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Gail Fraizer      10-7-99  
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## Introduction

The androgen pathway is central to prostate tumorigenesis. An increased risk of higher stage, more aggressive prostate cancer is associated with the inheritance of a more active androgen receptor (AR) (1-3). We are investigating an innovative transcription based mechanism that represses AR activity *in vitro*. The tumor suppressor gene, WT1, transcriptionally represses many growth control genes including AR. Our hypothesis is that WT1 may play a role in prostate tumorigenesis mediated by repression of AR gene expression. We have previously demonstrated that WT1 repression of the AR gene promoter construct (4) is mediated by DNA binding. To validate these exogenous AR promoter data we demonstrated that WT1 represses the endogenous AR promoter in androgen responsive cells (5). Now we have demonstrated that this AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. Additionally using Western blot analysis we confirmed the inverse relation between expression of WT1 and AR in prostate cancer cell lines, previously demonstrated by RT-PCR analysis. Androgen responsive cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR, suggesting a correlation with late-stage androgen independence. Thus, our hypothesis that WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells is being tested using both *in vitro* and *in vivo* approaches which assess both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. With our recent establishment of stably transfected LNCaP and MDAPCa2b lines we will now be able to determine their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. If the correlation of WT1 expression with higher grade disease is upheld in on-going studies and if WT1 represses AR expression *in vivo*, then we will have established a model of WT1-mediated progression of prostate tumors to androgen independence.

## BODY OF WORK

We have demonstrated an inverse correlation between WT1 and AR expression in several prostate tumor cell lines, suggesting that WT1 may play a role in prostate tumorigenesis. This hypothesis is supported by previous observations by others that in prostate tumor biopsies a significantly higher percentage of tumor cells (30%) expressed WT1 protein than did adjacent normal cells (12%) and normal prostate biopsies showed no significant WT1 expression (6). Our recent work (discussed below) has confirmed and extended these previous findings.

## PREVIOUS RESULTS:

### 1. WT1 and AR expression are inversely correlated

The expression of AR during fetal and postnatal development in androgen target tissues is inversely correlated to WT1 expression. Using RT-PCR we previously confirmed this inverse relationship of WT1 and AR expression in the prostate, an androgen responsive tissue. In collaboration with Dr. Nora Navone, we determined that WT1 is not expressed in LNCaP and MDAPCa2b, two androgen-responsive prostate tumor cell lines expressing AR mRNA. Conversely, WT1 is expressed in DU145 and PC3; two androgen unresponsive, highly tumorigenic prostate tumor cell lines that lack AR expression. If WT1 also represses the AR gene promoter *in vivo*, then inhibition of AR-induced transactivation of AR target genes may suppress tumor cell growth in androgen-responsive prostatic tumor cells. This work has been extended by examining protein expression levels in the prostate tumor cell lines and in the tumor progression cell lines derived from both LNCaP and PC3 prostate cancer cell lines.

**2. WT1 repression of the AR promoter is mediated by DNA binding**

In previous work we have demonstrated 3-10-fold repression of the AR promoter by overexpression of WT1(-KTS) isoforms in transient transfection assays in HeLa, T47D (Breast cancer), SaOS (osteosarcoma), 293 (kidney) and TM4 (Sertoli) cells. The specificity of this repression was confirmed by demonstrating the inability of a zinc-finger mutant WT1 expression construct to repress the AR promoter construct in HeLa cells. Thus, WT1 repression of the AR promoter requires a functionally intact DNA binding domain. This repression was demonstrated by electrophoretic mobility shift assay (EMSA) to be mediated by binding of the WT1 protein to at least two regions containing 6 potential WT1 binding sites in the AR promoter. This work has been extended by searching for other possible mechanisms of interaction between WT1 and AR, such as protein-protein interactions. We co-transfected both AR and WT1 expression constructs into HeLa and Cos cells and examined the effect on downstream targets as well as directly assessing physical interactions by co-labeling studies.

**3. WT1 repression of the AR pathway**

For a better understanding of the role played by WT1 in regulating the expression of AR and AR-target genes, we examined the effect of overexpression of WT1 in 293 kidney and TM4 Sertoli cells expressing AR. In these assays the ability of WT1 to directly repress the endogenous AR promoter resulted in an indirect repression of an AR-target gene construct containing four copies of the ARE binding site (GGTACAnnnTGTTCT), the E1B TATA box and the luciferase gene. WT1 over-expression in 293 and TM4 cells down-regulates AR and indirectly results in a dose-dependent reduction in ARE-activated luciferase activity. These experiments have been extended to prostate tumor cells, LNCaP and MDAPCa2b. This has allowed us to confirm this indirect mechanism by dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1. We are working towards reproducing these experiments in both LNCaP and MDAPCa2b in such a way that will allow quantitative western blot analysis of WT1 and AR expression in the transfected LNCaP and MDAPCa2b cells. Towards this end we have stably transfected a subline of LNCaP cells (obtained from our collaborator, Dr. G. Jenster) with WT1-FLAG expression constructs (obtained from another collaborator, Dr. C. Roberts). The stably transfected lines will allow quantitative western blot analysis using both WT1 and FLAG Ab. Stable transfections of the MDAPCa2b cell line are also being performed in collaboration with Dr. Navone. Concurrently we have established a TET-ON stably transfected LNCaP cell line in which we have transiently transfected WT1-FLAG expression constructs.

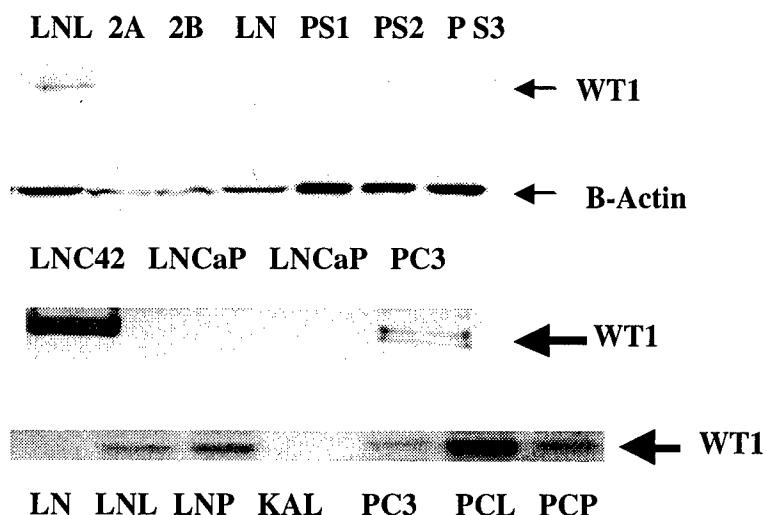
These previous results demonstrated that: 1) WT1 and AR expression are inversely correlated in androgen target tissues, 2) WT1 protein binds at least two of the WT1 binding sites in the AR promoter *in vitro*, 3) WT1 directly binds and represses exogenously added AR promoter constructs in kidney and gonadal cells, and 4) WT1 repression of the endogenous AR promoter in kidney and gonadal cells interferes with the androgen signal transduction pathway causing down-regulation of AR target gene transcription. In the past year these findings of inverse expression and downregulation of AR by WT1 have been confirmed in prostate cell lines.



**YEAR ONE RESULTS:**

**TECHNICAL OBJECTIVE I.** To determine the prognostic significance of WT1 expression in prostate tumors.

**WT1 protein is strongly expressed in androgen insensitive prostate cancer cell lines , but not in androgen sensitive prostate cancer cell lines.**



Nuclear extracts containing 30 ug of protein were separated by electrophoresis through a 12% SDS-polyacrylamide gel and analyzed by Western blotting using WT1 Ab (c19, Santa Cruz). Immunoreactive proteins were visualized by ECL-Plus luminescence and autoradiography. High salt extracts were prepared from the following cell lines: ; LNL, LNCaP-LN3; 2A, MDAPCa2a; 2B, MDAPCa2b; LN, LNCaP; PS1, primary cultures of mouse stromal cells ; PS2; PS3; LNC42, LNCaP-C42; LNCaP; ; P, PC3; LNP, LNCaP-Pro5;

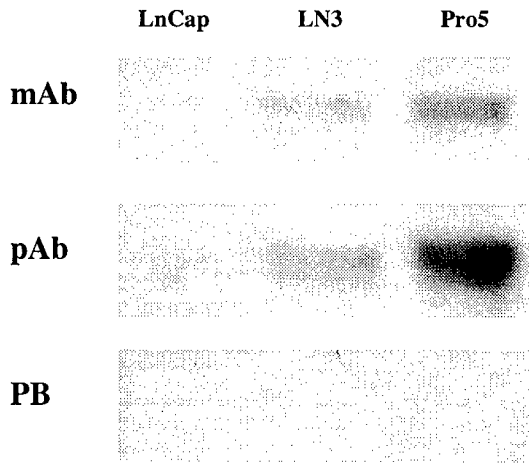
KAL, marker; ; PCL, PC3-LN; PCP, PC3-Pro. The position of the 45-47 kd WT1 proteins is marked with an arrow as is the position of the  $\beta$ -actin control. Note that WT1 expression is higher in the LNCaP sublines (LN3, Pro5 and C42) than in LNCaP.

These protein assays have now confirmed our previous RNA expression studies showing that WT1 expression patterns are inversely related to AR expression. Androgen responsive normal mouse prostate stromal lines and tumor cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR. The androgen independent LNCaP-C42 and LN-LN3 variants express WT1, while the androgen dependent LNCaP, MDAPCa2a and MDAPCa2b cells lack WT1, suggesting a correlation of WT1 expression with late-stage androgen independence.

Our results are in agreement with those showing primary cultures of normal epithelial cell strains lack significant WT1 expression (7). However, our results do not support the findings of others that some normal prostate stromal cell strains do express WT1, as detected by RT-PCR and RNase PA (7). In our primary cultures of mouse stromal cells, we saw no evidence of WT1 expression but did observe androgen response and strong AR expression. Similarly, the reported lack of WT1 expression in epithelial tumor cell strains, but presence in stromal tumor cell strains (7), is not supported by our immunohistochemistry results (see appendix). Indeed, the original finding was surprising as high WT1 expression levels seen in nephroblastomas are restricted to epithelial predominant tumors and are reduced in stromal predominant tumors (8). We have now observed WT1 expression in several epithelial prostate tumor cell lines, particularly the more aggressive, androgen unresponsive lines.

Interestingly LNCaP-Pro5, previously reported to be androgen sensitive (9), does express some WT1. However, using a dual labelling assay we have developed for WT1 and AR co-expression studies using TRITC-anti-rabbit and FITC anti-mouse antibodies we observed an inverse relation of WT1 to AR in these cells as well (Fig 12, Appendix). LNCaP cells express AR (FITC staining in left panel) and

5nM R1881 strongly induces AR immunofluorescence in LNCaP (FITC staining in right panel) But no WT1 staining is observed. In contrast LNCaP-Pro5 cells express AR only weakly (FITC staining in left panel) but do express WT1 in some cells (TRITC staining in left panel). Interestingly, in our hands, this androgen sensitive line is not highly responsive to R1881 induction. While 5nM R1881 strongly



induces AR immunofluorescence in LNCaP it only weakly induces AR in LNCaP-Pro5. (FITC staining in right panel). However, the AR positive LNCaP-Pro5 cells lack WT1 and the WT1 positive LNCaP-Pro5 cells lack AR expression. Thus, AR and WT1 are not co-expressed in the same cells as shown by the lack of yellow staining with the dual staining procedure. We confirmed these results by western blot analysis of LNCaP, LNCaP-LN3, and LNCaP-Pro5 progression lines. Initially we probed the filter with a mouse mAb (DAKO) which detects an amino terminus peptide of WT1. Then we stripped the western blot and reprobed with a polyclonal Ab which detects a carboxy terminus peptide. Finally we stripped and reprobed the western

blot with the polyclonal Ab which had been preincubated with blocking peptide (the immunogen). The mAb and the pAb gave identical results and the pAb was completely blocked by preincubation with the peptide. Thus, unlike LNCaP cells, the more aggressive LNCaP sublines express WT1, although on a single cell level, WT1 expression is inversely related to AR.

**WT1 protein is expressed in both nucleus and cytoplasm of androgen insensitive prostate cancer cell lines.** Western blot analysis of nuclear extracts and cytoplasmic remnants (supernatant removed from nuclear pellets) showed the presence of WT1 protein in both the nuclear and cytoplasmic fraction of androgen insensitive cell lines (DU145, LNCaP-LN3, PC3, and PC3 sublines). We confirmed the cytoplasmic location of WT1 protein using the same polyclonal anti-WT1 Ab (C19, Santa Cruz) in immunohistochemical analyses of prostate epithelial cell lines grown in 8-well chamber slides. We observed dark brown staining nuclei and light brown staining cytoplasm in the androgen insensitive cell lines (Data not shown). Specificity of staining was confirmed by treating Ab with WT1 blocking peptide prior to incubation of the polyclonal Ab with cell lines. While WT1 expression is not limited to the nucleus, its function (if any) in the cytoplasm is unknown.

#### **WT1 protein is focally expressed in both nucleus and cytoplasm of prostate tumor sections**

Using anti-WT1 Ab (C19, Santa Cruz) both dark brown staining nuclei and light brown staining cytoplasm are visible in prostate tumor epithelial cells (**Fig 6A** in appendix). **Fig 6B.** Focal cytoplasmic staining of epithelial cells of high grade prostate tumor sections. **Fig 6C.** Right panel shows specificity of staining by treating Ab with WT1 blocking peptide prior to incubation with tissues.

Stage and grade of prostate tumors were determined by medical record examination and correlated with WT1 expression. In limited analyses of 10 patients, WT1 expression correlates with high grade and stage tumors (Fig. 7). In samples of Gleason 5 grade tumors (**Fig 7C**) we observed no WT1 expression, while in Gleason 9 grade sections some displayed focal epithelial cytoplasmic staining (**Fig 7B shows high power view of Fig 7A**), sometimes accompanied by nuclear staining but often not. Normal prostate tissues and low grade PIN samples lacked WT1 expression. Additional evidence of correlation with grade using the TRAMP mouse model (10) confirmed these findings (Fig

8). WT1 expression was absent in normal tissues and PIN of all grades (**Fig 8C**), but was present in poorly differentiated mouse tumors (**Fig 8B**). Left panel (**Fig 8A**) shows specificity of staining by treating Ab with WT1 blocking peptide prior to incubation with tissues. As documentation of protein expression is more relevant to WT1 function we have focused our expression studies on immunohistochemistry and western blot assays. If unexpected isoform ratios are observed in mRNA isolated from cultured prostate tumor cells, then we will obtain frozen prostate tumor tissue for RNA analysis.

**TECHNICAL OBJECTIVE II.** To determine whether WT1 represses the androgen signal transduction pathway in prostate tumor cell lines.

Initially this work was extended by searching for other possible mechanisms of interaction between WT1 and AR, such as protein-protein interactions. We co-transfected both AR and WT1-FLAG expression constructs into HeLa and Cos cells and examined the effect on downstream targets as well as directly assessing physical interactions by immunoprecipitation assays (not shown) and co-labeling studies using immunofluorescent antibodies. Using a dual labelling assay we have developed for WT1 and AR co-expression studies we incubated the cotransfected monolayers (grown on glass slides) with monoclonal anti-FLAG Ab and polyclonal anti-AR Ab followed by incubation with TRITC-anti-mouse and FITC anti-rabbit antibodies. We detected TRITC-labeled and FITC-labeled cells and some dually-labeled cells (**Fig 11**, Appendix), however, on high power we observed primarily adjacent labelling without direct overlapping (as detected by the yellow color in far right panel).

Recently these dual labelling experiments have been extended to androgen responsive prostate tumor cells, LNCaP and MDAPCa2b (**Fig 12**). This has allowed us to exclude direct physical interaction and supports our earlier data suggesting an indirect mechanism of endogenous AR repression by WT1. Using dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1 we also demonstrated inverse expression. We are working towards reproducing these experiments in both LNCaP and MDAPCa2b in such a way that will allow quantitative western blot analysis of WT1 and AR expression in the transfected LNCaP and MDAPCa2b cells. Towards this end we have stably transfected a subline of LNCaP cells (obtained from our collaborator, Dr. G. Jenster) with WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts). These include the normal (-)KTS isoforms and the DDS zinc finger mutant (-)KTS isoforms which fail to bind DNA. The stably transfected lines will allow quantitative western blot analysis using both WT1 and FLAG Ab. Transfection efficiency in LNCaP cells is so low as to preclude bulk cell analysis of transfected monolayers. Thus we used a dual labelling FITC/TRITC Ab based assay to observe rare individual cells which were transfected with WT1 and were induced by hormone treatment.

Stable transfections of the MDAPCa2b cell line are also being performed in collaboration with Dr. Navone. Concurrently we have established a TET-ON stably transfected LNCaP cell line in which we have transiently transfected WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts).

**TECHNICAL OBJECTIVE III.** Establish a mouse model for prostate cancer progression.

With our recent establishment of stably transfected lines constitutively expressing WT1 or the zinc finger mutants we will now be able to determine their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. Towards this end we have established several stably transfected LNCaP cell lines. We have established a TET-ON stably transfected LNCaP cell line which we are able to transiently transfect with WT1-FLAG expression

constructs (obtained from our collaborator, Dr. C. Roberts). However, transfection efficiency in LNCaP cells is so low as to preclude selection of dually transfected monolayers. Thus the TET-On LNCaP cell lines which we have established respond to hormone but have not survived TRE-WT1 co-transfection with the TK-hygromycin selection construct. We are currently using an alternative drug selection marker (puromycin), but have also used an alternative approach to establishing stable lines. Thus we have established non-inducible WT1-FLAG tagged stably transfected LNCaP cells and stable transfections of the MDAPCa2b cell line are also being performed in collaboration with Dr. Navone. Because we anticipate poor growth of these lines (due to preliminary comparisons of FLAG vector transfected vs FLAG-WT1 transfected growth characteristics) we are also continuing our efforts in establishing regulatable WT1 expressing lines.

## **PLANS for YEAR TWO**

**TECHNICAL OBJECTIVE III.** Establish a mouse model for prostate cancer progression

- Task A. Assess growth characteristics of four stably transfected prostate tumor cell lines (months 12-18). Inducible overexpression of WT1 is expected to result in altered growth and tumorigenic characteristics.
- Task B. Each stably transfected prostate tumor cell line will be injected into nude mice to determine whether growth suppression in vitro correlates with tumor suppression or growth in vivo (18-24). We expect that if WT1 expression correlates with higher grade disease and AR repression, then it may play a role in the selection of androgen independent prostate tumor cells.

## KEY RESEARCH ACCOMPLISHMENTS

- **WT1 protein is strongly expressed in androgen insensitive prostate cancer cell lines , but not in androgen sensitive prostate cancer cell lines.**
- **WT1 protein is focally expressed in both nucleus and cytoplasm of prostate tumor sections**
- **WT1 expression correlates with high grade human tumors, poorly differentiated TRAMP mouse tumors.**
- **Normal prostate tissues and low grade PIN samples lacked WT1 expression in both human and mouse sections.**
- **TET-ON stably transfected LNCaP and WT1 stably transfected LNCaP and MDAPCa2b cell lines have been established.**

## REPORTABLE OUTCOMES

### **Presentations:**

REGULATION OF THE ANDROGEN RECEPTOR BY A TUMOR SUPPRESSOR GENE, WT1.  
Gail Fraizer. Fifty-first Annual Symposium on Fundamental Cancer Research, Poster Discussion Session II, Houston, Oct 28, 1998

REGULATION OF THE ANDROGEN RECEPTOR BY A TUMOR SUPPRESSOR GENE, WT1.  
Gail Fraizer. New Research Approaches in the prevention and cure of Prostate Cancer, AACR Special Conference, Dec, 1998

REGULATION OF THE ANDROGEN RECEPTOR BY A TUMOR SUPPRESSOR GENE, WT1.  
Gail Fraizer. American Urological Association Annual Meeting, Dallas, Texas, 1999

ROLE OF WT1 IN PROSTATE CANCER PROGRESSION, Urology Department Seminar Series,  
July 7, 1999

### **Published Abstracts:**

Annamaria Zaia, Ryuji Shimamura, Michael Badzioch, Grady Saunders, **Gail Fraizer**.  
Regulation of the Androgen Receptor by a Tumor Suppressor gene, WT1. AACR Special Conference in Cancer Research Abstract A#8, Indian Wells, CA, (1998)

Annamaria Zaia, Ryuji Shimamura, Grady Saunders, **Gail Fraizer**,  
Regulation of the Androgen Receptor by a Tumor suppressor gene, WT1. AUA 1999 Annual meeting, Dallas, Texas, 1999

### **Development of Cell Lines**

TET-ON stably transfected LNCaP cell lines have been established, clone #37 has been distributed to collaborators for use in other projects.

## CONCLUSIONS

If WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells, two possible predictions can be made. 1) Overexpression of a tumor suppressor and subsequent repression of the AR pathway could lead to tumor suppression. Or 2) If suppression of AR confers a growth advantage selecting AI tumor cells, then we would predict an outgrowth of aggressive hormone refractory tumor cells causing tumor progression. In the latter case, growth advantages conferred by the repression of the AR by WT1 lead to androgen independence. The hypothesis that WT1 can mediate androgen independent tumor progression is being tested using both in vitro and in vivo approaches which will assess both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. The first two specific aims have been performed concurrently and results will be confirmed and extended in the next few months. We will continue to examine WT1 expression for correlation with higher grade disease in an expanded analysis of metastatic human tumors and post-castration TRAMP mouse tissue sections. Stably transfected LNCaP and MDAPCa2b lines will be evaluated for their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression in the second year of this proposal. Additionally we will determine whether any tumors established by stably transfected LNCaP lines are androgen independent, ie, does WT1 also repress AR expression in vivo. Together these results will support our hypothesis that WT1 plays a role in the progression of prostate tumors to androgen independence.

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FIG. 6

cytoplasmic and nuclear  
WT1 expression in  
prostate tumors



FIG. 7

WT expression in Gleason 9 prostate cancer

A.



B.



WT expression in Gleason 5 prostate cancer

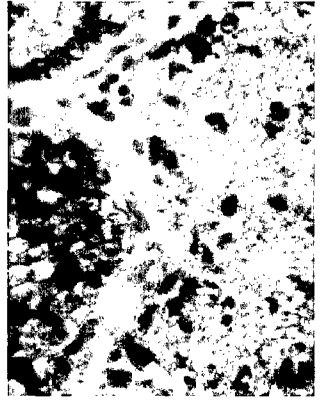
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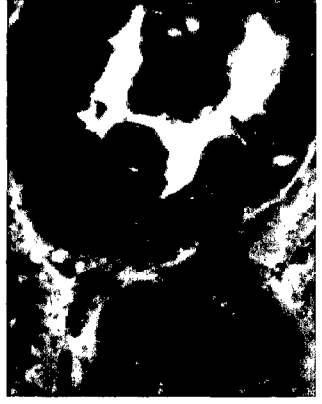
FIG. 8

WT1 expression in TRAMP model: poorly differentiated tumor

A.



B.



WT1 Expression in TRAMP Model: PIN

C.





Fig. 11

Co-localization of WT1 and AR in co-transfected cos cells

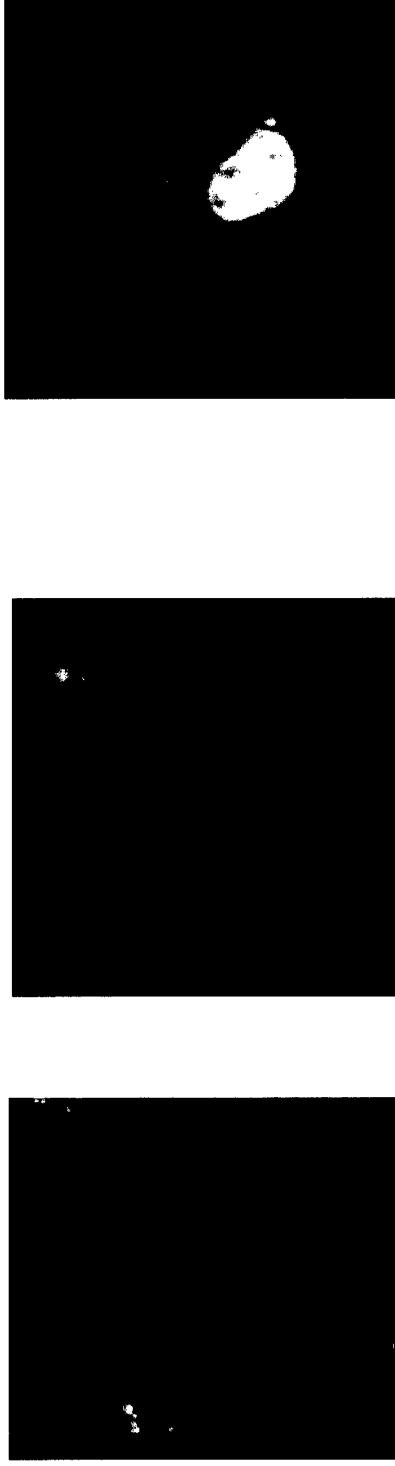


Fig. 12

Inverse expression of WT1 and AR in LNCap and LNCaP-Pro5 cells





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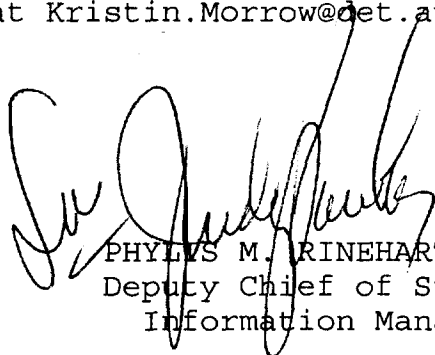
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