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Vascular Endothelial Growth Factor (VEGF) Is Suppressed in WT1-Transfected LNCaP Cells

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The Wilms' tumor suppressor gene product (WT1) regulates expression of growth control genes. Microarray analysis of gene expression profiles of hormone-treated LNCaP prostate cancer cell lines transfected with either wild-type *WT1* or a zinc finger mutant form, DDS (R394W), revealed significantly altered patterns of expression. Validation studies using quantitative real-time PCR confirmed the differential expression of the tumor progression gene, vascular endothelial growth factor (*VEGF*). WT1-LNCaP cells had significantly reduced levels of VEGF mRNA when compared to vector control cells; in contrast, DDS-LNCaP cells showed elevated levels of VEGF transcripts. To address a functional role for *WT1* overexpression, we investigated whether induction of *VEGF* expression, by the synthetic androgen R1881, would be disrupted in wild-type or mutant WT1-transfected LNCaP cells. Hormone treatment failed to elevate VEGF transcript levels above uninduced baseline levels in WT1-LNCaP cells, despite 48 h of treatment with 5 nM R1881. Consistent with our quantitative real-time PCR analysis, immunofluorescent staining of VEGF protein was reduced in WT1-LNCaP cells in both the presence and absence of R1881 treatment. Conversely, VEGF levels increased in vector control and DDS-LNCaP cells treated with 5 nM R1881. Not only do these studies point out the regulatory potential of WT1 for VEGF, but they also indicate an altered function for the mutant DDS isoform. Because VEGF is associated with neovascularization and promotion of metastasis in a variety of solid tumors including prostate cancer, a better understanding of the regulation of VEGF expression by transcription factors, such as WT1, is important for halting disease progression.

Key words: Prostate cancer; Wilms' tumor gene; Vascular endothelial growth factor; Denys-Drash syndrome; Microarray

PROSTATE cancer is the second leading cause of cancer death in men (16). To understand the progression of prostate cancer, gene expression studies have focused on identification of androgen-regulated genes or genes capable of regulating the effects observed during androgen treatment or withdrawal (7,20). Using microarray analyses we have compared gene expression patterns of LNCaP cells with those of LNCaP sublines stably transfected with a zinc finger transcription factor, *WT1*, the Wilms' tumor suppressor gene. We chose to examine the effect of WT1 overexpression because WT1 has been shown to transcriptionally repress several prostate cell growth regulators including the insulin-like growth factor (IGF)-1 receptor (34), the androgen receptor (28), and the pro-survival gene, *bcl-2* (5). Because WT1 transcriptionally represses both *IGF2* and the IGF2 receptor, *IGF1R*, this repression of the IGF axis can result in growth suppression (33). Conversely, increased IGF2 levels have been associated with loss of WT1 expression in primary breast cancer epithelial cells (30). Similar results have been reported for primary cultures of hyperplastic prostate stromal cells from patients with BPH (8). In this report we delineate the

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altered gene expression profiles of WT1-transfected LNCaP (WT-LNCaP) prostate cancer cells and describe the validation studies that confirmed the differential expression of the tumor progression gene, vascular endothelial growth factor (*VEGF*).

VEGF is a potent cytokine that functions in cell proliferation, vascular permeability, and angiogenesis (9) and has been associated with tumor progression and metastasis (11). VEGF expression and microvessel density (MVD) are increased in prostatic intraepithelial neoplasia (PIN), the precursor to prostate cancer, and are at higher levels in prostate cancer (18). Thus, VEGF is a biomarker for prostate tumor progression and androgen independence, and antiangiogenic therapies aimed at preventing tumor vascularity have emerged (12,37). Identification of regulatory genes, such as *WT1*, that can suppress angiogenic factors may also be important discoveries in the prevention of prostate cancer growth and progression.

VEGF expression is tightly regulated by many factors (e.g., oxygen tension, growth factors, cytokines, and steroid hormones) and mechanisms of VEGF regulation are complex (9,10). For example, hypoxia upregulates both VEGF transcriptional initiation (26, 27) and message stability (29). Similarly, hormone treatment is thought to increase VEGF levels through a combination of transcriptional and posttranscriptional mechanisms. In primary prostate stromal cells (14) and endometrial adenocarcinoma cells (19), androgen and estrogen treatment, respectively, increases transcriptional initiation of VEGF. However, increased stability has also been observed in LNCaP (32) and breast cancer cells (24) following androgen and estrogen treatment, respectively. Here we demonstrate that despite treatment with the androgen analog, R1881, VEGF levels were reduced in prostate cancer cells expressing the *WT1* gene.

Our approach was to identify transcripts modulated by WT1, using cDNA arrays to compare gene expression profiles of vector control LNCaP (V-LNCaP) cells with both the wild-type WT1- and a mutant (DDS)-transfected cells. The DDS (R394W)-WT1 used was a naturally occurring mutant form of WT1 associated with the nephropathy of Denys-Drash syndrome (DDS) (21). This mutation occurs as a single base substitution in the critical DNA binding region of the third zinc finger converting Arg394 to Trp. We asked whether expression of DDS-WT1 would alter LNCaP expression patterns, suggesting that DDS is not simply a transcriptionally inactive version of wild-type WT1. Preferentially expressed genes were initially identified as genes whose expression levels were changed by at least 1.8-fold compared to the V-LNCaP cells. To test the hypothesis that the DDS mutant has gained a function altered from wild-type WT1, genes were identified whose expression levels differed between wild-type WT1- and mutant DDS-transfected LNCaP cells. Finally, differentially expressed genes implicated in tumor growth and progression were identified. Using this rubric, *VEGF* was identified as a tumor progression gene whose expression varied between vector control, WT1-, and DDS-LNCaP cells. That is, LNCaP cells expressing wild-type WT1 had lower levels of VEGF mRNA available for competitive cDNA hybridization than did V-LNCaP cells. Surprisingly, we found that VEGF expression levels were enhanced in DDS-LNCaP cells relative to V-LNCaP cells. This differential expression was confirmed by quantitative real-time PCR and in DDS-LNCaP cells VEGF expression was hormone dependent. In contrast, suppressed VEGF expression in WT1-LNCaP cells was hormone independent, that is, in WT1-LNCaP cells basal VEGF levels were suppressed. This is significant as WT1 also transcriptionally regulates the androgen receptor (28) and could indirectly affect hormone induction of VEGF. Thus, WT1 could potentially regulate VEGF expression at two levels: indirectly through androgen signaling (28,36) in the presence of hormone and directly in its absence.

MATERIALS AND METHODS

Cell Culture

LNCaP prostate cancer cell lines were established as described previously (13). Briefly, the WT1-LNCaP cell line was established by transfection of LNCaP cells with the plasmid encoding the transcriptionally active isoform of *WT1* (isoform A) lacking both exon 5 and the KTS insertion. Similarly, the DDS-LNCaP cell line was established by transfection with the R394W mutant *WT1* (isoform A) plasmid and the V-LNCaP line by transfection with pcDNA3.1 vector. The cell lines reported here are three of the cell lines selected and characterized previously (13). Cells used in the initial microarray experiments were then grown for 48 h in charcoal stripped fetal calf serum in the presence or absence of 5 nM R1881 (methyltrienolone) synthetic androgen (NEN, Boston, MA). Cells used in subsequent hormone induction studies were grown identically except that cells were seeded into six-well plates, and subconfluent monolayers were synchronized by serum starvation for 8 h prior to incubation in the presence or absence of 5 nM R1881.

Array Construction

Custom spotted arrays contained 4027 cDNA clones corresponding to 2656 unigenes or unclustered

ESTs (17). The cDNA set included clones representing genes previously identified in cancer initiation and progression as well as housekeeping and control genes. Sequence verified clones were obtained from the 40K clone set or individually ordered (Research Genetics/Invitrogen, Carlsbad, CA). Plasmids were isolated from bacteria containing cDNA inserts and cDNAs were amplified using primer sequences shown in Table 1. Conditions for the AEKM13 primer set were: 20 mM Tris-HCl pH 8, 50 mM KCl, 1.5 mM $MgCl_2$ for 40 cycles of 94°C 30 s, 55°C 30 s, 72°C 1 min, and for the SP65 primer set: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ for 38 cycles of 94°C 30 s, 65°C 45 s, 72°C 30 s. Amplified products were then purified with size exclusion filter plates (Millipore, Billerica, MA) and electrophoresed on ethidium bromide-stained 1% agarose gels to verify quality. Microscope slides were stringently cleaned, poly-L-lysine coated, and aged for approximately 3 weeks as described (17). Then the cDNA (in 0.5 × DMSO) was printed in duplicate with the SDDC-2 microarrayer (Virtek Vision Inc., Waterloo, Ontario).

Array Hybridization and Analysis

RNA isolated from cells treated with R1881 was amplified prior to array hybridization. Briefly, 2 μg of total RNA was used for first-strand cDNA synthesis using T7-$(dT)_{24}$ primer and Superscript II (Invitrogen Carlsbad, CA). Second-strand cDNA synthesis was then performed; the cDNA was amplified using the T7 megascript kit (Ambion, Austin, TX) and purified with an RNeasy mini column (Qiagen, Valencia, CA). Labeling was done after annealing poly-$dT_{12\text{-}18}$ and random hexamers to 2 μg of amplified RNA and amino-allyl dUTP (aadUTP) was incorporated during the first-strand cDNA synthesis. Either Cy3 or Cy5 mono-reactive dye (Amersham Biosciences, Piscataway, NJ) was coupled to the aadUTP-labeled cDNA and unincorporated dye was removed using a Qiaquick (Qiagen, Valencia, CA) and the eluate was dried in a speed vacuum (Savaant). Prior to sample hybridization, printed array slides were prehybridized at 55°C in 2× SSC. The dye-labeled cDNAs for the paired test and control samples were combined and heat denatured before being mixed with hybridization buffer (Ambion, Austin, TX) and applied to each prewarmed slide. A coverslip was placed over the spotted area of the slide, and the locked hybridization chamber was incubated at 55°C overnight. After incubation, the array slides were removed from the hybridization chambers and washed sequentially in 2× SSC + 0.1% SDS, 2× SSC, and 0.2× SSC.

After washing, slides were scanned using a Gene Pix microarray scanner and GenePix software (Axon Instruments, Foster City, CA) while controlling for sample intensity in each of the channels. During visual inspection of each slide, spots of insufficient quality were flagged and eliminated from further analysis. The raw image data were analyzed using Gene Spring (Silicon Genetics, Redwood City, CA). Signal intensities were normalized to the controls present on each array and relative intensities were compared for each gene target.

RNA Isolation and Reverse Transcription

RNA used in the microarray experiments was isolated using Trizol reagent (Invitrogen, Carlsbad, CA)

TABLE 1
FORWARD AND REVERSE PRIMER SEQUENCES

Gene	Primer Sequences (5′-3′)	Amplicon (bp)
AEKM13*	F: GTT-GTA-AAA-CGA-CGG-CCA-GTG	NA
	R: CAC-ACA-GGA-AAC-AGC-TATG	
SP65R*	F: CCA-GTC-ACG-ACG-TTG-TAA-AAC-GAC	NA
	R: TGT-GGA-ATT-GTG-AGC-GGA-TAA-CAA	
Cyclophilin†	F: CTC-CTT-TGA-GCT-GTT-TCG-AG	325
	R: CAC-CAC-ATG-CTT-GCC-ATC-C	
VEGF†	F: CGA-AAC-CAT-GAA-CTT-TCT-GC	302
	R: CCT-CAG-TGG-GCA-CAC-ACT-CC	
TSC22‡	F: CCT-TGC-TGG-GGA-CTG-AAA-A	259
	R: AGC-TGG-GCC-TGA-AAC-TGG	
TSC22§	F: TCT-CTC-CTG-CTT-GCG-CTT-AGT	51
	R: AGC-TCC-ATC-GCT-TCA-CAA-CC	

*Primers designed to amplify 40K cDNA clone set or Individual Research Genetics/Invitrogen clones as described (17).
†Sequence of human *cyclophilin* and *VEGF* gene primers as described (31).
‡Sequence of *TSC22* primers as described (35).
§*TSC22* primer set designed using Primer Express software (Applied Biosystems).

as described (13). The quality of the RNA was evaluated by electrophoresis on a 0.8% agarose-formaldehyde denaturing gel (25) and by optical density ratios at 260 and 280 nm. RNA used for the hormone induction studies was harvested from six-well plates using the RNAqueous-4PCR Kit (Ambion, Austin, TX) according to the manufacturer's protocol. The optional DNAse treatment step was included after initial RNA isolation. First-strand cDNA was prepared from paired samples (grown in the presence or absence of R1881). RNA (1 μg) from each sample was reverse transcribed at 37°C for 2 h using Superscript II (Invitrogen, Carlsbad, CA) and random hexamers (Perkin Elmer, Boston, MA).

Quantitative Real-Time PCR

Microarray analyses were validated by quantitative real-time PCR using the ABI 7000 (Applied Biosystems, Foster City, CA). Primer sequences are shown in Table 1 (F, forward; R, reverse). RNA samples were treated with RNAse-free DNAse (Ambion, Austin, TX) prior to quantitation. First-strand cDNA was reverse transcribed (RT) from 1 μg of total RNA as described above. Real-time PCR amplification was performed using either 150 nM cyclophilin (31), 300 nM VEGF (31), or TSC22 primers (35) and the Sybr Green PCR MasterMix (Applied Biosystems, Foster City, CA). The amplification conditions were 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 1 min followed by the dissociation protocol beginning at 60°C. The 9600 emulation was used for all analyses. Quantitative analyses were performed according to manufacturer's recommendations (Applied Biosystems, Foster City, CA). Relative quantitation of PCR amplified products was determined using the comparative Ct analysis as described by Pfaffl (22) using cyclophilin as the internal reference (normalizer). For comparison of hormone-treated and untreated cells, cyclophilin-normalized VEGF mRNA levels in hormone-treated cells were calibrated against VEGF levels in untreated cells. Average fold change values (±SEM) following treatment were calculated for each cell line and significance was determined by two-tailed *T*-test ($p < 0.05$). For comparison of basal VEGF expression in different cell lines deprived of hormone (grown for 48 h in chFCS) normalized VEGF levels were calculated as 2^{-dCt}. Average normalized VEGF levels (+SEM) were calculated for WT1-, DDS-, and V-LNCaP cells and significance was determined by two-tailed *T*-test ($p < 0.05$).

Immunofluorescence

WT1-LNCaP, DDS-LNCaP, and V-LNCaP cells were seeded onto glass microscope slides, serum starved for 18 h to synchronize cells, and cultured for 48 h in RPMI medium (BioWhittaker, MD) with charocoal-dextran stripped serum (10% chFBS) in the presence or absence of 5 nM R1881. Subconfluent cell monolayers were fixed in cold acetone. Slides were incubated with polyclonal VEGF antibody (Santa Cruz CA, USA), then with FITC-labeled anti-rabbit antibodies (Santa Cruz CA, USA), and nuclei stained with DAPI (1 μg/ml) in Vectashield mounting media (Vector Laboratories, CA, USA). Slides were examined by epifluorescence microscopy (Leica) at 100–400× magnification, photographed digitally using a Polaroid digital camera and the Magnawire software program (Optronics, CA).

RESULTS

Microarray Analysis Comparing Wild-Type WT1- and Mutant DDS-Transfected LNCaP Cells to Vector Control LNCaP Cells

Amplified RNA from each of the hormone-treated *WT1*-transfected LNCaP cell lines was competitively hybridized with amplified RNA from the hormone-treated V-LNCaP cell lines on custom spotted microarray slides. Three biological replicates of each cell line were analyzed along with three corresponding dye-swapped technical replicates.

From the 4027 clones screened on the array, 56 showed altered expression levels 1.8-fold or greater in the normal WT1-LNCaP line when compared to the V-LNCaP. In the DDS-LNCaP line, 80 genes showed changes of 1.8-fold or greater when compared to the vector control. When the list of genes was compared by the use of Venn diagrams, the genes whose expression increased 1.8-fold in the wild-type WT1-LNCaP line did not include the same genes whose expression was increased 1.8-fold in the DDS-LNCaP line with the exception of the *WT1* gene itself (Fig. 1A). The same general relationship was true for the lists of genes whose expression was decreased 1.8-fold with the exception of a poorly characterized gene designated MCJ with J domain homology but of unknown function (Fig. 1B). A list of the fully annotated genes showing 1.8-fold or greater changes in expression (increased or decreased) is shown in Tables 2 and 3. Among the annotated genes we found that 12 were upregulated 2-fold or greater in the WT1-LNCaP line while 21 were downregulated 2-fold or greater. Overall more genes were suppressed than enhanced in the WT1-LNCaP line. Conversely, 14 annotated genes were unregulated 2-fold or greater in the DDS-LNCaP line while only 9 were downregulated 2-fold or greater. Unlike the trend in WT-LNCaP cells, more genes were enhanced than suppressed in

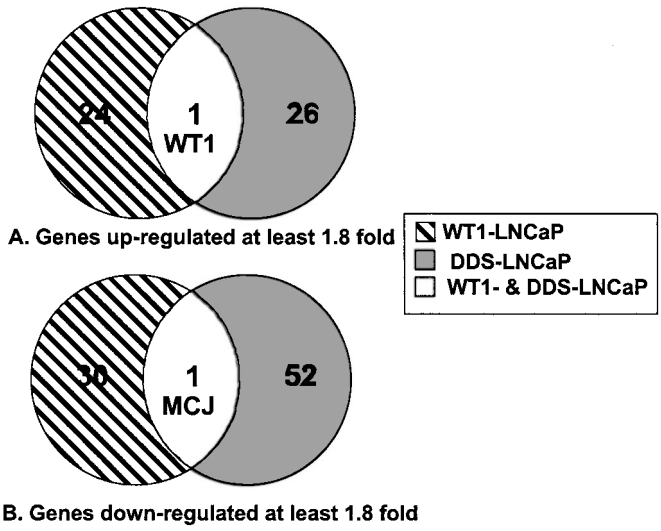

Figure 1. Venn diagram of genes with expression altered at least 1.8-fold relative to V-LNCaP cells. (A) Twenty-four genes were upregulated in the hormone-treated WT1-LNCaP cells (striped area) and 26 in DDS-LNCaP cells (gray area) compared to the vector control (V-LNCaP) cells. The only gene upregulated in both WT1-LNCaP and DDS-LNCaP cells was WT1. (B) Thirty genes were downregulated in the hormone-treated WT1-LNCaP cells (striped area) and 52 in DDS-LNCaP cells (gray area) when compared to the V-LNCaP cells. The only gene suppressed in both WT1- and DDS-LNCaP cells was MCJ, a gene with J domain homology and unknown function.

DDS-LNCaP cells. This suggests that mechanisms of gene regulation in DDS-LNCaP cells differ from those in WT-LNCaP cells.

Included in the list of genes potentially regulated by *WT1* expression were the tumor progression gene, *VEGF*, and transforming growth factor β-stimulated clone 22 (*TSC22*), two candidate genes with consistent and reproducible expression among the biological replicates and differential expression between V-LNCaP cells and WT1- or DDS-LNCaP cells (Tables 2 and 3). *VEGF* expression typifies the quantitative differences in gene expression between the WT-LNCaP and DDS-LNCaP cell lines, as *VEGF* expression was altered in both cell lines. *VEGF* expression in the WT1-LNCaP line was decreased (−2.2- and −2.6-fold) compared to the V-LNCaP cells, as demonstrated by reduced hybridization to two different *VEGF* clones spotted on the array (Table 2). Conversely, *VEGF* expression in the DDS-LNCaP cell line was increased between 2.3- and 3.2-fold relative to vector control for the same two cDNA clones (Table 3). Thus, *VEGF* was a common gene target for both WT-LNCaP and DDS-LNCaP cells, but its expression was suppressed in the former and enhanced in the latter. In contrast, TSC22 expression in the WT1-LNCaP line was increased 2.4-fold compared to the V-LNCaP cells (Table 2) with no significant change in the DDS-LNCaP cells.

Other genes differentially expressed inWT1-LNCaP cells compared to V-LNCaP cells include calnexin (repressed 2.6-and 2.9-fold in WT1-LNCaP) and tissue inhibitor of metalloproteinase-3 (TIMP-3) (increased 2-fold in WT1-LNCaP cells). Similarly, genes differentially expressed in DDS-LNCaP cells compared to V-LNCaP cells include *VCAM-1* (increased 2.4-fold in DDS-LNCaP cells) and *fibrinogen* (repressed 2.1- and 2.3-fold in DDS-LNCaP). Not only were gene expression patterns quantitatively different in WT-LNCaP and DDS-LNCaP cells, but they were qualitatively different, as well. The genes with altered expression of 1.8-fold or greater in the WT1- and DDS-LNCaP cell lines were classified according to their functions gleaned from Gene Ontology Annotations, GeneAtlas, GeneCards, or a compilation of the online databases (Fig. 2). The genes most greatly affected in WT1-LNCaP cells (Fig. 2, black bars) belonged to functional categories implicated with cellular transport and transcription. Conversely, in the DDS-LNCaP cells (Fig. 2, striped bars), genes most greatly affected tended to be associated with mitochondria, RNA binding and splicing, or translational processes.

Confirmation of Altered VEGF Expression by Quantitative Real-Time PCR

In order to validate the array results, we compared quantitative real-time PCR amplification and array data for the independent biological replicates of each cell line (Table 4). Quantitative real-time PCR amplification of *VEGF* mRNA was normalized to an internal reference, cyclophilin, a housekeeping gene. Comparative Ct analysis, as described by Pfaffl (22), was used to determine the significance of the relative

TABLE 2
GENES WITH ALTERED EXPRESSION IN WT1-LNCaP CELLS (AT LEAST 1.8-FOLD VS. CONTROL)

Unigene		Gene Definition	Avg. Fold Change
Hs.1145	WT1	Wilms' tumor protein	6.9
Hs.25209	MAPK10	Mitogen activated protein kinase 10	4.0
Hs.52620	ITGB8	Integrin beta-8	2.8
Hs.114360	TSC22	Transforming growth factor beta-stimulated protein TSC-22	2.4
Hs.1968	SEMG1	Seminal basic protein	2.4
Hs.76838	SERPINA7	Serine (or cysteine) proteinase inhibitor, clade a, member 7	2.2
Hs.76422	PLA2G2A	Phospholipase A2, membrane associated precursor	2.1
Hs.237658	APOA2	Aplolilpoprotein A-II precursor	2.1
Hs.94498	LILRA2	Leukocyte immunoglobulin-like receptor, subfamily A2	2.0
Hs.245188	TIMP3	Tissue inhibitor of metalloproteinases-3	2.0
Hs.458318	PSG4	Pregnancy-specific beta-1-glycoprotein 1 precursor, fetal liver non-specific cross-reactive antigen-2	2.0
Hs.184585	LMO2	LIM-only protein 2, cysteine rich protein TTG-2	2.0
Hs.8136	EPAS1	Hypoxia-inducible factor 2, alpha subunit	1.9
Hs.443409	ODC1	Ornithine decarboxylase pseudogene	1.9
Hs.93574	HOXD3	Homeobox protein HOX-D3	1.9
Hs.303649	SCYA2	Small inducible cytokine A2 precursor, monocyte chemotactic and activating factor	1.9
Hs.80642	STAT4	Signal transducer and activator of transcription 4	1.8
Hs.14637	CML1	Putative *n*-acetyltranserase camello 1, kidney specific gene	1.8
Hs.77578	USP9X	Ubiquitin-specific processing protease	−1.8
Hs.78482	PALM	Paralemmin+	−1.8
Hs.192221	ELL2	RNA polymerase II elongation factor ELL2	−1.8
Hs.83974	SLC21A2	Solute carrier family 21, member 2	−1.9
Hs.235069	RECQL	ATP-dependent DNA helicase Q1	−2.0
Hs.438830	MCJ	DNA J domain-containing	−2.0
Hs.231853	RDC1	G protein-coupled receptor RDC1 homolog	−2.0
Hs.438582	PRNP	Prion-related protein	−2.1
Hs.820	HOXC5	Homeobox protein HOX-C5	−2.1
Hs.73793	VEGF	Vascular endothelial growth factor	−2.2
Hs.116992	HGD	Homogentisate 1,2-dioxygenase	−2.2
Hs.172801	IARS	Isoleucyl-tRNA synthetase, cytoplasmic	−2.2
Hs.93728	PBX2	Pre-B-cell leukemia transcription factor-2	−2.2
Hs.78434	ZNF133	Zinc finger protein 133 (clone pHZ-13)	−2.3
Hs.231853	RDC1	G protein-coupled receptor RDC1 homolog	−2.3
Hs.78869	TCEA1	Transcription elongation factor A, 1	−2.3
Hs.135892	NCAM2	Neural cell adhesion molecule 2	−2.3
Hs.26208	COL16A1	Collagen alpha 1(xvi) chain precursor	−2.4
Hs.198443	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	−2.5
Hs.73793	VEGF	Vascular endothelial growth factor	−2.6
Hs.3059	COPB	Coatomer protein beta subunit	−2.6
Hs.155560	CANX	Calnexin	−2.6
Hs.389559	EIF3S10	Eukaryotic translation initiation factor 3 subunit 10	−2.7
Hs.78869	TCEA1	Transcription elongation factor A, 1	−2.8
Hs.155560	CANX	Calnexin	−2.9

quantitation, so that the cyclophilin-normalized VEGF levels in WT1-LNCaP or DDS-LNCaP cells were calibrated relative to levels in the V-LNCaP cells. Fold-change was calculated as 2^{-ddCt} (22) and is shown for each pair of samples. The microarray findings were validated by quantitative real-time PCR and 4- to 8-fold suppression of VEGF transcripts in WT1-LNCaP cells was observed (Table 4). The quantitative real-time PCR results confirmed the decreased expression of VEGF in all three biological replicates of WT1-LNCaP (Table 4) and increased expression of VEGF in two of three replicates of DDS-LNCaP (Table 4). In all cases, the quantitative real-time PCR results showed the same trend, as did the corresponding array result for the particular sample (Table 4).

Unlike the differential VEGF expression, differential TSC22 gene expression was not confirmed by quantitative real-time PCR (data not shown). While the array results demonstrated enhanced TSC22 expression in WT1-LNCaP cells, no significant increase was observed in two of three WT1-LNCaP RNA samples analyzed by quantitative real-time PCR. Similarly, while the microarray demonstrated no significant suppression of TSC22 expression in DDS-LNCaP cells,

a 2-fold increase in expression was observed in two of three RNA samples analyzed by quantitative real-time PCR. Although the original primers (35) were verified to specifically and efficiently amplify TSC22 in control samples, we obtained a second primer set designed for TSC22 amplification using the primer express software (ABI, Foster City, CA) and retested the RNA samples. However, these quantitative real-time PCR results were similar to those of the original primers (data not shown). Overall, no consistent elevation of TSC22 expression in WT1-LNCaP cells was observed using quantitative real-time PCR. Thus, while microarray results for VEGF expression were validated by quantitative real-time PCR they were not validated for TSC22.

Comparison of VEGF mRNA Expression Levels in Hormone-Induced and Uninduced Cells

Because wild-type *WT1* expression was inversely related to *VEGF* expression, we asked whether WT1-LNCaP cells might have a dampened response to hor-

TABLE 3
GENES WITH ALTERED EXPRESSION IN DDS-LNCAP CELLS (>1.8-FOLD VS. CONTROL)

Unigene		Gene Definition	Avg. Fold Change
Hs.1145	WT1	Wilms' tumor protein	53.8
Hs.858	RELB	V-REL avian reticuloendotheliosis viral oncogene homolog B	4.1
Hs.73793	VEGF	Vascular endothelial growth factor	3.2
Hs.78183	AKR1C3	Aldo-Keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	2.7
Hs.109225	VCAM1	Vascular cell adhesion protein 1 precursor	2.4
Hs.87773	PRKACB	cAMP-dependent protein kinase, beta-catalytic subunit	2.3
Hs.73793	VEGF	Vascular endothelial growth factor	2.3
Hs.78434	ZNF133	Zinc finger protein 133 (clone pHZ-13)	2.3
Hs.239489	TIA1	TIA1 cytotoxic granule-associated RNA-binding protein	2.2
Hs.351316	TM4SF1	Tumor-associated antigen L6, transmembrane 4 superfamily,1	2.1
Hs.445652	HSU53209	Transformer-2 alpha (HTRA-2 alpha)	2.1
Hs.184167	SFRS7	Splicing factor, arginine/serine-rich, 7	2.0
Hs.426142	PIGF	Phosphatidylinositol-glycan biosynthesis, class F protein	2.0
Hs.188879	RBM6	RNA binding motif protein 6	1.9
Hs.29877	TXK	Tyrosine-protein kinase TXK	1.9
Hs.158462	SLC12A3	Thiazide-sensitive sodium-chloride cotransporter, solute carrier family 12, member 3	1.8
Hs.79187	CXADR	Coxsackievirus and adenovirus receptor (CAR) protein	1.8
Hs.12246	RELN	Reelin	1.8
Hs.210367	SFRS2IP	Splicing factor, arginine/serine-rich, 2-interacting protein	1.8
Hs.310621	EIF5A	Eukaryotic translation initiation factor 5A	−1.8
Hs.351593	FGA	Fibrinogen-alpha polypeptide	−1.8
Hs.416801	RPL7A	60S ribosomal protein L7A, surfeit locus protein 3	−1.8
Hs.381072	PPIF	Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor	−1.8
Hs.512587	MST1	Macrophage stimulating protein, hepatocyte growth factor-like protein precursor	−1.8
Hs.184014	RPL31	60S ribosomal protein L31	−1.8
Hs.32916	NACA	Nascent-polypeptide-associated complex, alpha polypeptide	−1.8
Hs.512676	RPS25	40S ribosomal protein S25	−1.8
Hs.306791	POLD2	DNA polymerase, delta-2, regulatory subunit	−1.8
Hs.108957	RPS27	40S ribosomal protein S27 isoform	−1.8
Hs.432329	DKFZp564A176	Homo sapiens mRNA; cDNA DKFZp564A176 (from clone DKFZp564A176); complete CDS	−1.8
Hs.289271	CYC1	Cytochrome C1, heme protein precursor	−1.8
Hs.381126	RPS14	Ribosomal protein S14	−1.8
Hs.77385	MYL6	Myosin light chain polypeptide 6, non-muscle isoform	−1.9
Hs.441072	POLR2L	DNA-directed RNA polymerase II 7.6 KDA polypeptide L	−1.9
Hs.372960	RPS21	Ribosomal protein S21	−1.9
Hs.386384	RPS23	Ribosome associated RNA-binding protein	−1.9
Hs.310621	EIF5A	Eukaryotic tranlation initiation factor 5A	−2.0
Hs.397609	RPS16	40S ribosomal protein S16	−2.0
Hs.184877	SLC25A11	Mitochondrial 2-oxoglutarate/malate carrier protein, solute carrier family 25, member 11	−2.0
Hs.432607	PSMB2	Proteasome subunit, beta-type, 2	−2.0
Hs.432121	PRDX2	Thiol-specific antioxidant protein	−2.0
Hs.351593	FGA	Fibrinogen-alpha polypeptide	−2.1
Hs.351593	FGA	Fibrinogen-alpha polypeptide	−2.3
Hs.112259	TCRG	T-cell antigen receptor, gamma subunit	−2.4
Hs.438830	MCJ	DNA J domain-containing	−2.5

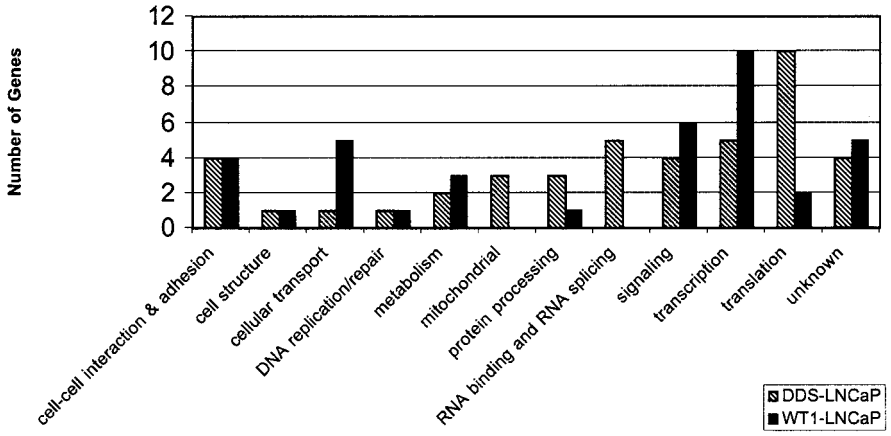

Figure 2. Functional classes of genes whose expression was altered in WT1-LNCaP and DDS-LNCaP cells. Classification by function of genes listed in Tables 2 and 3, using online databases such as Gene Ontology Annotations (see text). The height of the bar represents the number of genes with altered expression in DDS-LNCaP (striped) and WT1-LNCaP cells (black) compared to V-LNCaP cells. Genes affected in WT1-LNCaP cells were primarily involved in cellular transport and transcription. Conversely, a majority of genes affected in DDS-LNCaP cells were associated with mitochondrial processes, RNA splicing, and translational processes.

TABLE 4
ALTERED VEGF EXPRESSION IN WT1-LNCaP AND DDS-LNCaP CELLS

				Normalized VEGF Expression	
Set	Cell Line	VEGF (Avg. Ct*)	Cyclo (Avg. Ct*)	Real-Time PCR†	Array‡
WT1-LNCaP vs. V-LNCaP					
1	V-LNCaP	26.3	21.5	−3.9	−2.3
	WT1-LNCaP	27.9	21.4		
2	V-LNCaP	27.2	20.2	−7.7	−2.9
	WT1-LNCaP	28.7	21.1		
3	V-LNCaP	25.4	21.5	−7.7	−2.4
	WT1-LNCaP	31.8	23.0		
DDS-LNCaP vs. V-LNCaP					
1	V-LNCaP	27.2	20.2	2.9	2.7
	DDS-LNCaP	23.8	21.3		
2	V-LNCaP	25.4	21.5	13.1	5.0
	DDS-LNCaP	24.0	21.6		

*Average cycle threshold (Ct) values of triplicate amplifications of VEGF or cyclophilin control (normalizer) transcripts.
†Fold-change in VEGF expression in stably transfected cells (WTa-LNCaP or DDS-LNCaP). VEGF transcript levels were normalized to cyclophilin levels and calibrated against VEGF levels in vector control cells (V-LNCaP) using comparative Ct analysis (22) as described in text.
‡Signal intensities of competitive hybridization between RNA from test cell (WTa-LNCaP or DDS-LNCaP) and vector control (V-LNCaP) were normalized to the internal control genes on each array as described in text.

mone induction of VEGF. Paired samples of the three cell lines were grown for 24 and 48 h in the presence of 10% charcoal-stripped FCS with or without 5 nM R1881. RNA was isolated from three sets of biological replicates in the hormone-induced and uninduced states and samples were prepared for quantitative real-time PCR. Fold-change values following 24-h (Fig. 3A, solid bars) and 48-h (Fig. 3A, hatched bars) treatment with 5 nM R1881 were calculated as described above, based on average dCt values of 9 sample pairs (±R1881) normalized to cyclophilin. After 24 or 48 h of hormone induction, the vector control cells showed an average 2.3- or 2-fold increase, respectively, in *VEGF* expression. Asterisks denote significant differences between fold-change values of WT1-LNCaP or DDS-LNCaP cell lines versus the V-LNCaP control for the same time point ($p < 0.05$, two-tailed *T*-test). The WT1-LNCaP cells showed an initial response to hormone, but then VEGF levels returned to uninduced levels (1.1-fold) after 48 h of R1881 treatment, suggesting that WT1 expression maintained VEGF at baseline levels (only 55% of control LNCaP levels). This implied that WT1 expression interrupted hormone-induced VEGF upregulation. In contrast, VEGF levels in DDS-LNCaP cells were significantly elevated, 3.7- or 4.6-fold, respectively, at both 24 and 48 h after R1881 treatment ($p < 0.05$). This suggested that hormone treatment of LNCaP cells induced elevated VEGF levels in the presence of the DDS mutant but not wild-type WT1.

We then asked whether VEGF levels in WT1-LNCaP cells were modulated independently of hormone induction. This was important to determine as

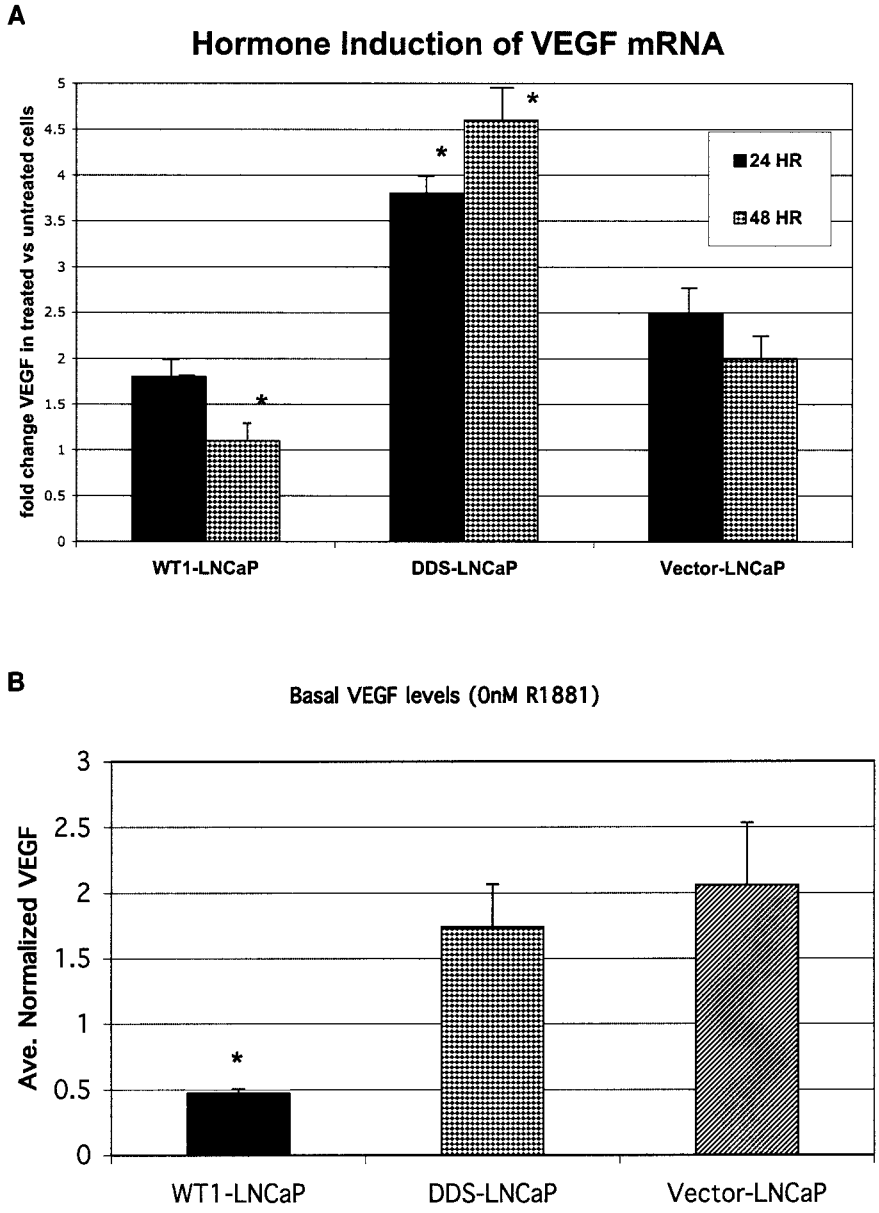

Figure 3. Hormone induction of VEGF mRNA in WT1-LNCaP, DDS-LNCaP, and V-LNCaP cells. (A) Results of quantitative real-time PCR are shown as the fold-change in VEGF mRNA levels in cells at 24 h (black bars) and 48 h (striped bars) after treatment with 5 nM R1881. Fold-change values (relative to untreated cells) were calculated as described in the text. Error bars depict the SEM and asterisks denote significant differences between ddCt values of hormone-treated and untreated cells for the same time point. Significance was determined by the two-tailed *T*-test ($p < 0.05$). (B) Basal VEGF transcript levels in hormone deprived WT1-, DDS-, or V-LNCaP cells were compared by quantitative real-time PCR. Average normalized VEGF levels are shown with SEM depicted by error bars. Asterisks denote significant differences from V-LNCaP as determined by the two-tailed *T*-test ($p < 0.03$).

WT1 transcriptionally regulates AR (28) and disrupts hormonal signaling in some cells (36) and thus could potentially alter VEGF levels indirectly. However, VEGF suppression in WT1-LNCNaP cells appeared independent of AR and hormone interaction, as VEGF levels in the absence of hormone were significantly lower in WT1-LNCNaP cells than V-LNCaP cells ($p < 0.03$, two-tailed *T*-test) (Fig. 3B). Thus, VEGF levels in WT1-LNCaP cells (relative to V-LNCaP cells) were suppressed 4.9-fold or 3.6-fold in the presence or absence of R1881, respectively (Table 5). This suggested that WT1 expression in WT1-LNCaP cells suppressed uninduced, basal VEGF expression as well as hormone-induced VEGF expression. In contrast, while VEGF levels were 5.9-fold greater in hormone-treated DDS-LNCaP cells (relative to V-LNCaP cells), no significant increases in VEGF levels were observed in DDS-LNCaP cells in the ab-

TABLE 5
HORMONE-INDUCED VEGF EXPRESSION IN WT1- AND DDS-LNCaP CELLS RELATIVE TO V-LNCaP CELLS*

	5 nM R1881†		0 nM R1881†	
Cells	24 h	48 h	24 h	48 h
WT1-LNCaP	1.9-fold decrease	4.9-fold decrease	1.2-fold decrease	3.6-fold decrease
DDS-LNCaP	2.7-fold increase	5.9-fold increase	0.56-fold increase	1.2-fold decrease

*Using quantitative real-time PCR, VEGF transcript levels were normalized to cyclophilin levels as described in Table 4. Normalized VEGF mRNA levels in transfected cells (WT1-LNCaP or DDS-LNCaP) were calibrated against VEGF levels in V-LNCaP cells using comparative Ct analysis (22) as described in the text.
†Cells were synchronized by serum starvation then treated with 0 or 5 nM R1881 for 24 or 48 h prior to RNA isolation.

sence of hormone treatment (Fig. 3B and Table 5). This suggested that DDS expression enhanced hormone induction of VEGF expression but not basal VEGF levels. Thus, while VEGF levels in WT1-LNCaP cells were modulated independently of hormone treatment, VEGF levels in DDS-LNCaP cells were hormone dependent.

VEGF Protein in DDS-LNCaP Cells

To assess VEGF expression in transfected LNCaP cells, immunofluorescence was performed (Fig. 4). Cells cultured on microscope chamber slides were cultured as described above in RPMI with 10% chFBS and treated for 48 h with either 0 or 5 nM R1881. After fixation slides were incubated with polyclonal VEGF antibody (Santa Cruz), then FITC labeled anti-rabbit antibodies (Santa Cruz) and DAPI to stain the nuclei. Slides were examined by epifluorescence microscopy (Leica) at 100–400× magnification and photographed digitally. Consistent with our quantitative real-time PCR analysis, we observed strong FITC staining in most DDS-LNCaP cells (Fig. 4A, top panel) and little staining in the WT1-LNCaP cells (Fig. 4A, bottom panel). VEGF protein was found predominantly in the cytoplasm. We compared VEGF expression in cells cultured in either the presence or absence of 5 nM R1881 (Fig. 4B). DAPI-stained nuclei show the location of cells in these fields (Fig. 4B, insets). Consistent with our quantitative real-time PCR results, VEGF expression was high in hormone-treated DDS-LNCaP cells (Fig. 4B, top right panel) and low or undetectable in WT1-LNCaP cells cultured with (Fig. 4B, bottom right panel) or without hormone (Fig. 4B, bottom left panel).

DISCUSSION

Using microarray analyses we have compared gene expression patterns of LNCaP cells with those of LNCaP sublines stably transfected with the *WT1* gene. We examined the effect of WT1 overexpression in LNCaP cells because WT1 has been shown to transcriptionally repress several prostate cell growth regulators (5,28,34). Comparison of the differentially expressed gene lists reported here with those reported in previous studies (7,20) show several similarities, including VEGF, and TSC22, mitogen activated kinases, SERPINs, ODC, and members of the solute carrier (SLC) family. VEGF and TSC22 have been identified as hormone-responsive genes in R1881-treated parental LNCaP cells [(7,20,32), this report]. Because earlier studies indicated that androgen treatment altered the expression of these families of genes, we asked whether WT1 expression would suppress expression of the R1881-responsive genes.

VEGF expression is affected by many factors, including steroid hormones, so *VEGF* regulation is complex and not well understood. Here we demonstrate that VEGF mRNA levels are suppressed in LNCaP cells expressing wild-type WT1. In contrast, LNCaP cells expressing the mutant DDS (R394W)-WT1 showed increased VEGF mRNA levels compared to V-LNCaP cells. Both of these microarray findings were validated by quantitative real-time PCR. Even after 48 h of hormone treatment, VEGF levels in the WT1-LNCaP cells did not increase significantly above basal levels. These results demonstrated an association of WT1 expression with a disruption of hormone-induced VEGF expression.

Additionally, we demonstrated that in WT1-LNCaP cells VEGF levels were modulated independently of hormone induction, unlike in DDS-LNCaP cells. This was important because WT1 transcriptionally regulates AR in vitro (28) and can disrupt hormonal signaling in some cells (36). However, after 48 h of hormone depletion (by growth in medium containing chFBS), VEGF levels were still 3.6-fold lower in WT1-LNCaP cells than V-LNCaP cells (Table 5). Thus, repression of VEGF expression was not depen-

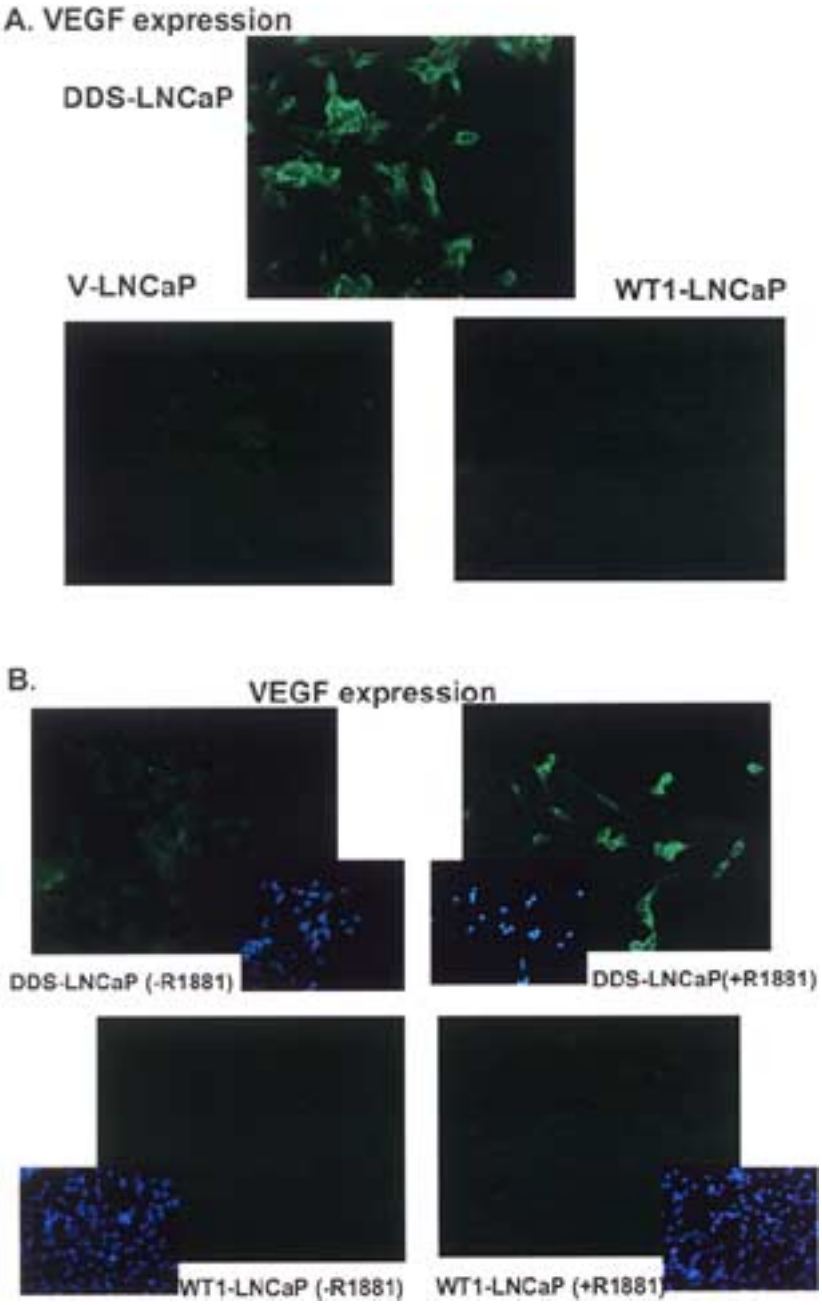

Figure 4. DDS-LNCaP cells grown in 5 nM R1881 overexpress VEGF protein. (A) To compare VEGF expression in WT1- and DDS-LNCaP cells with V-LNCaP cells, cells were cultured on microscope chamber slides and treated with 5 nM R1881 as described in the text. After fixation slides were incubated with VEGF antibody (Santa Cruz), then FITC labeled anti-rabbit antibodies and DAPI. Slides were examined by epifluorescence microscopy (as described in the text) and VEGF protein was found predominantly in the cytoplasm. Consistent with our quantitative real-time PCR analysis, we observed strong FITC staining in most DDS-LNCaP cells (top panel) and little staining in the WT1-LNCaP cells (bottom panel). (B) To assess the effect of hormone treatment on VEGF expression, DDS-LNCaP cells (upper panels) and WT1-LNCaP cells (lower panels) were cultured in the presence (right panels) or absence (left panels) of 5 nM R1881 as described. Slides were incubated with antibodies and DAPI to stain the nuclei (see insets) as described. Consistent with our quantitative real-time PCR analysis, VEGF expression was high after hormone treatment of DDS-LNCaP cells (top right panel) and low in WT1-LNCaP cells cultured with (bottom right panel) or without hormone (bottom left panel).

dent on hormone treatment, indicating that suppression of basal VEGF expression by WT1 is unlikely to require the AR. In contrast, while VEGF levels were elevated in hormone-treated DDS-LNCaP cells, in the absence of hormone treatment VEGF levels in DDS-LNCaP cells were equivalent to those in V-LNCaP cells. This suggested that only hormone-induced VEGF expression was enhanced in DDS-LNCaP cells, but not basal VEGF levels. Overall, because VEGF levels in WT1-LNCaP cells were suppressed independently of hormone while elevated VEGF levels in DDS-LNCaP cells were hormone dependent, this implies different mechanisms of regulation of VEGF by wild-type than mutant WT1.

Consistent with our quantitative real-time PCR analysis, we observed strong FITC staining in most DDS-LNCaP cells, less staining in the V-LNCaP cells, and little staining in the WT1-LNCaP cells. VEGF protein was found predominantly in the cytoplasm and in some of the strongly staining DDS-LNCaP cells appeared in a perinuclear pattern. Immunofluorescent staining of VEGF protein was reduced in WT1-LNCaP cells in both the presence and absence of R1881 treatment, as seen for VEGF mRNA. The finding of VEGF suppression in WT1-LNCaP cells independent of hormone induction was consistent throughout these studies.

Other genes shown by microarray analysis to be differentially expressed in WT1- and DDS-LNCaP cells include *TIMP-3*, *calnexin*, *VCAM-1*, and *fibrinogen*. Though not androgen targets, understanding the regulation of these genes may give insight into the molecular mechanisms of tumor progression. TIMP-3 expression in WT1-LNCaP cells was increased and TIMP-3 protein is thought to suppress angiogenesis by blocking VEGF binding to its receptor (23). Thus, an inverse relation between TIMP-3 and VEGF expression is consistent with overall reduction of angiogenic signals in tumor-suppressed WT1-LNCaP cells. TIMP-3 expression is also associated with increased survival in a study of lethal Wilms' tumors showing relapsed tumors expressed significantly more VEGF and less TIMP-3 than tumors from patients who survived (2).

While repression or activation of WT1 gene targets is likely mediated through the zinc finger DNA binding domain, it is less clear how or whether the DDS mutant form of WT1 might regulate transcription of its target genes. Nonetheless, gene expression patterns were significantly altered in DDS-LNCaP cells. Fourteen genes (including fibrinogen) were upregulated 2-fold or greater and 9 genes (including VCAM) downregulated 2-fold or greater compared to V-LNCaP cells. Both these potential gene targets could affect tumor progression. With the exception of VEGF, the genes with altered expression in DDS-LNCaP cells are a unique subset of genes that do not overlap those potential WT1 targets. Thus, this report supports the hypothesis that the DDS mutant is not simply inactive due to the Arg $\rightarrow$ Trp mutation (R394W) in the third zinc finger, but appears to have an altered function (33). In fact, when differentially expressed genes from Tables 2 and 3 were grouped by functional classes (Fig. 2), some of the most greatly affected genes in the DDS-LNCaP cells were genes associated with RNA binding, splicing, and translational processes. This list highlights the intriguing possibility that the DDS (R394W) mutant may bind RNA, analogous to the WT1 (+) KTS isoforms that bind DNA poorly but play a role in RNA processing (1,3,15). Work in progress is addressing the function of the DDS (R394W) mutant in LNCaP cells, but the upregulation of VEGF transcription in hormone-treated DDS-LNCaP cells is unlikely to be mediated indirectly by activation of the AR gene, as DDS (R394W) does not significantly modulate transcription of the AR promoter in vitro (28). Additionally, R1881 treatment of DDS-LNCaP cells does not induce ARE-Luciferase reporters (S. Priyadarshini and Fraizer, unpublished), nor are ARE-Luciferase reporters affected by DDS (R394W) cotransfection of kidney cells (36). Thus, upregulation of VEGF expression in the DDS-LNCaP cells is unlikely to involve either direct DNA binding by DDS (R394W) or indirect modulation of AR.

Neovascularization and angiogenic factors, such as VEGF, are integral to the ability of tumors to grow and progress to metastatic disease (4). VEGF has been implicated as a facilitator of prostate metastasis, migration, and establishment in bone, a primary site of prostate cancer metastasis (6). If abnormal angiogenesis can be halted or slowed, the tumors will not be able to grow beyond the confines of the prostate or metastasize. We have previously demonstrated that WT1-LNCaP cells are growth suppressed in nude mice (13). The reduction in WT1-LNCaP tumor cell growth observed in mice may be related to inhibition of neovascularization in the tumor. However, DDS-LNCaP cells were also growth suppressed in nude mice (13) and, paradoxically, cultured DDS-LNCaP cells expressed elevated VEGF levels in vitro. To address this paradox we compared tumor growth suppression of DDS-LNCaP cells to growth suppression of WT1-LNCaP cells. While the end result of DDS-LNCaP and WT-LNCaP tumor cell growth suppression was the same, the time course of tumor suppression differed. The early tumor cell growth of DDS-LNCaP cells (<6 weeks after SC injection) was significantly

enhanced compared to WT1-LNCaP cells. In fact, for the first 5 weeks small tumor nodules formed at 28–40% of DDS-LNCaP cell injection sites, at 22–38% of V-LNCaP cell injection sites, but at only 9–15% of WT1-LNCaP cell injection sites. Thus, early on the DDS-LNCaP cells formed as many tumor nodules as did the V-LNCaP cells. In addition to tumor incidence, tumor volumes at these early time points were also significantly different between the DDS-LNCaP and WT1-LNCaP cells (13). Thus, these results suggest that while WT-LNCaP tumor cells may be growth suppressed from the earliest time points, DDS-LNCaP growth suppression did not occur until 3–4 weeks later. The mechanisms of growth suppression of WT1- and DDS-LNCaP cell lines in nude mice are unknown, but they are likely different. We are focusing our efforts on understanding the relationship between transcriptional repression in vitro and growth suppression in vivo for both the WT1-LNCaP and DDS-LNCaP cells.

Based on our model of WT1 growth suppression in LNCaP cells in vivo (13) we predict that WT1 expression in prostate tumor tissue would be reduced compared to normal prostate. Consistent with the expectation that normal prostate expresses WT1, primary cultures of normal prostate stromal cells (although not epithelial cells) express WT1 mRNA (8). Additional evidence of WT1 expression in normal prostate tissues can be found in public genomic databases using the Unigene EST ProfileViewer, Unigene Accession number Hs555896 (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.555896). Additionally the Cancer Genome Anatomy project (CGAP) lists the WT1 transcript as present in prostate (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=5674815) and the virtual northern summarizing SAGE and EST Data list WT1 as present in normal prostate but absent in prostate cancer (GenBank accession number AI935945).

If WT1 expression were lost in prostate cancer cells, it would be significant as WT1 transcriptionally regulates prostate cell growth regulators including IGF1R and IGF2 (33,34). Indeed, the loss of WT1 expression in both breast epithelial and prostate stromal cells has been associated with increased expression of the WT1 target genes of the IGF axis (8,30).

However, we and others have observed paradoxical elevated WT1 expression in aggressive androgen-insensitive prostate cancer cell lines (5,36). This paradoxical expression of tumor suppressor genes in tumor tissue is often described but poorly understood. To address the issue of WT1 expression levels in tumor tissue we are exploring differences in WT1 expression relative to Gleason grade and clinical stage.

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