analysis in LNCaP cells (101, 104, 114). AR and Sp1 formed a nuclear complex and were shown to bind to the VEGF core promoter in hormone-treated CWR22Rv1 PC cells (104). Suppression of Sp1 binding in the VEGF core promoter by mutation of a specific Sp1-binding site abrogated VEGF promoter activation by androgen. Additionally, treatment with mithramycin A, which blocks access of proteins to GC-rich DNA, significantly reduced Sp1 binding and VEGF expression. Together, these results indicated that another mechanism of androgen-mediated induction of VEGF expression in PC cells involved interaction of AR with a specific, critical Sp1-binding site in the VEGF core promoter region (104) similar to that described here for WT1 interaction at the proximal promoter region. Overall hormone activation of the VEGF promoter region is enhanced by interaction of AR with transcription factor-binding partners in PC cells.

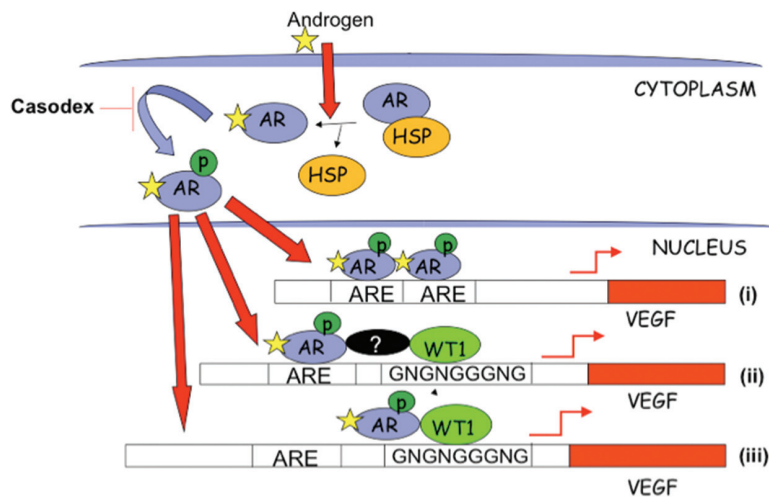


Figure 3. Proposed models of androgen regulation of VEGF in prostate cancer. Three potential ways that androgen is proposed to bind AR and regulate VEGF in prostate cancer: (i) AR binding to androgen response elements (AREs) as a dimer, (ii) monomeric AR binding to half-site ARE and bridged by unknown factor (?) to WT1 at its binding site, or (iii) AR tethering to WT1 at WT1-binding site, but not bound to ARE.

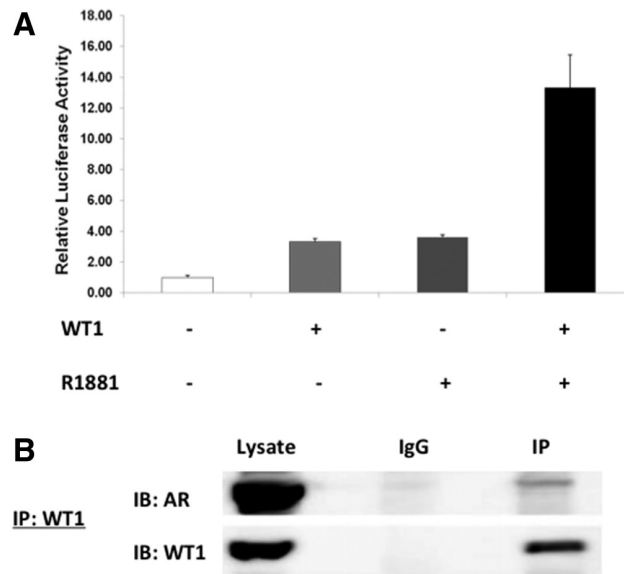


Figure 4. (A) WT1 and AR interact to activate the VEGF proximal promoter. (A) Serum-starved LNCaP cells were cotransfected as described above with V411 reporter and either GFP-WT1 or pCDNA3.1 vector control DNA. Transfected cells were treated with 0 or 5 nM R1881 in media containing 10% charcoal-stripped FCS. Values shown represent mean normalized luciferase activity (and SEM) relative to pCDNA3.1 empty vector control in the absence of hormone treatment (white). Each experiment was performed in triplicate and repeated twice. (B) The interaction of AR and WT1 in transfected LNCaP cells was demonstrated by coimmunoprecipitation of nuclear protein lysates by either WT1 Ab (Epitomics) or serum IgG. Immunoprecipitated (IP) proteins were electrophoresed and immunoblotted (IB) with either AR (top) or WT1 (bottom) antibodies. Left lanes show nuclear lysates, middle and right lanes show proteins immunoprecipitated by IgG or WT1 Ab, respectively.

Conclusion

Here, we review evidence that WT1 is expressed in PC epithelial cells and transcriptionally regulates PC critical genes. The relevance of WT1 to PC has been shown by finding that WT1 mRNA and protein are more often expressed in high-grade, invasive PC than low-grade localized tumors and that WT1 is not expressed in BPH or non-neoplastic prostate tissue (49). The identification of potential WT1-binding sites in the regulatory sequences of cancer-critical genes expressed in PC epithelial cells, together with the demonstration of WT1 protein bound to these gene promoters in native chromatin of transfected LNCaP cells, supported the notion that elevated WT1 expression in prostate epithelial cells affects transcriptional modulation of homeostatic genes important for PC (101). That WT1 may

promote metastatic disease is consistent with previous findings that WT1 suppressed E-cadherin, thereby increasing motility and metastatic potential of PC cells (48). The fact that WT1 transcriptionally upregulated VEGF expression and enhanced hormone induction of VEGF (102) suggested that WT1 could activate an angiogenic switch in PC cells. Taken together, the potential for WT1 to promote tumor angiogenesis and PC cell migration would suggest that WT1 regulates genes that enhance tumor growth and promote progression to lethal metastatic disease. These functions of WT1 are consistent with an oncogenic, not a tumor-suppressive, role and suggest that WT1 expression might serve as a marker for PC progression (53). Furthermore, therapies targeting WT1 in PC may block metastatic spread and increase the overall survival.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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