

Hormone treatment enhances WT1 activation of *Renilla* luciferase constructs in LNCaP cells

Julie Hanson, Jennifer Reese, Jacquelyn Gorman, Jennifer Cash, and Gail Fraizer

Department of Biological Sciences, Kent State University, Kent, OH 44242 USA

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1. ABSTRACT

The zinc finger transcription factor, WT1, regulates many growth control genes, repressing or activating transcription depending on the gene and cell type. Based on earlier analyses of the effect of WT1 on androgen responsive genes, we hypothesized that there may be an interaction between the androgen signaling pathway and WT1, such that the commonly used *Renilla* luciferase control vectors were activated in LNCaP prostate cancer cells. Using co-transfection assays we tested the effects of WT1 and/or the androgen analog, R1881, on two *Renilla* luciferase vectors, pRL-SV40 and the promoter-less pRL-null. To determine whether the zinc finger DNA binding domain was required, the zinc finger mutant DDS-WT1 (R394W) was tested; but it had no significant effect on the *Renilla* luciferase vectors. To determine whether the androgen signaling pathway was required, WT1 was co-transfected with *Renilla* vectors in cells with varied hormone responsiveness. The WT1 effect on pRL-null varied from no significant effect in 293 and PC3 cells to very strong enhancement in LNCaP cells treated with 5nM R1881. Overall, these results suggest that hormone enhanced WT1 mediated activation of *Renilla* luciferase and that these interactions require an intact WT1 zinc finger DNA binding domain.

2. INTRODUCTION

During the process of studying transcriptional regulation of prostate cancer genes, we observed unexpected results regarding control vectors. The use of control *Renilla* luciferase vectors as normalizers requires that they not be influenced by any variables in the experiment. However, we and others have shown that at *Renilla* luciferase control vectors such as pRL-TK and phRL-SV40 can be modulated by hormones (1, 2) and by transcription factors (3, 4). Androgen induction of control vectors has been previously documented; in LNCaP cells R1881 increased phRL-SV40 vector 9-fold (1). Other control vectors regulated by TK or CMV promoters such as pRL-TK and phRL-CMV have shown similar 8- and 11-fold increases respectively when exposed to R1881 (1). In addition to activation by R1881 and DHT, pRL-TK can also be suppressed by dexamethasone (DEX) (2). Androgen induction of *Renilla* luciferase has been ascribed to the presence of two putative androgen responsive elements in the RL vector series (1). In addition, binding sites exist within the *Renilla* luciferase gene (accession number AF025845) for zinc finger transcription factors such as SP1 and GATA-4 or GATA-6 and they modulate *Renilla* luciferase activity (3, 4). Both the pRL-TK and the pRL-SV40 control vectors have increased (up to 8-fold)

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Renilla luciferase activity when either GATA-4 or GATA-6 is present (4). *Renilla* luciferase activity increased 12-fold when the transcription factor Sp1 is present (3).

Following similar observations, we asked whether the zinc finger transcription factor Wilms' Tumor gene, WT1 altered *Renilla* luciferase activity in LNCaP cells, a human prostate cancer cell line. WT1 is critical for kidney development; and mutations or loss of this gene can result in the development of tumors (5). WT1 protein is found in several isoforms and the isoform lacking the KTS insertion (-KTS) is the most transcriptionally active (6). The WT1 (-/-) isoform lacking exon 5 and KTS regulates the promoter regions of several growth factor genes and their receptors (5, 7). Target genes for WT1 include those important for growth control such as growth factors and their receptors (8, 9, 10). It is known that WT1 binds to the promoter regions of many genes and can repress or activate transcription depending on the cell type and the target gene (5). Based on earlier analyses of the effect of WT1 on androgen responsive genes, we hypothesized that there may be an interaction between the androgen signaling pathway and WT1 (7, 11, 12). This type of interaction has recently been reported for WT1 and estrogen receptor in breast cancer cells (13). Recently we have examined regulation of vascular endothelial growth factor (VEGF), a growth factor essential for angiogenesis in tumors. Since VEGF expression is hormone responsive (14, 15, 16, 17) and is important in prostate cancer, we investigated its regulation in LNCaP cells. Our previous studies indicated that VEGF was differentially expressed in LNCaP cells expressing either the wild-type WT1 or the DDS-WT1 mutant (R394W) (11) and that hormone treatment affected expression of VEGF in these engineered lines (11). However, regulation of VEGF is complex and the VEGF promoter can be induced by both dihydrotestosterone (DHT) (17), and estradiol (18) *in vitro*. While we examined the transcriptional regulation of VEGF by WT1, we discovered that WT1 and R1881 together enhanced *Renilla* luciferase activity.

Since potential interaction of WT1 with androgen signaling might involve zinc finger interaction, we asked whether up-regulation of the pRL-null construct would require a functional zinc finger domain. To determine the importance of the DNA binding domain for the activation of the *Renilla* luciferase control vectors, we examined the effect of a mutant form of WT1, DDS-WT1 (R394W) bearing a mutation in the third zinc finger, affecting its ability to bind DNA (19). Previous studies have suggested that for some promoter/regulatory regions DDS-WT1 can function similarly to the wild-type WT1 (20). Tajinda *et al.* demonstrated that the DDS-WT1 mutant functions similarly to wild-type WT1, repressing the IGF-I receptor promoter (20). In fact, when wild type WT1 was co-transfected with DDS-WT1 an additive effect on IGF-I receptor promoter repression was observed (20). However for other promoters, DDS-WT1 mutants lack DNA-binding capabilities (19) and can actually impede the function of wild type WT1 (21).

To explore the mechanism of WT1-hormone interaction we asked whether the SV40 promoter was

essential for hormone enhancement. We repeated these experiments with a promoter-less *Renilla* luciferase control vector, pRL-null, similar to those used successfully by others to normalize transfections (22). Overall, these results suggested that hormone enhanced WT1 mediated activation of *Renilla* luciferase and that these interactions required an intact WT1 zinc finger DNA binding domain.

3. MATERIALS AND METHODS

3.1. Plasmids

Cytomegalovirus (CMV) promoter driven WT1 (-/-) (lacking both KTS insertion and exon 5) expression construct and pCB6+ empty expression vector were previously described (7, 12). The mutant DDS -WT1 expression construct contains the mouse wt1 gene with an Arg to Trp mutation (R394W) in the third zinc finger (19). In this study two different *Renilla* luciferase reporters were tested, the pRL-SV40 with an SV40 promoter and the pRL-null lacking a promoter (Promega Madison, Wisconsin). These assays were performed in the presence of VEGF promoter-luciferase reporter constructs obtained from Dr. K. Xie (23). The minimal (VEGF 88), full length (VEGF 2274), and deleted (VEGF 411) promoter constructs were tested. All DNA was purified by the Qiagen plasmid Maxi Kit.

3.2. Transfection and reporter assays

LNCaP prostate cancer cells (ATCC CRL 1740 from the American Type Culture Collection, Rockville, MD) and PC3 cells (ATCC CRL 1435), an androgen-independent prostate cancer cell line, were grown in RPMI-1640 media (Invitrogen) with 10% fetal calf serum (FCS). HEK-293 cells (ATCC CRL 1573), a kidney cell line, were maintained in DME media supplemented with 10% FCS as previously described (12). In preparation for the transfections, the cells were cultured in 12-well plates. When the cells reached 80% confluency they were transfected as described (12) using lipofectamine 2000 (Invitrogen, Carlsbad CA) in serum- and antibiotic-free media. Increasing concentrations of the WT1 (-/-) or DDS-WT1 (0, 0.25, 0.5 micrograms) were added to the wells. DNA levels were held constant by the addition of the empty CMV expression vector CB6+ (0, 0.25, or 0.5 micrograms per well). 5ng of pRL-null *Renilla* control plasmid or 2.5ng of pRL-SV40 (Promega Madison, Wisconsin) was added to each well to assess the effects of WT1 or DDS-WT1 on the *Renilla* luciferase constructs. For LNCaP transfection, medium was removed after 5-6 hours and fresh RPMI with 10% ChS FCS or full serum RPMI was added. For those plates that received ChS, half of the wells were treated with 0nM and half with 5nM R1881 (methyltrienolone). For PC3 transfections, medium was removed after 5-6 hours and replaced with full serum RPMI. For HEK-293 cells, DME media containing 10% FCS was replaced 18 hours after transfection. After 72 hours cells were harvested and both firefly and *Renilla* luciferase activity was measured as per manufacturer's recommendations using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin) and a 20/20n luminometer (Turner Sunnyvale, California). The protein concentration of 10 microliter samples of cell

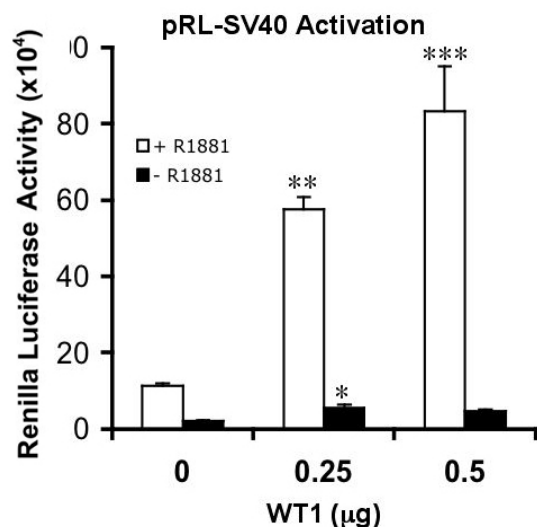


Figure 1. Both androgen and WT1 induce *Renilla* luciferase activity of the pRL-SV40 control in LNCaP cells. Cells were cultured in RPMI with ChS +/-5nM R1881 for 72 hours following transfection with 2.5ng pRL-SV40, VEGF 88 promoter construct and increasing amounts (0 to 500 ng) of WT1 (-/-) expression construct as described in the text. DNA levels were held constant by addition of empty vector pCB6+. Each experiment was performed in quadruplicate and results are given as mean +/-SEM. Significant differences between cells transfected with WT1 or vector control, CB6+, were determined by ANOVA ($p < 0.0001$) for cells treated with 0nM R1881 (black) or 5nM R1881 (white) and ChS-RPMI. Asterisks indicate significant differences by the Tukey-Kramer Multiple Comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

extract was determined using the Micro BCA Protein Assay Reagent kit (Pierce), and absorbance was read at 570nm on a Dynex Technologies MRX Revelation plate reader (Chantilly, Virginia). Average protein concentration was determined using a BSA standard and normalized luciferase activity was reported relative to the protein concentration of the cell extracts ($\times 10^5$).

3.3. Statistical analysis

Each transfection was performed in quadruplicate and repeated at least three times. Standard errors of the mean were determined using the GraphPad InStat statistical software program (San Diego, CA). Significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer Multiple Comparison Test. Two-way analysis of variance (ANOVA) (GraphPad Prism; San Diego, CA) was used to demonstrate significance of the interaction between WT1 and R1881.

4. RESULTS AND DISCUSSION

4.1. WT1 activates *Renilla* luciferase reporters

To address the effect of hormone on WT1 mediated transcriptional regulation in LNCaP reporter assays, we tested both SV40 promoter driven (pRL-SV40) and a promoter-less (pRL-null) *Renilla* luciferase reporter construct. We verified that hormone treatment induced

pRL-SV40 (5-fold) in LNCaP cells (Figure 1), and WT1 also up-regulated the pRL-SV40 construct 2.5-fold (Figure 1). This induction is consistent with previous research, which concluded that WT1 binds to the SV40 promoter/enhancer element (24). However, the novel observation that hormone treatment greatly increased WT1 mediated activation of the pRLSV40 construct was a surprising result as 0.5 micrograms of WT1 significantly enhanced *Renilla* luciferase values 7-fold in cells treated with 5nM R1881, $p < 0.0001$ using ANOVA (Figure 1). Two-way ANOVA analysis determined that treatment with hormone had a significant effect ($p < 0.0001$). In addition, the interaction between hormone and the dose of WT1 was also determined to be significant ($p < 0.0001$). Similar up-regulation was observed in LNCaP cells co-transfected with WT1 and VEGF 2274, full length VEGF promoter (data not shown).

A potential explanation for this strong activation in the presence of hormone is that both WT1 and the androgen receptor bind to SV40 promoter in the pRLSV40 *Renilla* luciferase construct and increase its activity. Sequence analysis reveals potential binding sites for both in the SV40 promoter region (1, 24). To test the requirement for the binding sites within the SV40 promoter, we investigated the use of a promoter-less *Renilla* luciferase control vector, pRL-null, to determine whether it is also regulated by WT1 and hormone. Initially we cultured cells in RPMI with FCS to assess the effect of WT1 itself (without R1881 treatment). Surprisingly, WT1 enhanced the *Renilla* luciferase activity 3-fold in the presence of full serum in LNCaP cells co-transfected with VEGF 88 (Figure 2A). Similar results were obtained in LNCaP cells co-transfected with the larger VEGF promoter construct (VEGF 411) in which WT1 increased *Renilla* luciferase activity 2-fold $p < 0.0001$ using ANOVA (Figure 2B). Significant enhancement in both VEGF 88 and VEGF 411 co-transfected LNCaP cells was demonstrated using ANOVA ($p < 0.0001$) and verified by the Tukey-Kramer Multiple Comparison post-test.

Despite the absence of the SV40 promoter binding sites, WT1 activated the pRL-null luciferase vector. To better understand the mechanism whereby WT1 affects pRL-null, we asked whether WT1 activation of *Renilla luciferase* expression required a functional DNA binding domain. LNCaP cells co-transfected with DDS-WT1 (R394W), the mutant form of the WT1 gene, had no significant affect on *Renilla* luciferase (Figure 2A and 2B). Even when DDS-WT1 transfected LNCaP cells were treated with 5nM R1881 no significant enhancement of luciferase activity was observed (data not shown). This suggests that without a functional binding domain, WT1 is unable to modulate *Renilla* luciferase activity in LNCaP cells, and this lack of activity cannot be rescued by hormone treatment.

4.2. Hormone enhances WT1 activation of *Renilla* luciferase reporter

To determine whether ARE binding sites within the SV40 promoter were required for the hormone enhanced activation by WT1, we transfected cells with pRL-null and treated with ChS FCS in the presence or

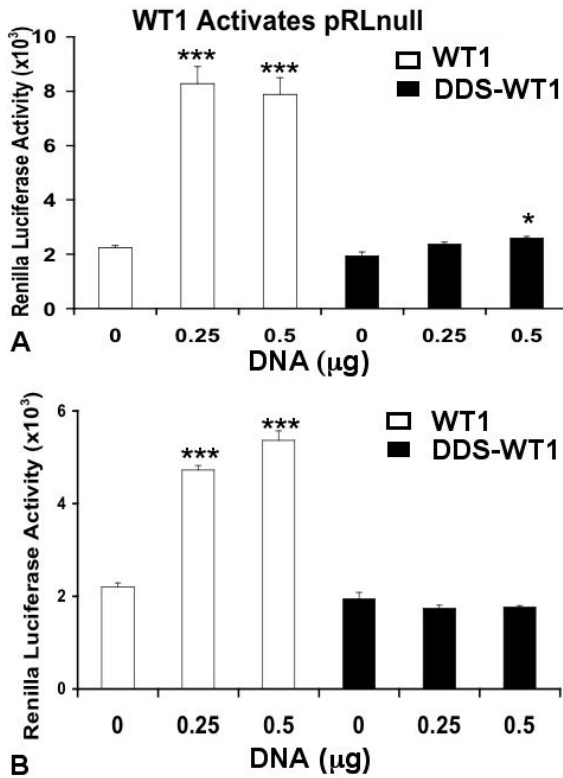


Figure 2. WT1, but not DDS-WT1, enhances *Renilla* luciferase activity of pRL-null controls in LNCaP cells. Cells were cultured in RPMI with FCS for 72 hours after transfection with 5ng pRL-null and increasing amounts of WT1 (-/-) or DDS-WT1. DNA levels were held constant by addition of empty vector pCB6+. In panel A, cells were co-transfected with a minimal VEGF promoter construct, VEGF 88 (as described in the text). In panel B, cells were co-transfected with a larger VEGF promoter construct (VEGF 411). Each experiment was performed in quadruplicate and results of experiments are given as mean +/-SEM. WT1 (white) greatly increased *Renilla* luciferase activity as determined by ANOVA ($p < 0.0001$) whereas DDS-WT1 (black) had little or no effect ($p = 0.0434$ for figure 2A and $p = 0.5787$ for figure 2B). Asterisks indicate significant differences determined by the Tukey-Kramer Multiple Comparison Test as described in Figure 1.

absence of 5nM R1881. Promoter-less vectors lack enhancer and promoter elements, so are not expected to be hormone responsive as they lack known ARE binding sites. Unlike pRL-SV40, the pRL-null was not induced by hormone treatment alone in LNCaP cells co-transfected with either a VEGF 88 or VEGF 411 reporter construct (Figure 3 panel A and panel B respectively). In the absence of hormone, WT1 modestly increased pRL-null *Renilla* luciferase activity in LNCaP cells co-transfected with 0.5 micrograms of WT1 and either VEGF 88 (1.64-fold) or VEGF 411 (2.22-fold) (Figure 3, panel A and panel B). This slight, but significant activation of *Renilla* luciferase in the absence of hormone ($p < 0.05$, ANOVA), was verified by the Tukey-Kramer Multiple Comparison Test. In contrast, in the presence of hormone, WT1 greatly

enhanced *Renilla* luciferase activity of the pRL-null vector 11-fold and 10-fold in LNCaP cells co-transfected with VEGF 88 and VEGF 411, respectively, $p = 0.0002$, ANOVA (Figure 3, panel A and panel B). Two-way ANOVA analysis demonstrated that the interaction between the dose of WT1 and R1881 was significant ($p < 0.0001$) for both VEGF constructs. This enhancement in the presence of both hormone and WT1 was similar to that observed for pRL-SV40 transfected cells.

The simplest explanation for the enhanced activation of the pRL-null construct by the combination of hormone and WT1 would be that WT1 protein levels are elevated in the presence of hormone. Since R1881 could activate the CMV promoter of the WT1-CB6+ expression construct, we measured WT1 protein levels in transfected LNCaP cells. However western blot analysis showed that WT1 protein levels were not increased by 5nM R1881 treatment of transfected LNCaP cells (data not shown).

A second possibility is that in LNCaP cells hormone-AR complexes stabilize WT1 binding to the pRL-null vector. Since this potential interaction could only occur in hormone responsive cells, we co-transfected the hormone insensitive PC3 cells with WT1, VEGF 411 and pRL-null (Figure 4A). As predicted WT1 did not significantly activate pRL-null in transfected PC3 cells, as determined by ANOVA ($p = 0.9061$). We confirmed this result in another hormone insensitive cell line, HEK-293, that was co-transfected with WT1, VEGF 411 and pRL-null (Figure 4B). Once again, WT1 in the absence of hormone did not significantly activate the pRL-null construct, as determined by ANOVA ($p = 0.2395$). (Figure 4B). Although the pRL-null reporter construct was unaffected by WT1, and therefore an adequate normalizer for WT1 in both PC3 cells and HEK-293 cells, it was not suitable for LNCaP cells.

Since pRL-null is not an adequate control for WT1 transfected LNCaP cells, cellular protein concentration was used to normalize *Renilla* luciferase activity. The cellular protein concentration depends upon cell viability and has been used as a normalizer in previous transfection studies (25, 26). The total cellular protein content was determined for each sample and was found to remain relatively constant (varying only 19-27%) indicating little variation between numbers of cells. Consequently, when *Renilla* luciferase values were normalized with total cellular protein (Figure 5) we obtained similar results to those described above (Figures 2 and 3). In LNCaP cells cultured in FCS, WT1 up-regulated *Renilla* luciferase activity 3- or 2-fold in cells co-transfected with VEGF 88 or VEGF 411, respectively, $p < 0.01$, ANOVA (Figure 5 Panel A and B). Similarly in LNCaP cells treated with 5nM R1881, WT1 up-regulated normalized *Renilla* luciferase activity 12-fold and 9-fold in cells co-transfected with VEGF 88 or VEGF 411, respectively (Figure 5 Panel A and B). Significant differences in the means of each group treated with hormone were demonstrated by ANOVA ($p < 0.001$) and validated by the Tukey-Kramer Multiple Comparison post-

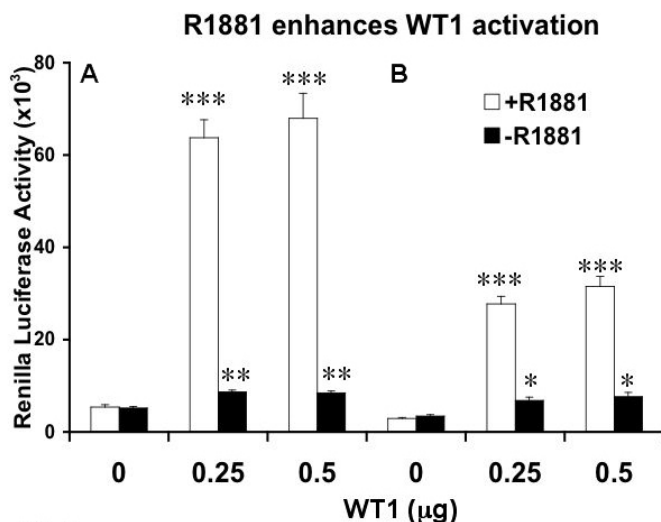


Figure 3. WT1 activation of pRL-null is greatly enhanced by hormone treatment. LNCaP cells were cultured in RPMI with ChS +/- 5nM R1881 for 72 hours after transfection with 5ng pRL-null, increasing amounts of WT1 (-/-) along with the minimal VEGF promoter construct VEGF 88 (A, left side) or VEGF 411 (B, right side) as described in Figure 2. Each experiment was performed in quadruplicate and results are given as mean +/- SEM. Significant differences between cells transfected with WT1 or CB6+ were determined by ANOVA for cells treated with 0nM R1881 (black) or 5 nMR1881 (white). Asterisks indicate significant differences by the Tukey-Kramer Multiple Comparison post-test as described in Figure 1.

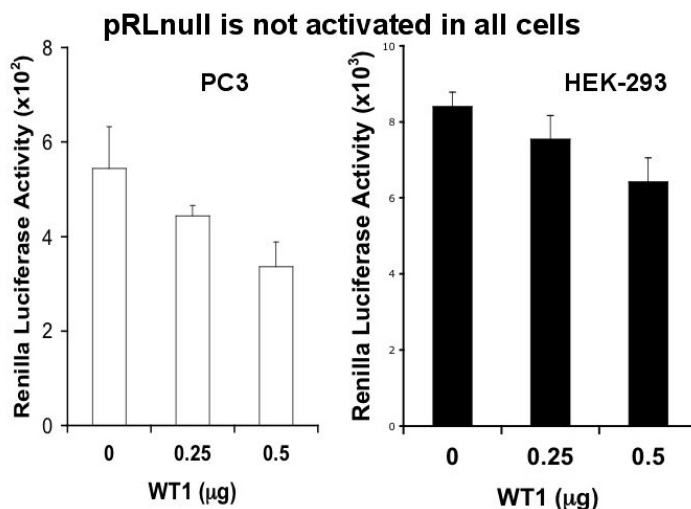


Figure 4. WT1 does not enhance *Renilla* luciferase activity in androgen insensitive cells. The androgen-independent PC3 prostate cancer cells (left panel) and human embryonic kidney cells, HEK-293 (right panel) were co-transfected with 5ng pRL-null, increasing amounts of WT1 (-/-) and the VEGF 411 reporter as described in Figure 2. Each experiment was performed in triplicate and results are given as mean +/-SEM. No significant differences were observed using the Tukey-Kramer Multiple Comparison Test.

test. In contrast, in the absence of hormone, WT1 only slightly increased normalized *Renilla* luciferase activity (about 2-fold) in LNCaP cells co-transfected with either VEGF 88 or VEGF 411, $p < 0.05$, ANOVA (Figure 5). Two-way ANOVA analysis verified that the interaction between WT1 and R1881 was significant ($p < 0.0001$) for both VEGF constructs. Overall, the BCA normalized results shown in Figure 5 were similar to those shown in Figures 2 and 3. Thus these results support our conclusion that hormone alone did not affect the promoter-less pRL-null and WT1 only modestly induced luciferase activity,

but their combined action dramatically increased *Renilla* luciferase activity.

In this study, we have demonstrated that hormone strongly enhanced WT1 mediated activation of both the pRL-SV40 and pRL-null vectors in cells with intact androgen signaling pathways. Both WT1 and hormone enhanced *Renilla* luciferase activity of the pRL-SV40 construct in LNCaP cells. In contrast, the promoter-less control vector, pRL-null was not induced by hormone alone, but was strongly enhanced by the combination of

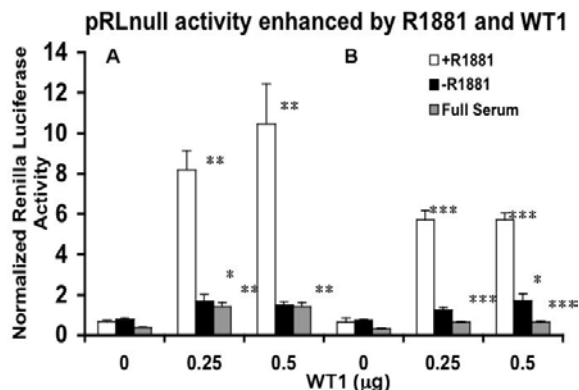


Figure 5. Hormone enhancement of WT1-mediated activation of pRL-null validated by cellular protein normalization. LNCaP cells were treated with 0nM R1881 (black) or 5 nMR1881 (white) and ChS-RPMI or FCS (gray) for 72 hours after transfection with 5ng pRL-null, increasing amounts of WT1 (-/-) along with the minimal VEGF promoter construct VEGF 88 (A, left side) or VEGF 411 (B, right side) as described in Figure 2. Each experiment was performed in quadruplicate and activity in WT1 transfected cells was compared to cells transfected with CB6+. *Renilla* luciferase values were normalized by protein concentration as described in the text and are given as mean +/-SEM. Significant increases were determined by ANOVA followed by the Tukey-Kramer Multiple Comparison post-test (significance indicated by asterisks as described in Figure 1). Protein normalization gave similar results to Figure 3.

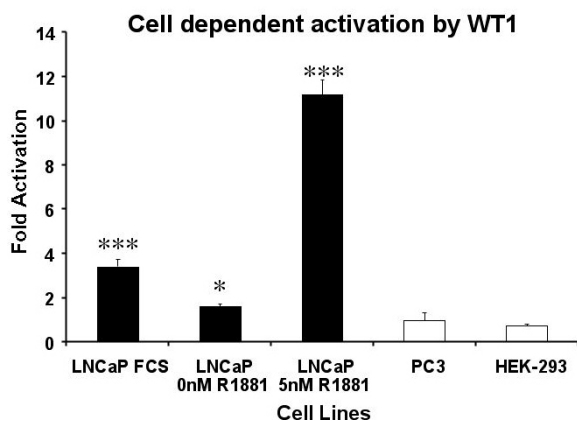


Figure 6. WT1-mediated activation of pRL-null correlates with hormone responsiveness of transfected cells. Cells were transfected with either 500ng WT1 or CB6+ vector DNA and treated with FCS or ChS with or without R1881, as described in the text. Each experiment was performed in triplicate and repeated at least three times. Results are given as fold activation by WT1 transfection and shown relative to CB6+ vector control for each cell line. Asterisks indicate significant differences between the mean luciferase activities of transfected vector control and WT1 as determined by the Tukey-Kramer Multiple Comparison post-test as described in Figure 1. The effect of WT1 on *Renilla* luciferase activity varied from non-significant (PC3 and HEK-293) to strong activation (LNCaP cells treated with R1881).

WT1 and hormone. We also discovered that the mutant DDS-WT1 had no significant effect on the *Renilla* luciferase activity of the pRL-null control vector, suggesting that a functional binding domain is necessary to modulate *Renilla* luciferase activity. In addition, WT1-mediated induction of the pRL-null appeared to be androgen responsive since *Renilla* luciferase activity was not induced in the androgen insensitive PC3 and HEK-293 cells. A comparison of WT1 transfected cells showed that WT1 mediated activation of *Renilla* luciferase correlated with hormone responsiveness of transfected cells and was greatly enhanced by R1881 treatment in androgen responsive LNCaP cells (Figure 6). The WT1 effect on pRL-null varied from no significant effect in 293 and PC3 cells (0.7-fold and 0.9-fold, respectively) to significant activation in LNCaP cells cultured in FCS (3.5-fold) and strong enhancement in LNCaP cells treated with 5nM R1881 (10.5-fold upregulation compared to the vector CB6+ transfected cells).

Overall the combined mechanism of activation of pRL-null by WT1 and R1881 depends upon the WT1 zinc finger DNA binding domain and an intact androgen signaling pathway. Thus both WT1 and hormone are necessary, but alone neither are sufficient for the high level induction we observed in LNCaP cells. R1881 alone had no effect on pRL-null, but WT1 alone only weakly activated the null *Renilla* control (similarly to the pRLSV40 *Renilla* construct). The importance of these results is that the WT1 zinc finger DNA binding domain appears to play a role in hormone enhanced WT1-mediated regulation of transcriptional targets. Future studies are aimed at defining the hormone WT1 interaction domains.

5. ACKNOWLEDGMENTS

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Send correspondence to: Dr Gail Fraizer, Dept Biological Sciences, Kent State University, Kent, OH, 44242, Tel: 330-672-1398, Fax: 330-672-3713, E-mail gfraizer@kent.edu

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