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Award Number: W81XWH-07-1-0022

TITLE: Enhancement of Vitamin D Action in Prostate Cancer through Silencing of CYP24

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REPORT DATE: February 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-02-2011	Final	15 Jan 2007 - 14 Jan 2011

4. TITLE AND SUBTITLE

Enhancement of Vitamin D Action in Prostate Cancer through Silencing of CYP24

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-07-1-0022

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study focuses on the enzyme, CYP24 which hydroxylates vitamin D acting to catalyze the first step in the breakdown of Vitamin D, effectively limiting this growth inhibitory signaling pathway. We are testing the hypothesis that hrough the inhibition of CYP24 using an siRNA approach we can convert prostate cancer cells that are resistant to the antiproliferative actions of Vitamin D to cells that are growth inhibited at low concentrations of Vitamin D. Inhibition of 1,25(OH)2D3 CYP24 mediated metabolism to potentiate Vitamin D actions in prostate cancer shows great potential for both a chemopreventative approach and the treatment of advanced hormone refractory cancer in patients. We have tested CYP24 siRNA constructs, ketoconazole and silencer control siRNA on three cell lines (LNCaP, PC3 and DU145) and evaluated CYP24 protein expression, mRNA expression, and growth inhibition. We are in the process of developing the stable transfected cell lines and optimal approach to enhance Vitamin D action in resistant cells.

15. SUBJECT TERMS

Prostate cancer, vitamin D, CYP-24 (24-hydroxylase), growth inhibition, apoptosis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	42	**19b. TELEPHONE NUMBER** *(include area code)*

FINAL PROGRESS REPORT

Specific Aim #1 To enhance Vitamin D inhibition of prostate cancer growth through inhibition of CYP24.

The summary below describes our progress towards meeting all aims on our funded proposal.

1. CYP24A1 is Over-expressed In Human Prostate Cancer: It was important to first assess the mRNA levels of endogenous CYP24A1 in adenocarcinomas of the human prostate. cDNAs obtained from pathologist-verified human prostate biopsies representing different TNM (Tumor, Node and Metastasis) stages of prostate cancer, as well as, benign prostatic hyperplasia (BPH) were analyzed by quantitative PCR. Despite the variability observed between individuals, the benign tissues expressed low levels of CYP24A1 while the majority of the analyzed adenocarcinomas (73%) had CYP24A1 highly expressed (Figure 1A). When the relative levels of mRNA were examined as mean values by TNM stage, a marked elevation of CYP24A1 was observed with increasing pathological grades of prostate cancer, with some heterogeneity at T3 (Figure 1A). Consistently, CYP24A1 protein was over expressed in adenocarcinomas of prostate compared to normal human prostate (Figure 1B). While normal cells demonstrated faint cellular staining for the catabolic enzyme, immunodetection of CYP24A1 revealed intense cytoplasmic staining in epithelial cells of both low and high Gleason grade adenocarcinomas (Figure 1B).

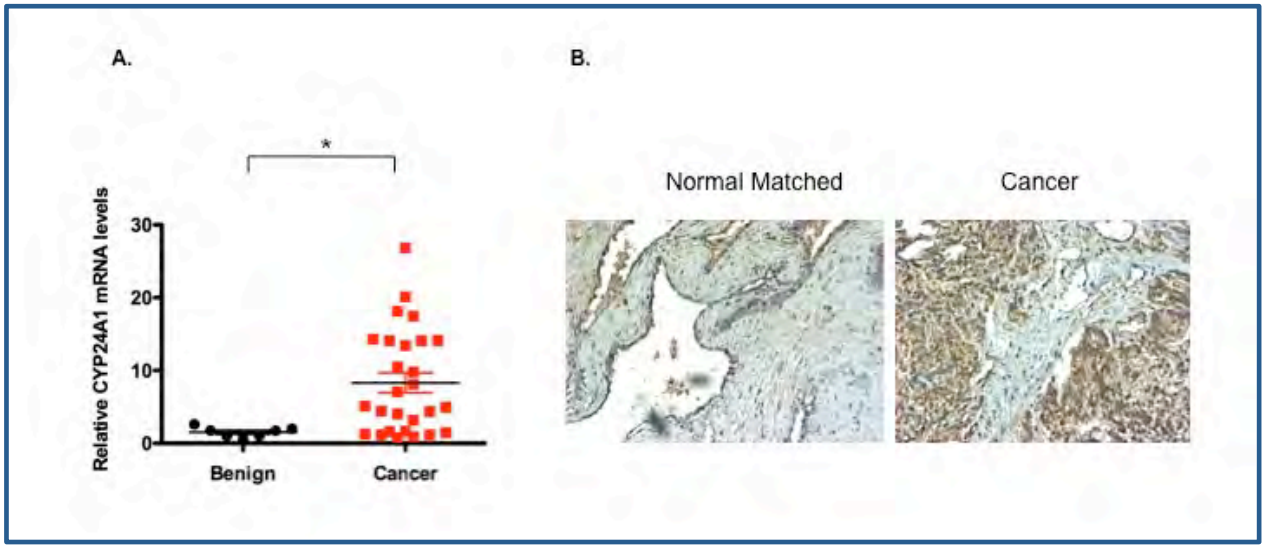


Figure 1: Over expression of CYP24A1 in men with prostate cancer. **A. CYP24A1 gene expression levels across the stages of human prostate cancer progression.** TaqMan QPCR was performed using specific CYP24A1 primers. β actin served as an internal control. Patients' cDNA were prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels. Clinicopathological information for each patient was provided by the supplier (Origene, MD, USA). Data were analyzed using the comparative CT method with the values normalized to β actin levels. Figure in inset is a scatter plot presenting qPCR results as averages of relative CYP24A1 mRNA levels in individuals presenting identical TNM stage**. B. Marked increase of cytoplasmic CYP24A1 immunostaining (brown) in representative human prostate adenocarcinomas compared to normal human prostate.**

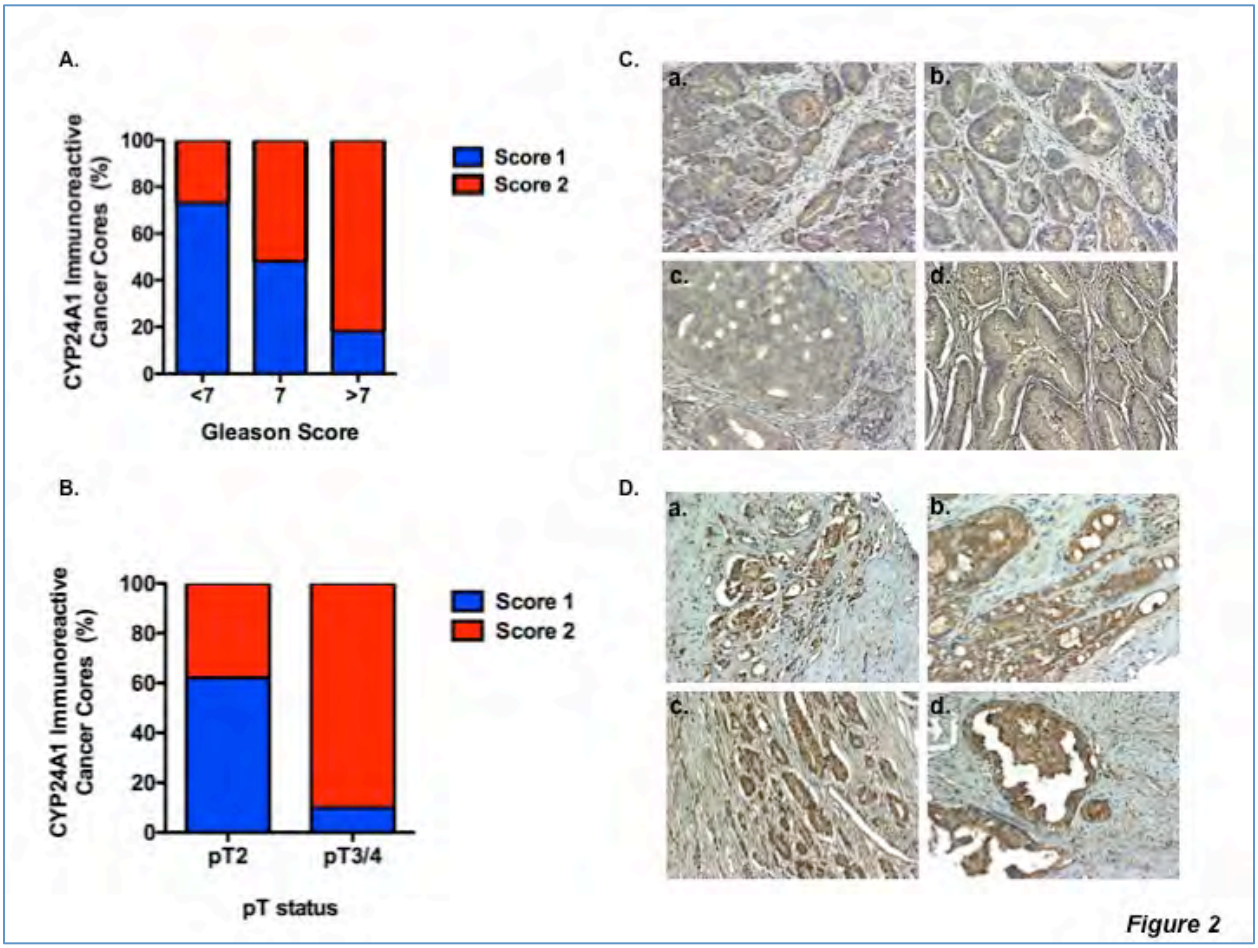


Figure 2: Correlation between high protein levels of CYP24A1 and high tumor grade and late stage of human prostate cancer. A-B. Distribution of the relative expression levels of CYP24A1 and Gleason score (A) and tumor stage (B) categories. Semi-quantitative scoring of the quantity of CYP24A1 expression in 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease, was performed (score 1= moderate; score 2=strong). Cases are shown as percentages of total immunoreactive cancer cores on the y-axis. C-D. Immunostaining of CYP24A1 in representative adenocarcinomas of different grades. **In C., low grade lesions of prostate cancer showing moderate intensity of the staining of the catabolic enzyme** (a. Gleason 2+2 and T2NxM0; b. Gleason 2+3 and T2NxM0; c. Gleason 2+3 and T2NxM0; d. Gleason 1+1 and T2bNxM0). **In D., intense staining in malignant prostate tissues presenting with high Gleason score and late stage tumor progression** (a. Gleason 9 and T3bN0M0; b. Gleason 7 and T3bN0M0; c. Gleason 7 and T4N0M0; d. Gleason 9 and T3bN0M0). Magnification x400.

Indeed, this is seen clearly in Figure 2 that shows the correlation of CYP24A1 with high tumor grade and late stage prostate cancer. We analyzed tissue samples from 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease and Gleason scores ranging from 4 to 10. The percentage of immunoreactive cases with CYP24A1 was high (98% of cancer cores). However, staining intensity varied between patients, ranging from intense immunoreactivity of the entire cytoplasm to more discrete granular staining. Importantly, high protein levels of CYP24A1 positively correlated with high-grade cancer (Figure 2; $P < 0.0001$, Pearson's Chi-square), as well as with late stage tumor progression (Figure 2; $P < 0.0001$, Pearson's Chi-square). Increased CYP24A1 staining intensity was indeed, frequently observed in cancer cores with Gleason scores higher than 7 and in lesions presenting with 3 or 4 TNM stages (Fig 2A-D). Taken together, these data demonstrate for the first time, the

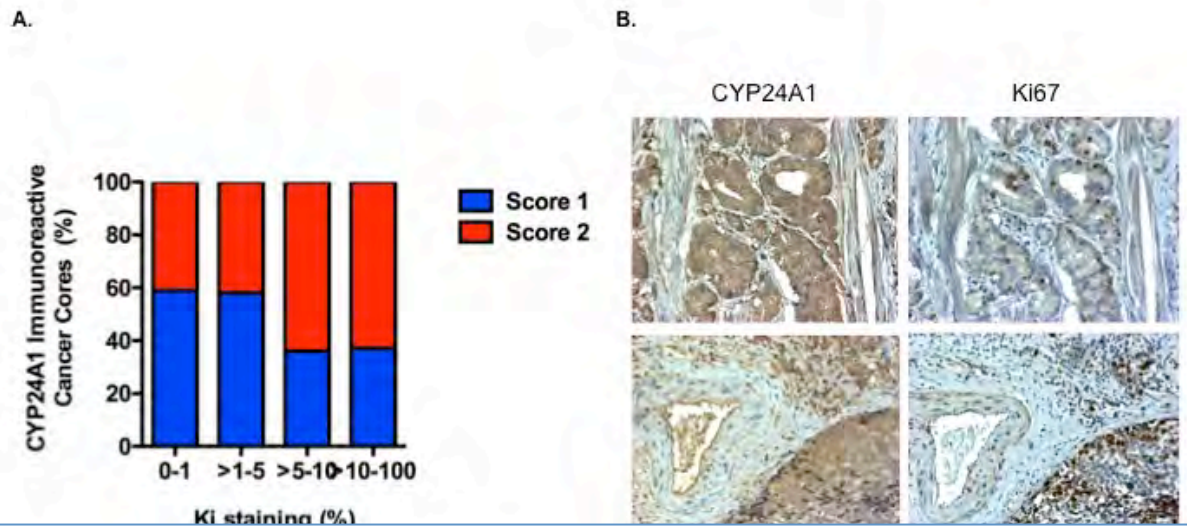


Figure 3 Distribution of the percentage of Ki67-positive cells between cancer cores expressing high (2) and moderate levels (1) of CYP24. Semi-quantitative scoring of the CYP24A1 staining (1= moderate, 2=strong) was independently performed by two investigators who were blinded to the data. Scoring for semi-quantitative analysis for Ki67 staining was evaluated by the percentage of cells showing positively labeled nuclei: 0 (0-1% positivity), 1 (1-5 % positivity), 2 (5-10% positivity), 3 (10-100% positivity). Magnification x400.

changes of expression of CYP24A1 in patients with prostate cancer and highlight the potential key role of CYP24A1 in prostate cancer.

2. Basal Levels of CYP24A1 mRNA Correlate With The Growth Response To Vitamin D In Prostate Cancer Cells: Next, to examine whether increased CYP24A1 expression reflects increased tumor proliferation, we determined its relationship to the expression of the proliferation marker Ki67. High expression of CYP24A1 positively correlated with parallel increased staining of Ki67 in the same human prostate cancer tissues (Figure 3; $P<0.0002$, Pearson's Chi square). This observation supports the hypothesis that overexpression of CYP24A1 in prostate cancer cells may reduce the ability of 1,25-dihydroxyvitamin D_3 to inhibit proliferation.

As the steady-state levels of cellular vitamin D_3 in prostate tumor tissue of patients is difficult to measure, we analyzed the expression levels of endogenous CYP24A1 in three human prostate cancer cell lines (LnCAP, PC3 and DU145) and monitored their respective cell proliferation in presence of vitamin D_3. Consistent with the findings of previous reports on CYP24A1 enzymatic activity in these prostate cell lines, we found an inverse correlation between the constitutive cellular levels of CYP24A1 mRNA and the growth response to vitamin D_3. Indeed, LnCAP, with the lowest constitutive expression levels of CYP24A1 (Figure 4A), is most responsive to the anti-proliferative effect of calcitriol (Figure 4B). In contrast, PC3, which displays the highest basal levels of CYP24A1 (Figure 4A), is the most resistant to the inhibitory effect of calcitriol on cell growth (Figure 4D). Thus, the differential prostate cell response to calcitriol is due to their inherent constitutive gene expression levels of 1,25$(OH)_2$ vitamin D_3 catabolic enzyme, CYP24A1. These data support the role of vitamin D_3 as a paracrine hormone to inhibit the growth of the prostate and provide a rationale basis to the use of specific inhibitors of CYP24A1 to enhance vitamin D-based therapies for prostate cancer management.

2.a Optimal Transcript Design for Efficient CYP24A1Inhibition: We first assessed by qRT-PCR, the efficacy of inhibition of CYP24A1 expression. siRNA oligonucleotides were

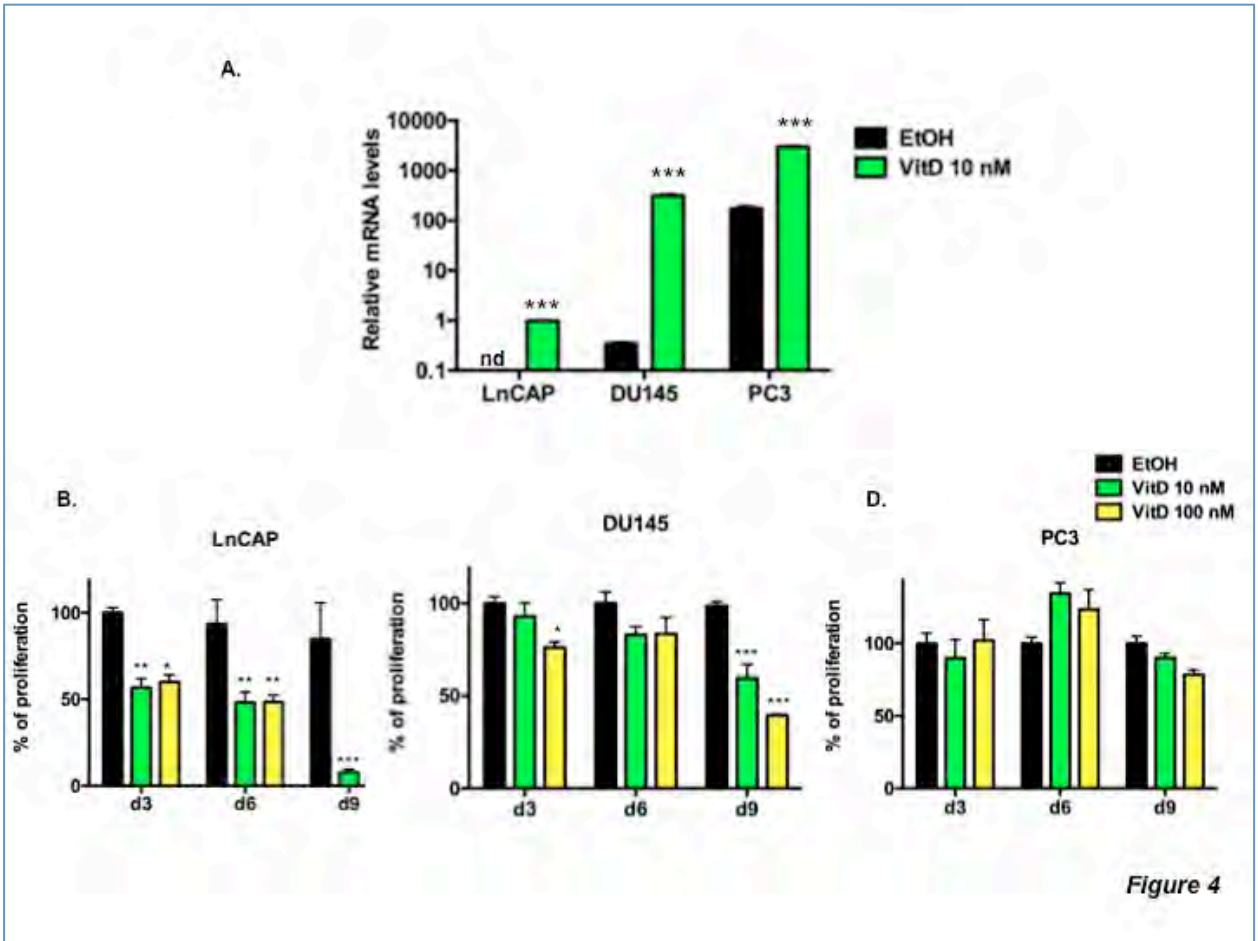


Figure 4: Inverse correlation between endogenous levels of CYP241 mRNA and growth abrogation action of vitamin D_3.

A. qRT-PCR analysis of endogenous CYP24A1 mRNA levels in presence of 10nM Vitamin D_3 or vehicle (ethanol; EtOH) in LnCAP, DU145 and PC3 prostate cancer lines. GAPDH served as the internal control. **B-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 3, 6 and 9 days of treatment in LnCAP (B), DU145 (C) and PC3 (D) (* if P< .05; ** if P< .01; *** if P< .001, ANOVA).

synthesized by Dharmacon Research, In. (Lafayette, CO). Cells were transfected with CYP24A1siRNA oligos or scrambled siRNA. The optimal approach used CYP24A1 ON TARGETplus SMART pool at a concentration of 20-40 ng/ml

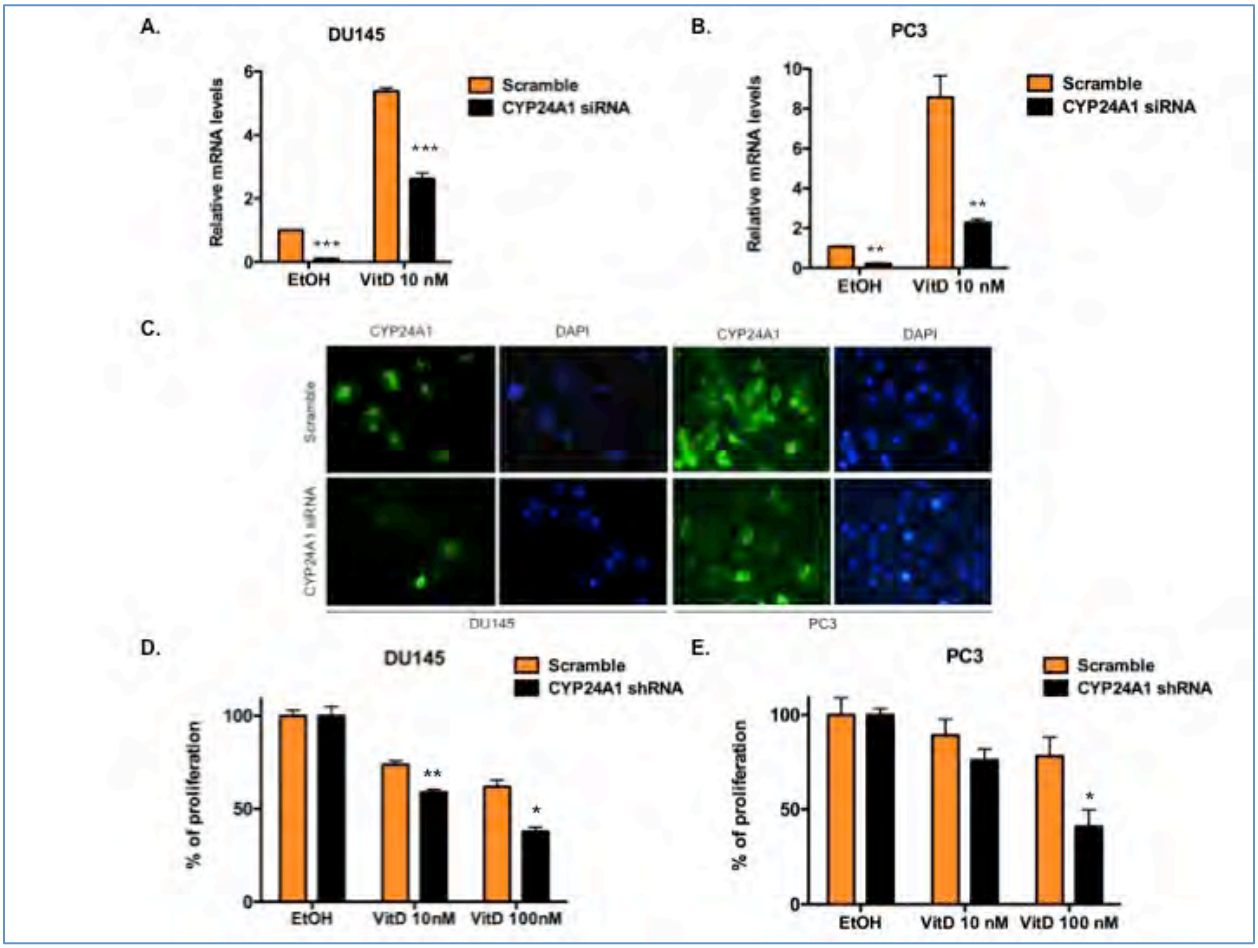


Figure 5: Effect of selective inhibition of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D_3.
A. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D_3 or vehicle (EtOH) in DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble). GAPDH served as the internal control. **B.** Immunofluorescent staining of CYP24A1 protein of DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble) and incubated in presence of Vitamin D_3 10 nM. DAPI was used for cell nuclei visualization. **C-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 6 days of treatment in DU145 (C) and PC3 (D) transiently transfected with CYP24A1 siRNA or non targeting siRNA (Scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if P< .05; ** if P< .01; *** if P< .001.

(http://www.dharmacon.com/CatalogSearch/ConsolidatedSearch.aspx?searchTerm=cyp24a1&searchTarget=1591_+CYP24A1).

2.b. Selective inhibition of CYP24A1 Expression in Human Prostate Cancer Cell Lines: Effect on Vitamin D Action on Cell Proliferation: Using this approach, we were able to selectively inhibit CYP24A1expression in prostate cancer cell lines that are resistant to vitamin D_3 using RNA interference. The relative mRNA levels of CYP24A1 were efficiently knocked down by siRNA in absence or presence of vitamin D_3 and asked whether the alteration of CYP24A1 gene expression would enhance growth inhibition by vitamin D_3 (Figure 5A). When

compared to cells transfected with the non-targeting siRNA, the abrogation of expression by CYP24A1 siRNA in PC3 cells was about 80% in constitutive conditions and 74 % in presence of 10 nM vitamin D_3 (Figure 5A). The knockdown of CYP24A1 mRNA translated into reduced levels of CYP24A1 protein, as reflected by the attenuation of the punctuated staining seen in cells transfected with CYP24A1 siRNA compared to control cells transfected with scramble (Figure 5C). To assess the cellular consequences of siRNA-mediated silencing of CYP24A1 gene expression, proliferation assays were performed. While PC3 and DU145 cells were not responsive to the growth inhibition mediated by vitamin D_3 (Figures 5D and 5E), CYP24A1 siRNA significantly enhanced the anti-proliferative action of vitamin D_3 (Figures 5D and 5E). Transient transfection of CYP24A1 siRNA also produced a significant enhancement of the growth inhibitory action of calcitriol in the LNCaP cell line which is responsive to vitamin D_3 .This study shows the role of vitamin D_3 as a paracrine hormone to inhibit the growth of the prostate and provide a rationale basis to the use of specific inhibitors of CYP24A1 to enhance vitamin D-based therapies for prostate cancer management.

3. Selective Inhibition of CYP24A1 Enhances The Anti-Proliferative Effect of 1,25(OH)$_2$D$_3$ in Stably Transduced Prostate Cancer Cell Lines: To assess the impact of a sustained expression of CYP24A1 siRNA on the growth abrogation mediated by vitamin D3, we generated LnCAP, DU145 and PC3 stable cell lines expressing CYP24A1shRNA or non-targeting shRNA. The random integration into the genome of the vector-based shRNA led to a significant knock down of the CYP24A1expression in presence or absence of calcitriol (Figures 6A and 6C). Indeed, when compared to cells with non-targeting shRNA, relative CYP24A1 mRNA levels were reduced by 75% in PC3 and 85% in DU145 in presence of ethanol, and the knockdown was about 90% for PC3 and 60% for DU145 in presence of vitamin D3. In these clonal conditions, PC3 showed an improvement of its growth response to vitamin D3, reaching significance at 100 nM (Figure 6B). In DU145, a significant reduction of cell proliferation was already achieved at 10 nM of vitamin D3 (Figure 6D). Hence, the prolonged abrogation of CYP24A1 gene expression enhanced the 1,25(OH)2D3-mediated growth inhibition in prostate cancer cells.

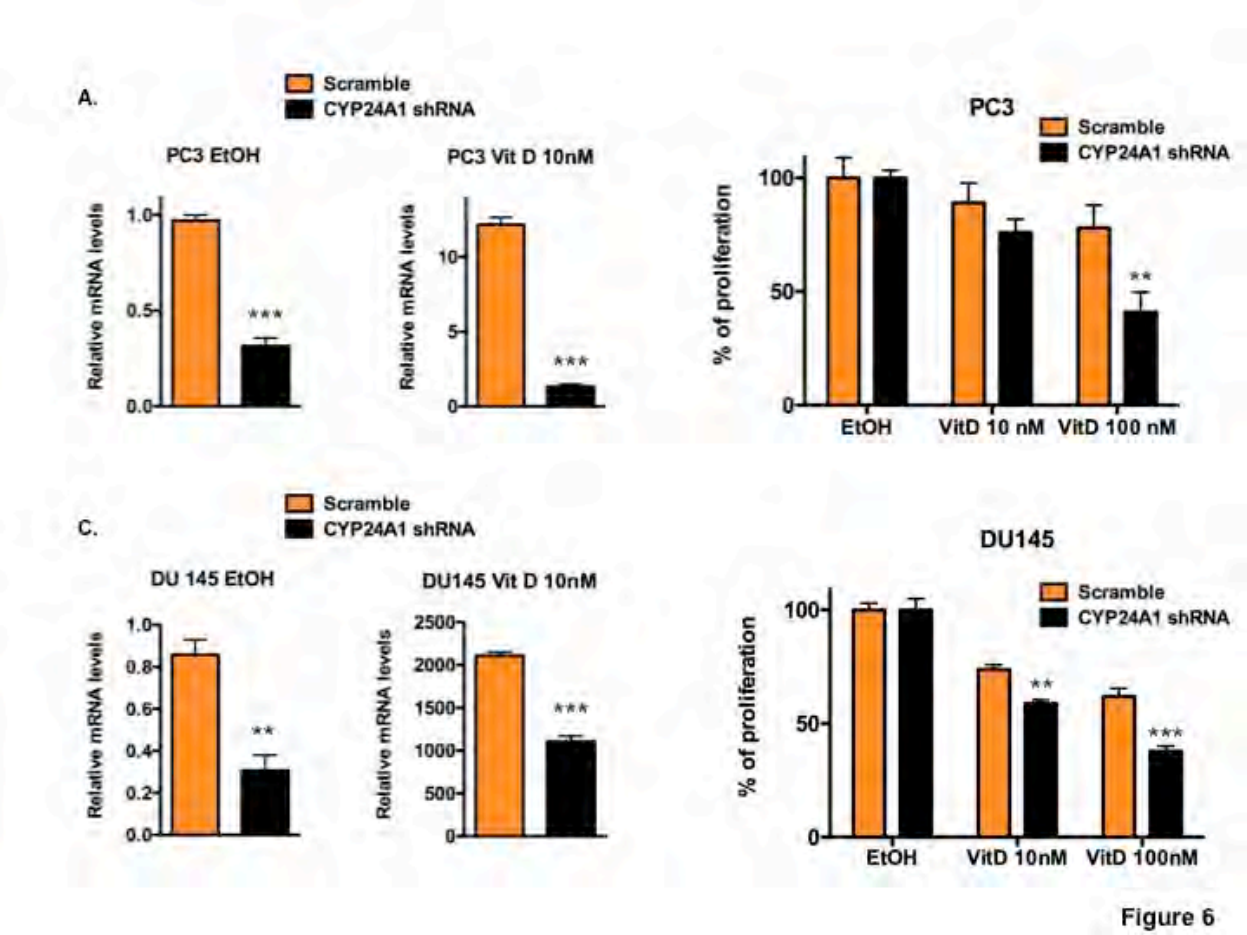


Figure 6: Generation of stable clones expressing shRNA and impact of sustained alteration of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D3.
A and C. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D3 or vehicle (EtOH) in PC3 (A) and DU145 (C) stably transfected with CYP24A1 shRNA or a non targeting shRNA (Scramble). GAPDH served as the internal control. **B and D**. Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol for 6 days in stable PC3 (B) and DU145 (D) expressing CYP24A1 shRNA or non targeting shRNA (scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if P< .05; ** if P< .01; *** if P< .001.

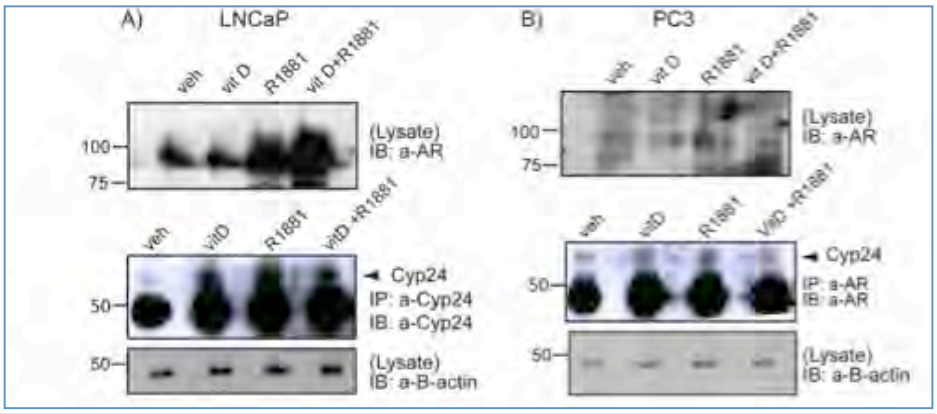


Figure 7 Expression of AR and CYP24A1in androgen-dependent and independent cell lines. Cells were plated in 6 well plate to 60% confluence and treated with 0.1% ethanol, 10nM 1α25(OH)2 D3 and /or 10nM R1881 for 48 hr. Cell lysates were immunoprecipitated with anti CYP24A1and probed for AR,CYP24A1and ß-actin antibody.

This work shows that CYP24A1 was overexpressed in patients with prostate cancer, particularly in high-stage cases. In addition to highlight the endogenous anti-cancer action of vitamin D_3 in human prostate, this deregulation may be a predictive marker of vitamin D_3 clinical efficacy in patients with advanced prostate cancer as an adjuvant therapy. Our findings also provide for the first time, a proof of principle that targeting of CYP24A1 by siRNA, a more selective and specific approach and as a result, a potentially less toxic alternative, is efficient to enhance the anti-proliferative action of vitamin D_3 in prostate cancer cells, offering a promising combination therapy for vitamin D-based therapies for advanced prostate cancer.

4. Androgen Administration Enhances the Growth Inhibitory Effect of Vitamin D_3: We studied the actions of androgen (alone or together with vitamin D_3) administration on cell using WST-1 colorimetric assay (Fig.7). In LNCaP (expresses a mutant AR and displays androgen-regulated growth) and PC3AR cells (stablely transfected expressing the androgen receptor (AR)), R1881 (a synthetic androgen) administration caused 43% decrease in the cell growth. Similarly $1,25(OH)_2D_3$ simultaneously inhibited LNCaP cell growth by 33%. The combined effect of R1881 and Vitamin D_3 were more pronounced, showing almost a 50% inhibition by 72 hr. The synergistic effect of the androgens and Vitamin D_3 is more potent, exhibiting a strong anti proliferative effect in AR-dependent cells within 72 hrs. In contrast, AR-independent PC3 (lacking androgen receptor) was not significantly growth inhibited by R1881 and Vitamin D_3. LNCaP_AR cells (androgen receptor present but growth resistant to androgens) showed a pattern similar to LNCaP. The cell line lacking AR (PC3) was resistant to Vit D3 or R1881 administration and this was reversed when androgen receptors were stably transfected (PC3AR). Studies have shown that DHT has a biphasic effect on LNCaP cells, at low doses (0.1nM- 1 nM), it has a growth stimulatory effect and at higher doses there is a gradual loss of the growth stimulatory action of DHT. Our study was done at 100nM R1881, where we see a growth inhibition.

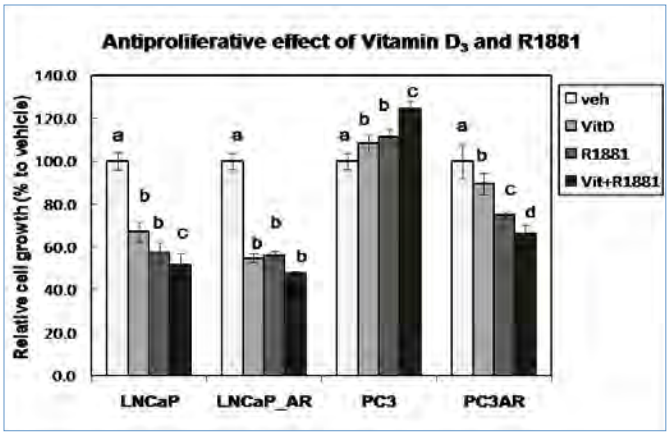


Figure 8 Effect of androgen on the growth inhibitory effect of $1,25(OH)_2D_3$in LNCaP, LNCaP_AR, PC3 and PC3AR cells. Cells were grown to 50% confluence, treated with 0.1% ethanol, 10nM $1,25(OH)_2D_3$ and /or 100nM R1881 and harvested after 72 hr. Colorimetric cell proliferation assay by WST-1 reagent was used to measure the cell proliferation (n=6, 2 exp. replications). Two-way ANOVA shows statistically significant interaction ($p<0.05$) between $1,25(OH)_2D_3$ and R1881 in LNCaP and PC3AR cell lines

5. Effect of Synthetic Androgen (R1881) on CYP24A1and Androgen Receptor Gene Expression: Androgen-dependent and -independent human prostate cancer cell lines (as described above) were pretreated with 10nM $1,25(OH)_2D_3$in absence or presence of 10nM R1881 and were harvested after 48 hr. As expected (18,21), vitamin D_3 induction significantly enhanced the expression of CYP24A1mRNA with the highest level of induction being found in the LNCaP cell line (Fig.8). Similar results were reported by others. In contrast, pre-incubation with 10nM R1881 together with 10nM $1,25(OH)_2D_3$ significantly suppressed the expression of CYP24, indicating that R1881 at physiological concentration protects Vitamin D_3 from catabolism. In androgen-independent cell lines, PC3, DU145 and LNCaP_AR (C-42, a stable androgen independent cell line), the expression of CYP24A1mRNA induced by $1,25(OH)_2D_3$ was not significantly inhibited by androgens. Thus, androgens down-regulate CYP24A1 gene expression in androgen-dependent cell lines, there by enhancing the antiproliferative functions of Vitamin D_3. Western blot analysis demonstrated a robust expression of AR in LNCaP and PC3AR cells (not shown) with no expression in PC3 cells (Fig.7). AR was stabilized and showed a graded response with induction of 10nM $1,25(OH)_2D_3$and 10nM R1881, whereas, in PC3, DU145 and LNCaP_AR cells there was little or no expression of AR. CYP24A1protein is over-expressed in all human prostate cancer cell lines. CYP24A1expression is enhanced with the administration of 10nM $1,25(OH)_2D_3$ in both AR-dependent (LNCaP) and AR independent (PC3) cell line.

R1881 administration to androgen-responsive prostate cancer cells enhances the

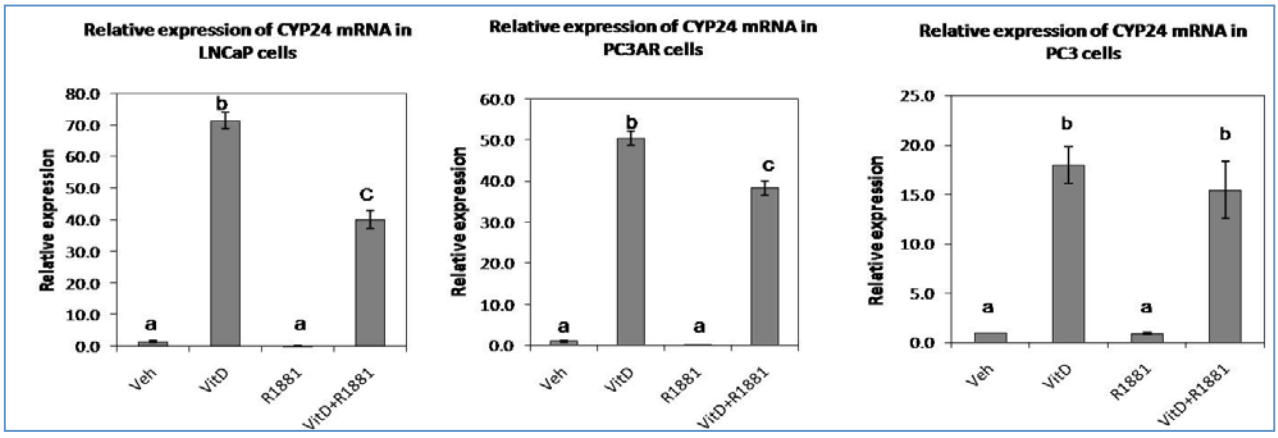


Figure 9 Effect of androgen on the level of 24-hydroxylase mRNA in LNCaP (A), PC-3 AR (B) and PC3 (C) cells. Cells were treated with 0.1% ethanol, 10nM $1,25(OH)_2D_3$and /or 10nM R1881 for 48 hr. Total RNA was isolated and CYP24A1mRNA estimated by real-time RT PCR (n=3). Two-way ANOVA shows statistically significant interaction between 1α25(OH)2 D3 and R1881 in LNCaP and PC-3 AR cell lines .

antiproliferative activity of Vitamin D_3 (Fig.9) and protects Vitamin D_3 from inactivation by suppressing CYP24A1expression. ***Results suggest that androgen ablation actually converts the prostate cancer cells to a state of vitamin D resistance.***

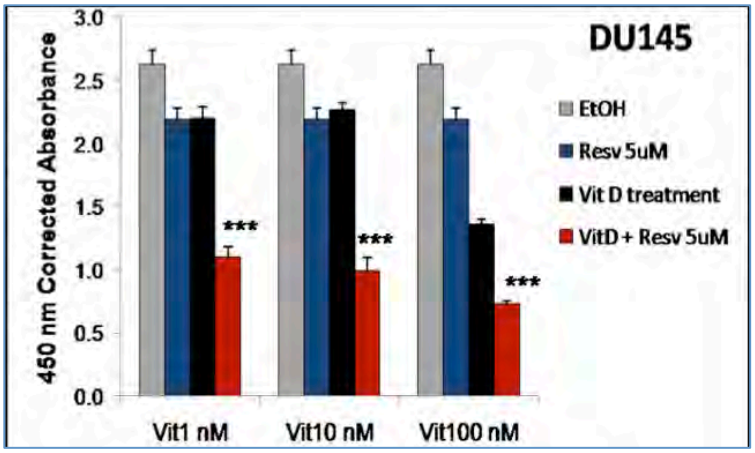


Figure 10. Effect of resveratrol on the growth inhibitory effect of $1,25(OH)_2D_3$ in DU145 cells. Cells were treated with 0.1% ethanol, 1, 10nM or 100 nM $1,25(OH)_2D_3$ and resveratrol (5µM) and harvested after 6 days. Colorimetric cell proliferation assay by WTT reagent was used to measure the cell proliferation (Values represent the mean of at least three separate experiments). One-way ANOVA shows statistically significance of the interaction ($p \leq 0.001$) between $1,25(OH)_2D_3$ and resveratrol in DU145 cells.

6. Resveratrol Potentiates The Anti-Proliferative Effects of Calcitriol by Enhancing The Transcriptional Activity of the VDR:

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenol highly enriched in grapes, berries, peanuts and other dietary sources. Resveratrol exerts inhibitory effects on the initiation, promotion, and progression of carcinogenesis by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis. Treatment with low-dose resveratrol and calcitriol in combination drastically inhibits cell proliferation. resveratrol used at low doses (5 µM) potentiates the anti-proliferative effects of calcitriol. While resveratrol (5µM) or calcitriol (1-10 nM) alone were not effective in suppressing the growth of the prostate cancer cells, DU145, combined treatment with resveratrol and calcitriol drastically inhibited cell proliferation as assessed by MTT proliferation assays (Figure 10).

Genes involved in the control of cell proliferation such as GADD45 (growth arrest and DNA-damage-inducible gene), IGFBP3 (insulin growth factor binding protein 3) and CDKN1A that encodes the cyclin dependent kinase inhibitor p21, were transcriptionally activated by 1α,25(OH)2D3. Their levels of expression increased with resveratrol as well. However, when the cells were treated with both resveratrol and calcitriol, their levels of mRNA were significantly increased compared to cells subjected to single treatment (Figure 11).

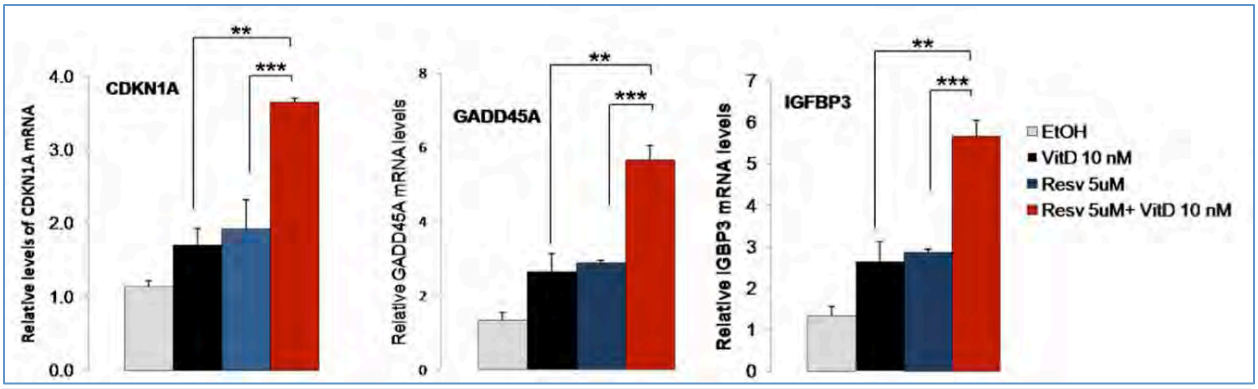


Figure 11 Synergistic transcriptional effect of resveratrol and calcitriol on vitamin D responsive genes. Taqman qRT-PCR analysis of CDKN1A (p21), GADD45A and IGFBP3 mRNA levels in DU145 cells treated for 24 h with 10 nM Vitamin D3 and/or resveratrol (5) or vehicle (EtOH). GAPDH served as the internal control. **p<0.01, ***p<0.001.

However, the transcriptional synergistic effect on the vitamin D responsive genes could not be

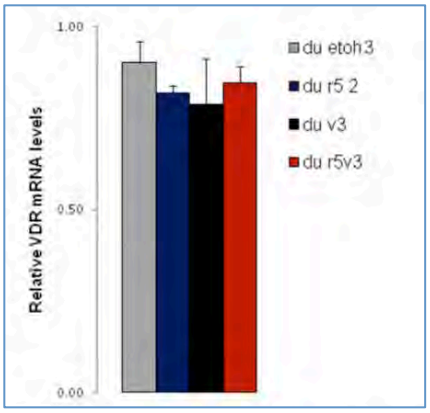


Figure 13 Gene expression levels of VDR are not affected by combined treatment of resveratrol and vitamin D

due to the increase of mRNA levels of the vitamin D receptor (VDR) since these agents used alone or in combination did not induce VDR gene expression (Figure 13). Potentiation occurs through a VDRE element as shown by luciferase assays (Figure 14). Indeed, a synergistic effect was obtained when both resveratrol and calcitriol were used on the VDRE. To define the molecular mechanisms underlying this potentiation, we tested whether the interaction of VDR with coactivators is enhanced in presence of both components. One important interacting cofactor with VDR is the p160 family member, SRC-1. Transfection with an SRC-1 expression vector enhanced the transcriptional activity on a VDRE in presence of resveratrol and calcitiol.

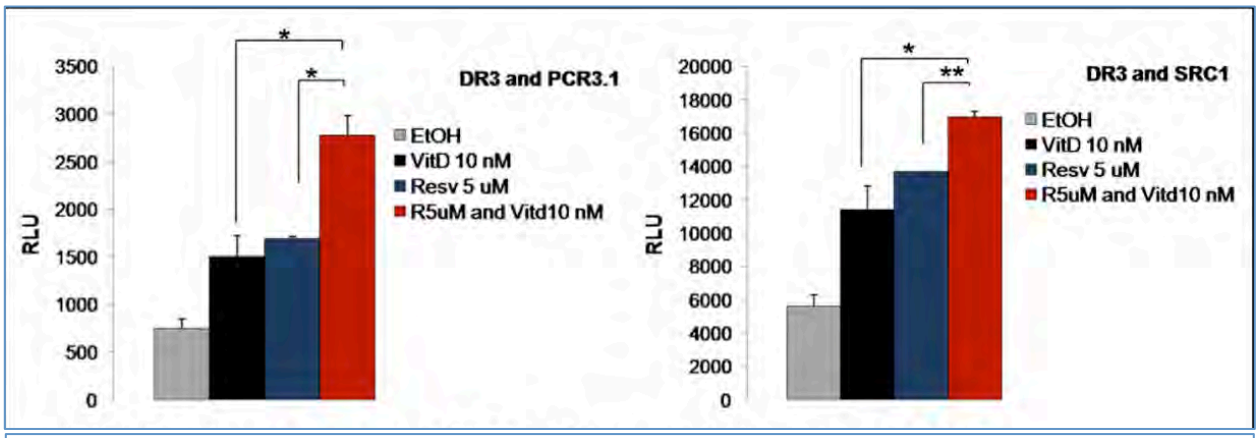


Figure 14 Luciferase assay using VDRE luciferase reporter in the presence or absence of 10 nM Vitamin D and/or 5 µM resveratrol. Left pane shows co-transfection with an empty vector. Right panel shows co-transfection with an SRC-1 expression vector

Specific Aim #2 To Enhance Vitamin D inhibition of prostate cancer proliferation by CYP24A1siRNA in vivo.

We generated stable CYP24A1shRNA expressing prostate cancer cell lines (section C.3) and enhanced the anti-proliferative effect of calcitriol when compared to control cells expressing non-targeting shRNA. The control shRNA (a commercially available general purpose 21 mre, 48% GC) has no homology to human, mouse or rat mRNA. SCID mice were maintained on a vitamin D_3 deficient diet containing 0.5% calcium (#5826-CI, Purina) for 2 weeks prior to use. The recipient mice were anesthetized and injected intraprostatically with 1 X 10^6 stably transfected prostate cancer cells. This cell concentration achieves consistent local tumor growth within 7 days of implantation. Two weeks after injection, mice were separated into groups of 8 animals each and treated with control- sesame oil with 2% ethanol or 0.5 µg/kg 1,25(OH)2D3 (in sesame oil with 2% ethanol) every other day by oral gavages with a 20 gauge intragastric feeding tube.

In these studies, MR images were obtained using a Bruker Biospin Pharmascan 7.0T spectrometer (Bruker Biospin, Billerica, MA) in the Mouse Phenotyping Core at Baylor College of Medicine so that we could non-invasively follow xenograft tumor growth over time. The average size of the prostate prior to intraprostatic injection of prostate cancer cells was 24 mm^3.

In animals receiving DU145 cells stably transfected with the CYP24A1siRNA (DU41 cells), the combined volume of the prostate gland and tumor is 14.7 mm^3 at D2 and decreased in size to 3.07 mm^3 on D18 of vitamin D treatment. There are a total of 8 animals (out of 40 total in the study; n = 2 mice / treatment group) that underwent repeated MR imaging in-order-to monitor tumor growth. In the remaining mice (as well as the imaged mice), the tumors were removed at day 60 for analysis. Baseline volume (D0) of the prostate gland was determined and averaged.

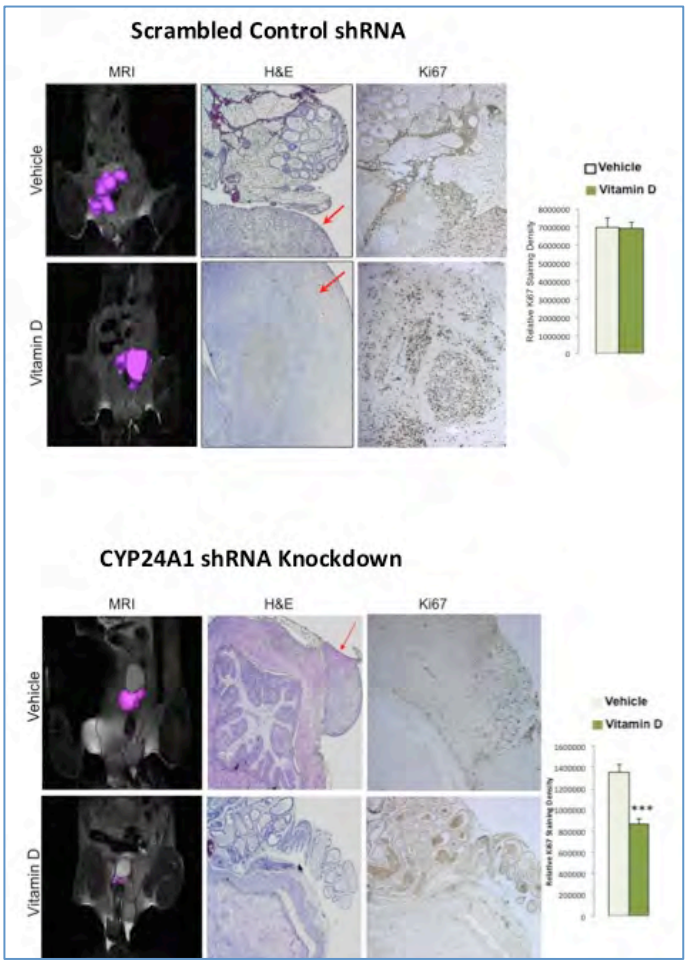


Figure 15 Vitamin D inhibition of human prostate cancer xenografts after CYP24A1 knockdown. CYP24A1 knockdown (lower panels) and scrambled shRNA (top panels) in DU145 cells injected orthotopically in the prostate using male SCID mice receiving 1, 25$(OH)_2D_3$ DU145 cells stably transduced with CYP24A1siRNA (lower) or scrambled controls (upper) (1 x 10^6 DU41 cells) were injected intraprostatically after surgical exposure of the dorsal prostate gland. Mice are treated with 0.5ug/kg 1,25$(OH)_2D_3$ (in sesame oil with 2% ethanol) every other day by oral gavage. Purple indicates tumor. MRI imaging of SCID mice at 60 days after xenograft injection (left panels). MRI images were obtained in the Mouse Phenotyping Core at Baylor College of Medicine. The images are T2-weighted (TR=3000ms and TE=36.61ms). The slice thickness is 0.5 mm (no interslice gaps) and the field of view (FOV) is 40 mm x 40 mm. The right panels show H&E staining of the prostate cancer xenograft and further right is shown the Ki67 staining. Ki67 staining shows cell growth inhibition by 1,25$(OH)_2D_3$ only in the cancer cells after CYP24 knockdown. The bar graphs show the relative degree of proliferation based on the Ki67 staining.

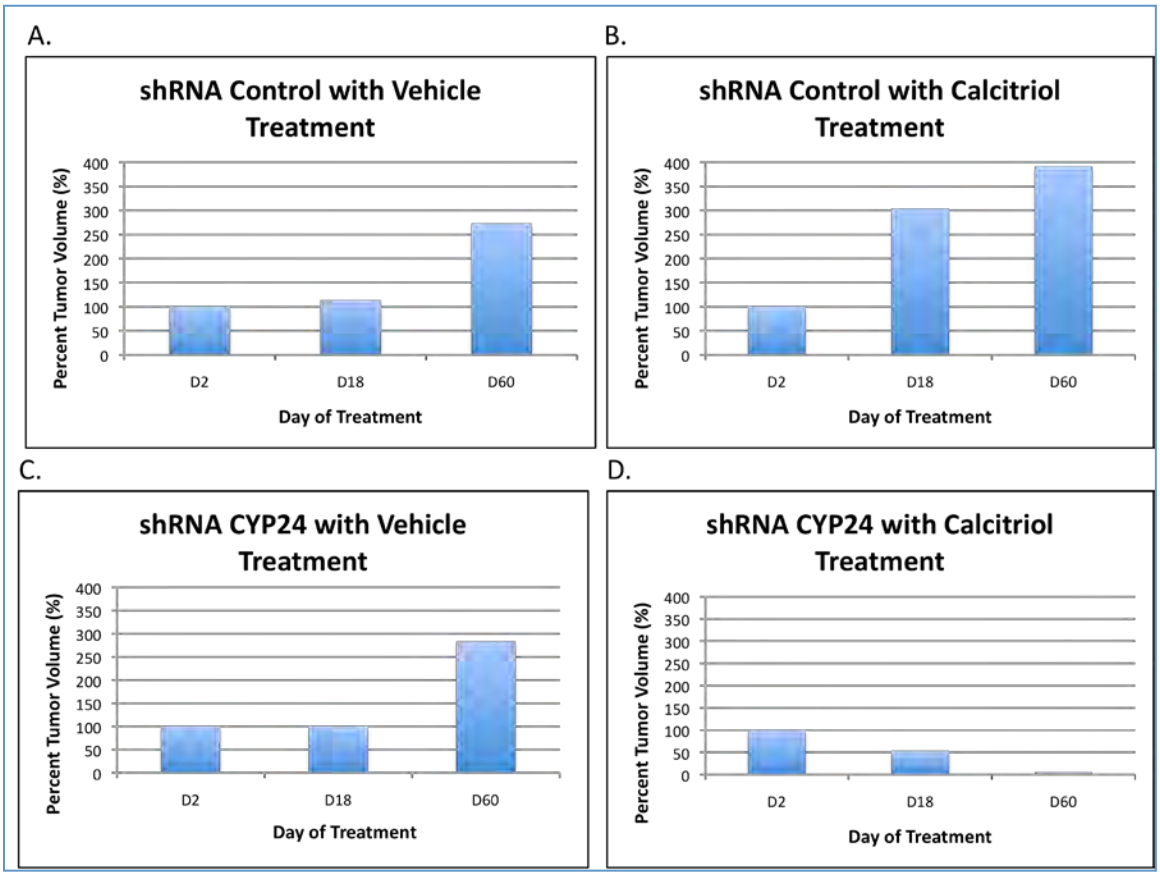
Figure 15 simply shows representative results at day 60 of treatment expressed as tumor volume calculated by MRI. The tumors grew at similar rates in the presence or absence of calcitriol with control (scrambled) shRNA (upper figures) cells and with CYP24 shRNA and vehicle only (lower panel). Importantly, the mice with the tumor xenograft with CYP24 knockdown showed highly significant inhibition of tumor growth over the same time period. This growth inhibition was also evident when the tumors histology was assessed with decreased ki67 staining apparent which correlates with proliferative index (right panels and bar graphs).

Figure 16 shows the tumor volumes of representative individual mice over time beginning with treatment day 2. It is apparent that that control xenograft cells (scrambled shRNA) continue to growth over the study period ending at 60 days whereas the growth of the shRNA CYP24A1 cells was markedly inhibited by Vitamin D_3 over the 60-day study period.

These studies provide evidence that CYP24A1 knockdown in human prostate cancer cells tested in a xenograft model enhances the growth inhibiting/apoptotic actions of Vitamin D_3 administration in prostate cancer. We performed additional histological and phenotypic assays to further define the effect of CYP24A1knockdown on prostate cancer cell growth. Once total tumor burden was 1.5 cm, the mice were anesthetized and cardiac puncture performed to

collect serum. Tumor growth and sites of metastasis especially pulmonary and retroperitoneal metastases, commonly observed in advanced prostate cancer were assessed. Average tumor size and weight were measured and tumor volume calculated using the equation: tumor volume (cm3) = 0.523 x [length (cm) x width2 (cm2)]. Specimens were fixed in formalin and embedded in paraffin for hematoxylin-eosin staining or for proliferation markers immunostaining

Figure 16: Graphs A-D show tumor volumes over the course of treatment as a percentage of the starting tumor volume at treatment day 2. Each graph shows data from representative treatment groups in this orthotopic xenograft experiment where mice were injected intraprostatically with DU145 prostate cancer cells stably transduced with either CYP24 targeted or control shRNA. The mice were then given treatments every other day with calcitriol or vehicle.



(Proliferating Cell Nuclear Antigen) and apoptosis using the TUNEL assay. Specimens were cryopreserved for further protein and/or RNA analysis of proliferation and apoptosis markers. Serum collected by cardiac puncture were used for calcium measurement to detect hypercalcemia (normal calcium values being about 8 mg/dl) and kidney sections were stained for calcium using the von Kossa stain or Alizarin Red S.

Major Findings of This Final Progress Report Studies are:

a) CYP24A1(RNA and Protein) is over-expressed in prostate cancer and increases with TNM

b) CYP24A1siRNA blocks CYP24A1expression and caused vitamin D_3 resistant prostate cancer to become vitamin D_3 sensitive and to once again be growth inhibited by 1,25$(OH)_2D_3$.

1. Growth inhibition is seen in vivo and in vitro

2. This is a drugable pathway (for future studies)

c) Androgens enhance the antiproliferative action of vitamin D_3 in prostate cancer by inhibiting CYP24A1expression

d). Resveratrol markedly enhanced vitamin D_3 action working through SRC-1 and VDR

Publications Resulting from these Investigations:

One paper (enclosed) is submitted and under review at Cancer Research. This paper includes the work in Specific Aim #1 on the expression of CYP24A1 in human prostate cancer and prostate cancer cell lines, evidence of reversible vitamin D resistance by CYP24A1 knockdown and the production of stably transfected cell lines. This manuscript is included with this report.

Three other manuscripts are nearing completion for submission: The second manuscript focuses on the requirement for androgens and the androgen receptor for efficient vitamin D action. A third paper focuses on CYP24A1 expression in metastatic disease (using tissue arrays from warm autopsy specimens), xenograft studies of the stably transfected cell lines and the enhancement of vitamin D action in vivo in cells that are otherwise Vitamin D resistant. A fourth paper focuses on the use of resveratrol to modify vitamin D action in prostate cancer cell lines through an effect on SRC-1.

Selective Targeting of CYP24A1 as a Rational Approach for Vitamin D_3 Based Therapies in Human Prostate Cancer

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Running Title: Overexpression of CYP24A1 in Prostate Cancer

Key words: Prostate Cancer, Vitamin D, CYP24A1, Biomarker

ABSTRACT

Purpose: Clinical trials to develop vitamin D_3-based therapies for advanced prostate cancer were hindered by the risk of hypercalcemia and the limited antitumor benefit. We aimed to define factors predictive of the tumor growth response to vitamin D_3 in patients with prostate cancer and specifically examine the role of its major catabolic enzyme, CYP24A1 in prostate tumorigenesis.

Experimental Design: Real-time PCR and immunostaining were performed using 36 benign and cancer biopsy samples and tissue microarrays with 112 prostate cancer cores derived from 56 patients. The functional role of CYP24A1 and the impact of selective inhibition of its expression on the anti-proliferative action of vitamin D_3 were examined in human prostate cancer cell lines.

Results: CYP24A1 mRNA was elevated in malignant human prostate tissues compared to benign lesions. High CYP24A1 protein levels were seen in poorly differentiated and highly advanced stages of prostate cancer ($P<0.0001$) and correlated with parallel increased staining of Ki67, a marker of proliferation ($P<0.0002$). While levels of CYP24A1 mRNA in prostate cancer cells inversely correlated with their respective growth response to vitamin D_3, abrogation of CYP24A1 expression using specific siRNA significantly enhanced the vitamin D_3-mediated growth inhibition.

Conclusions: Increased CYP24A1 expression may restrict the vitamin D_3-mediated growth inhibition in patients. The use of CYP24A1 siRNA renders cancer cells more sensitive to the anti-proliferative action of vitamin D_3 and could be used in combination with therapeutic doses of vitamin D_3 for prostate cancer treatment.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy in men. Treatments include radical prostatectomy followed by radiation or hormone ablation therapy. Unfortunately, durable clinical responses are not always obtained and as prostate carcinoma progresses, systemic metastasis to distant areas lead to patient morbidity and mortality. Therefore, development of effective therapeutic strategies are needed for the management of castrate-resistant prostate cancer. One promising alternative is the use of the hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or calcitriol, which exerts pro-differentiative, anti-proliferative, anti-angiogenic and pro-apoptotic effects on prostate cancer cells *in vitro* and *in vivo* (see for review (1)). Phase I and II trials of calcitriol, either alone or in combination with other therapeutic agents have been conducted in patients with castrate-resistant prostate cancer (2-8). However, the clinical use of vitamin D_3 based therapies was hampered by the risk of hypercalcemia and/or inadequate design for the optimal administration of calcitriol.

In order to improve the clinical efficacy of exogenous vitamin D_3 in the treatment of prostate cancer, it may be useful to define predictive factors for the growth suppressive response of vitamin D_3 in patients. In studying the action of $1,25(OH)_2D_3$ in prostate cancer, it is essential to examine the potential pathways that control local tissue levels of $1,25(OH)_2D_3$. The cytochrome P450 enzyme, 24-hydroxylase, encoded by CYP24A1 is central to the catabolism of $1,25(OH)_2D_3$. While its constitutive expression levels are low, CYP24A1 is strongly induced by $1,25(OH)_2D_3$ administration to produce less active vitamin D metabolites. Overexpression of CYP24A1 was reported in various human tumors including colon (9-11), cervical (12), ovarian (9, 12), esophageal (13), lung (14) and basal cell carcinomas (15). Changes of the expression or activity of vitamin D_3 catabolic enzyme CYP24A1 may affect the bioavailability of $1,25(OH)_2$ vitamin D_3 at the tumor site and abrogate the anti-proliferative action of vitamin D_3. This may lead to failure of any vitamin D_3 - based therapy in patients. Therefore, determining local CYP24A1 expression levels would allow for individualized treatments and higher clinical efficacy of exogenous vitamin D_3.

To date, no study examined the gene expression levels of endogenous CYP24A1 in the human prostate. In this present article, we evaluated the *in situ* expression of CYP24A1 in human

adenocarcinomas and benign prostate lesions. We analyzed its possible association with tumor cell proliferation and tested in human prostate cancer cell lines, whether the inhibition of CYP24A1 expression by RNA interference, could enhance the abrogating vitamin D- mediated growth control.

MATERIALS AND METHODS

Cell lines and chemicals

Human prostate cancer cell lines (LnCAP, PC3 and DU145) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI, DMEM or MEM containing 10% fetal bovine serum, respectively. All cells were maintained in a humidified 37^{o}C incubator with 5% CO_2. Calcitriol (Sigma-Aldrich, St. Louis, MO, USA) was reconstituted in 100% ethanol, stored at -20ºC and protected from light.

Transfection with small interfering RNA

Cells were plated in 6-well plates (1 x 10^5/well) for RNA extraction or in 96 well plates (1-1.5 x 10^3/well) for cell proliferation assays. The next day, cells were transfected for 24 hours with 40 nM of nonspecific (Scramble) or siRNA for human CYP24A1 (Dharmacon, Lafayette, CO, USA) using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's procedure. Following transfection, the cells were treated with either vehicle (0.1% v/v ethanol) or calcitriol (10-100 nM), for 24 h (RNA extraction) or 6 days (for cell proliferation).

Cell proliferation assays

Briefly, 1-1.5 x 10^3 cells per well were seeded into 96-well plates and treated with or without CYP24A1 siRNA for 24 h, then cultured in the presence or absence of 1,25$(OH)_2D_3$ at concentrations ranging from 1 nM to 100 nM. Viable cell number was determined after 3, 6 or 9 days of treatment using the WST1 Cell proliferation (Roche Applied Science, IN, USA) according to the manufacturer's instructions. The plates were read using a microplate spectrophotometer at a wavelength of 450 nm and corrected to 630 nm. Each independent experiment was performed at least three times.

Creation of stable cell lines harboring CYP24A1 shRNA

To generate stable prostate cancer cells expressing human CYP24A1 or non-targeting shRNA, cells were incubated with lentiviral transduction particles producing a non-target control or human

CYP24A1 shRNA (Sigma-Aldrich, St. Louis, MO, USA), in presence of polybrene, according to the manufacturer's protocol. Stable clones were selected with 1 µg/mL puromycin for three weeks.

Quantitative RT-PCR analysis of CYP24A1 gene expression

Total RNA was purified using the RNeasy Kit including an optional DNase I treatment according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). cDNA was prepared from 500 ng of total RNA by reverse transcription. Patients' cDNA were prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels (Origene, MD, USA). The supplier provided clinicopathological information for each patient. Real-time PCR was performed with TaqMan PCR Master Mix on an ABI StepOnePlus Realtime PCR System (Applied Biosystems, USA). PCR conditions were: 50ºC for 2 min, 94ºC for 2 min, followed by 40 cycles of 94ºC for 15 s and 60ºC for 30 s. For each experimental sample, the relative abundance value was normalized to the value derived from the endogenous control (beta-actin or GAPDH) of the same sample. Relative mRNA levels were quantified by the comparative ΔΔCT method.

CYP24A1 Immunofluorescence

Non-confluent prostate cells were fixed in paraformaldehyde 4% for 5 min on ice. Labeling with CYP24A1 antibody (Santa-Cruz, CA USA) was carried out in the presence of 0.1% Triton X-100, followed by Alexa Fluor 488 goat anti-rabbit IgG secondary antibody. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for cell nuclei visualization.

Tissue Microarrays and CYP24A1 immunohistochemical detection

Tissue microarrays containing 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease and Gleason scores ranging from 4 to 10 (Folio, OH, USA) were used in this study. Slides of paraffin-embedded adenocarcinomas samples were deparaffinized and dehydrated. Antigen retrieval was performed by heat inactivation in 0.1 M sodium citrate for 30 min. CYP24A1 (Santa Cruz, CA, USA) or Ki67 (DAKO, CA USA) primary antibodies were

used for immunodetection. The staining was performed using the avidin-biotin peroxidase system (ABC-peroxidase), and positive signals were visualized as brown precipitates using 3,3'-diaminobenzidine tetra-hydrochloride. Control staining was conducted by omission of the primary antibody. Hematoxylin was used for counterstaining. Two investigators, blinded to the data, performed light microscopy (Axioplan 2, Carl Zeiss, USA) and scored semi-quantitatively the quantity of protein expression in the whole section (0=none, 1= moderate, 2=strong). Scoring for semi-quantitative analysis for Ki67 staining was evaluated by the percentage of cells showing positively labeled nuclei: 0 (0-1% positivity), 1 (1-5 % positivity), 2 (5-10% positivity), 3 (10-100% positivity).

Statistical analysis

The data were obtained from at least two different experiments and are presented as mean± S.E.M. All statistical analyses were performed using the one-way or two-way analysis of variance (ANOVA). Contingency tables with Chi-square analysis were done using GraphPad (La Jolla, CA, USA). Values were considered statistically significant if $P < .05$ (* if $P< .05$; ** if $P< .01$; *** if $P< .001$).

RESULTS

CYP24A1 Was Overexpressed In Patients With Prostate Cancer

To assess the mRNA levels of endogenous CYP24A1 in adenocarcinomas of the human prostate, cDNAs obtained from pathologist-verified human prostate biopsies representing different pathological stages of prostate cancer as well as benign prostatic hyperplasia (BPH) were analyzed by quantitative PCR. Comparison of the CYP24A1 gene expression profile showed statistically significant mRNA overexpression (4 fold; P= 0.03, Wilcoxon rank-sum test) in prostate cancer samples (n= 29) compared with benign tissue (n=7) (Figure 1*A*). Consistently, malignant tissues displayed higher levels of CYP24A1 protein than the paired cancer-adjacent normal tissues (n=8) (Figure 1B).

To gain insight into specific changes associated with cancer progression, we analyzed tissue samples from 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease and Gleason scores ranging from 4 to 10. The percentage of immunoreactive cases with CYP24A1 was high (98% of cancer cores). However, the staining intensity varied between patients, ranging from intense immunoreactivity of the entire cytoplasm to more discrete granular staining. Importantly, high protein levels of CYP24A1 positively correlated with high-grade cancer (Figure 2; P< 0.0001, Pearson's Chi-square) as well as with late stage tumor progression (Figure 2; P< 0.0001, Pearson's Chi-square). Increased CYP24A1 staining intensity was indeed, frequently observed in cancer cores with Gleason scores higher than 7 and in lesions presenting with 3 or 4 TNM stages (Fig 2A-D).

Next, to examine whether increased CYP24A1 expression reflects increased tumor proliferation, we determined its relationship to the expression of the proliferation marker Ki67. High expression of CYP24A1 positively correlated with parallel increased staining of Ki67 in the same human prostate cancer tissues (Figure 3; P<0.0002, Pearson's Chi square). This observation supports the hypothesis that overexpression of CYP24A1 in prostate cancer cells may reduce the ability of 1,25-dihydroxyvitamin D_3 to inhibit proliferation.

Collectively, we demonstrate for the first time, the changes of expression of CYP24A1 in human patients with prostate cancer and highlight the positive correlation between elevated CYP24A1

protein levels and tumor progression and proliferation status. Our observations provide support for the potential key role of the vitamin D catabolizing enzyme CYP24A1 in human prostate cancer.

Inverse Correlation Between Basal Levels Of Human CYP24A1 mRNA And Growth Response To Vitamin D In Prostate Cancer Cells

Since CYP24A1 is the main catabolic enzyme determining the biological half-life of vitamin D_3, increased CYP24A1 expression in human prostate cancer tissues would presumably decrease the intra-tumor $1,25(OH)_2D_3$ levels, effectively counteracting its anti-proliferative effects. As the steady-state levels of cellular vitamin D_3 in prostate tumor tissue of patients is difficult to measure, we analyzed the expression levels of endogenous CYP24A1 in three human prostate cancer cell lines (LnCAP, PC3 and DU145) and monitored their respective cell proliferation in presence of vitamin D_3. Consistent with the findings of previous reports on CYP24A1 enzymatic activity in these prostate cell lines (16), we found an inverse correlation between the constitutive cellular levels of CYP24A1 mRNA and the growth response to vitamin D_3 (Figures 4A-D). Indeed, LnCAP, with the lowest constitutive expression levels of CYP24A1 (Figure 4A), is most responsive to the anti-proliferative effect of calcitriol (Figure 4B). In contrast, PC3, which displays the highest basal levels of CYP24A1 (Figure 4A), is the most resistant to the inhibitory effect of calcitriol on cell growth (Figure 4D). Thus, the differential prostate cell response to calcitriol is due to their inherent constitutive gene expression levels of $1,25(OH)_2$ vitamin D_3 catabolic enzyme, CYP24A1. These data support the role of vitamin D_3 as a paracrine hormone to inhibit the growth of the prostate and provide a rationale basis to the use of specific inhibitors of CYP24A1 to enhance vitamin D-based therapies for prostate cancer management.

Specific Inhibition Of CYP24A1 Expression By siRNA Enhanced The Anti-Proliferative Effect Of $1,25(OH)_2D_3$ In Prostate Cancer Cells.

To investigate functional consequences of CYP24 overexpression, we used a siRNA-based approach. We chose to specifically target CYP24A1 expression with small interfering RNA (siRNA) in

human prostate cancer cells that are resistant to vitamin D_3 (PC3 and DU145) and examine whether the alteration of CYP24A1 gene expression would enhance the growth inhibition mediated by vitamin D_3. We first assessed by qRT-PCR, the efficacy of inhibition of CYP24A1 expression. The relative levels of CYP24A1 mRNA were efficiently knocked down by siRNA in absence or presence of vitamin D_3 (Figure 5A). When compared to cells transfected with the non-targeting siRNA, the abrogation of expression by CYP24A1 siRNA in DU145 and PC3 cells was about 90% and 80% in presence of vehicle (ethanol) and 60 and 74 % in presence of 10 nM vitamin D_3, respectively (Figures 5A-B). The knockdown of CYP24A1 mRNA translated into reduced levels of CYP24A1 protein, as reflected by the attenuation of the punctuated staining seen in cells transfected with CYP24A1 siRNA compared to control cells transfected with the scrambled control construct (Figure 5B). To assess the cellular consequences of siRNA-mediated silencing of CYP24A1 gene expression, proliferation assays were performed. While PC3 and DU145 cells were not responsive to the growth inhibition mediated by vitamin D_3 (Figures 2C and 2D), CYP24A1 siRNA significantly enhanced the anti-proliferative action of vitamin D_3 (Figures 5C and 5D). Taken together, knockdown of CYP24A1 gene expression by siRNA renders prostate cancer cells more sensitive to the growth-suppressive effect of vitamin D_3.

To assess the impact of a sustained expression of CYP24A1 siRNA on the growth abrogation mediated by vitamin D_3, we generated PC3 and DU145 cell lines stably expressing CYP24A1 shRNA or non-targeting shRNA. The random integration into the genome of the vector-based shRNA led to a significant knock down of the CYP24A1 expression in absence and presence of calcitriol (Figures 6A and 6C). Indeed, when compared to cells with non-targeting shRNA, relative CYP24A1 mRNA levels were reduced by 75% in PC3 and 85% in DU145 cells in presence of vehicle, and the knockdown was about 90% for PC3 and 60% for DU145 cells in presence of vitamin D_3. In these clonal conditions, PC3 cells showed an improvement of their growth inhibitory response to vitamin D_3, reaching significance at 100 nM (Figure 6B). In DU145 cells, a significant reduction of cell proliferation was already achieved at 10 nM of vitamin D_3 (Figure 6D). Hence, the prolonged abrogation of CYP24A1 gene expression enhanced the 1,25$(OH)_2D_3$-mediated growth inhibition in prostate cancer cells and essentially changed vitamin D_3 resistant cells into vitamin D_3 sensitive cell lines.

DISCUSSION

Our study is the first to compare the mRNA and protein levels of the major vitamin D_3 catabolizing enzyme, CYP24A1, in benign and cancerous human prostate tissues. Changes in the expression of this enzyme are associated with poor differentiation status and prognosis of other types of cancer including colon (9-11), cervical (12), ovarian (9, 12), esophageal (13), lung (14) and basal cell carcinomas (15). The chromosomal locus 20q13.2-20q13.3 encompassing CYP24A1 was found amplified in breast (17), ovarian (18), gastric (19, 20) and colorectal cancers (21). The increased expression of the rate-limiting catabolic enzyme, CYP24A1, is likely to restrict *in situ* $1,25(OH)_2D_3$ growth abrogation activity, presumably by decreasing local $1,25(OH)_2D_3$ levels. The significant correlation found between high CYP24A1 expression levels and increased proliferation rate in human prostate tumors supports this hypothesis. The fact that the suppression of CYP24A1 gene expression led to an enhanced $1,25(OH)_2D_3$ anti-proliferative action in prostate cancer cells, provides an additional argument in favor of the *in situ* role of CYP24A1 in the control of prostate proliferation. Consistent with the notion that a selective growth advantage is given to the prostate tumor cells expressing high levels of CYP24A1, we found that overexpression of this catabolizing enzyme was associated with a more aggressive tumor behavior in prostate cancer. This latter observation is of clinical importance as CYP24A1 may represent a tissue biomarker of prostate tumorigenesis. Staining using a CYP24A1 immunohistochemistry-based test of prostate needle biopsies might be combined to existing tests in order to specifically stratify prostate cancer and predict the biological behavior of tumors. In addition to increase the accuracy in predicting prostate cancer disease outcome, CYP24A1 may serve to monitor treatment efficacy using exogenous vitamin D_3 as an adjuvant therapy for advanced prostate cancer.

Our findings indirectly point at the potential role for vitamin D_3 as a hormone acting locally to inhibit and perhaps to delay or prevent the progression to cancer. Epidemiological studies remain often-conflicting regarding an inverse association between sun exposure, serum levels of 25(OH)D, and risk of developing prostate cancer. Nevertheless, none of these studies assessed the intra-prostatic $1,25(OH)_2D_3$ levels. By examining the tissue expression of the main enzyme responsible for

the breakdown of vitamin D_3, our present data shed light on the *in situ* dynamic importance of calcitriol bioavailability, thus enriching a large body of preclinical research and clinical trials evidence supporting the biological relevance of vitamin D in cell growth restriction and prostate cancer treatment (22).

This study offers a rational basis for the use of specific inhibitors of CYP24A1 in the management of prostate cancer. The inverse relationship between CYP24A1 expression and differentiation status in prostate cancer calls for the development of CYP24A1 selective inhibitors that can be used either as single entities delivered to the tumor site to increase or extend endogenous calcitriol function or in combination with exogenous doses of $1,25(OH)_2D_3$ to achieve very localized high concentrations of vitamin D_3 resulting in optimal growth abrogation of advanced tumors with minimum systemic toxicity.

The use of cytochrome P450 inhibitors such as liarazole (23) and ketoconazole (24) or the phytoestrogen, genistein (25) synergistically enhanced the growth abrogation-mediated by vitamin D_3 by inhibiting the activity of CYP24A1 in prostate cancer cells. However, all these compounds lack selectivity and specificity and are likely to inhibit other cellular targets leading to long-term toxicity, thus rendering them incompatible or inefficient for clinical use. Ketoconazole and liarazole inhibit other P450 enzymes, including the enzymes involved in the steroidogenic pathways for testosterone, cortisol and aldosterone biosynthesis and may lead for instance, to adrenal insufficiency that would exacerbate the hypercalcemia of any vitamin D treatment. This present work explored the application of RNAi technology to selectively silence the expression of CYP24A1 in prostate cancer cells. RNAi-mediated degradation of homologous mRNA allows for a selective inhibition of key target molecules and offers a high specificity lacking in current drug treatments. Significant enhancement of the growth inhibitory action of vitamin D_3 achieved by combinatorial treatment with CYP24A1 siRNA holds a promise in the field of prostate cancer therapy. Nevertheless, significant obstacles, such as *in vivo* delivery, incomplete suppression of target genes need to be overcome before this technology can successfully be translated into the clinical arena.

This work shows that CYP24A1 was overexpressed in patients with prostate cancer, particularly in high-stage cases. In addition to highlight the endogenous anti-cancer action of vitamin

D_3 in human prostate, this dysregulation may be a predictive marker of vitamin D_3 clinical efficacy in patients with advanced prostate cancer as an adjuvant therapy. Our findings also provide for the first time, a proof of principle that targeting of CYP24A1 by siRNA, a more selective and specific approach and as a result, a potentially less toxic alternative, is efficient to enhance the anti-proliferative action of vitamin D_3 in prostate cancer cells, offering a promising combination therapy for vitamin D-based therapies for advanced prostate cancer.

Acknowledgments

This work was supported by W81XWH-07-1-0022 PC061154 Idea Development Award from the Department of Defense, Prostate Cancer Research Program and the Prostate Cancer Research Initiative to DJL.

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FIGURE LEGENDS

Figure 1: Overexpression of CYP24A1 in human prostate cancer.

A. Determination of CYP24A1 gene expression levels in men with prostate cancer. TaqMan qPCR was performed on patients' cDNA, prepared from well-documented cancer biopsy samples, normalized and assembled into ready-to-use gene expression panels. Data were analyzed using the comparative C_T method with the values normalized to β-actin levels. **B.** Representative immunohistochemical detection of CYP24A1 revealing higher staining in malignant prostate tissue than in paired cancer-adjacent normal tissue. Magnification x40.

Figure 2: Correlation between high protein levels of CYP24A1 and high tumor grade and late stage of human prostate cancer. A-B. Distribution of the relative expression levels of CYP24A1 and Gleason score (A) and tumor stage (B) categories. Semi-quantitative scoring of the quantity of CYP24A1 expression in 112 prostate cancer cores, derived from 56 patients with localized and metastatic prostate cancer disease, was performed (score 1= moderate; score 2=strong). Cases are shown as percentages of total immunoreactive cancer cores on the y-axis. **C-D.** Immunostaining of CYP24A1 in representative adenocarcinomas of different grades. In **C.**, low grade lesions of prostate cancer showing moderate intensity of the staining of the catabolic enzyme (**a.** Gleason 2+2 and T2NxM0; **b.** Gleason 2+3 and T2NxM0; **c.** Gleason 2+3 and T2NxM0; **d.** Gleason 1+1 and T2bNxM0). In **D.**, intense staining in malignant prostate tissues presenting with high Gleason score and late stage tumor progression (**a.** Gleason 9 and T3bN0M0; **b.** Gleason 7 and T3bN0M0; **c.** Gleason 7 and T4N0M0; **d.** Gleason 9 and T3bN0M0). Magnification x40.

Figure 3: Distribution of the percentage of Ki67-positive cells between cancer cores expressing high (2) and moderate levels (1) of CYP24. Semi-quantitative scoring of the CYP24A1 staining (1= moderate, 2=strong) was independently performed by two investigators who were blinded to the data. Scoring for semi-quantitative analysis for Ki67 staining was evaluated by the percentage of cells showing positively labeled nuclei: 0 (0-1% positivity), 1 (1-5 % positivity), 2 (5-10% positivity), 3 (10-

100% positivity). Magnification x40.

Figure 4: Inverse correlation between endogenous levels of CYP241A1 mRNA and growth abrogation action of vitamin D_3.

A. qRT-PCR analysis of endogenous CYP24A1 mRNA levels in presence of 10nM Vitamin D_3 or vehicle (ethanol; EtOH) in LnCAP, DU145 and PC3 prostate cancer lines. GAPDH served as the internal control. **B-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 3, 6 and 9 days of treatment in LnCAP (**B**), DU145 (**C**) and PC3 (**D**) (* if $P< .05$; ** if $P< .01$; *** if $P< .001$, ANOVA).

Figure 5: Effect of selective inhibition of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D_3.

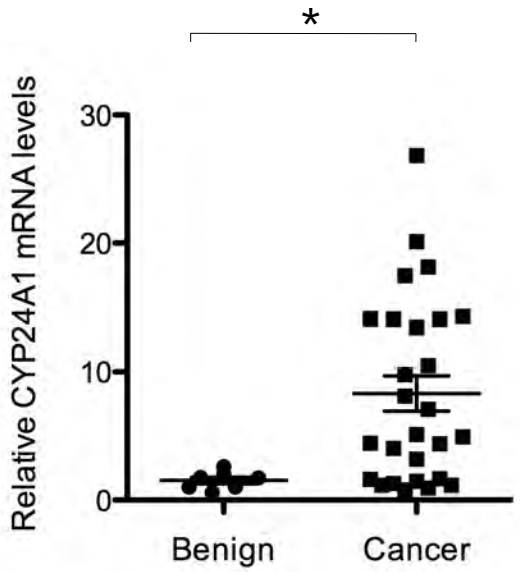
A. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D_3 or vehicle (EtOH) in DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non targeting siRNA (Scramble). GAPDH served as the internal control. **B.** Immunofluorescent staining of CYP24A1 protein of DU145 and PC3 transiently transfected with CYP24A1 siRNA or a non-targeting siRNA (Scramble) and incubated in presence of Vitamin D_3 10 nM. DAPI was used for cell nuclei visualization. **C-D.** Cell proliferation assays performed in presence of 10 nM or 100 nM calcitriol or ethanol after 6 days of treatment in DU145 (**C**) and PC3 (**D**) transiently transfected with CYP24A1 siRNA or non targeting siRNA (Scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if $P< .05$; ** if $P< .01$; *** if $P< .001$.

Figure 6: Generation of stable clones expressing shRNA and impact of sustained alteration of CYP24A1 gene expression on prostate cancer cell growth response to vitamin D_3.

A and C. qRT-PCR analysis of CYP24A1 mRNA levels in presence of 10nM Vitamin D_3 or vehicle (EtOH) in PC3 (**A**) and DU145 (**C**) stably transfected with CYP24A1 shRNA or a non targeting shRNA (Scramble). GAPDH served as the internal control. **B and D.** Cell proliferation assays performed in

presence of 10 nM or 100 nM calcitriol or ethanol for 6 days in stable PC3 (**B**) and DU145 (**D**) expressing CYP24A1 shRNA or non targeting shRNA (scramble). The values represent the mean of at least three separate experiments. Significance was evaluated by ANOVA, * if $P < .05$; ** if $P < .01$; *** if $P < .001$.

A.


*
Relative CYP24A1 mRNA levels
30
20
10
0
Benign
Cancer

B.

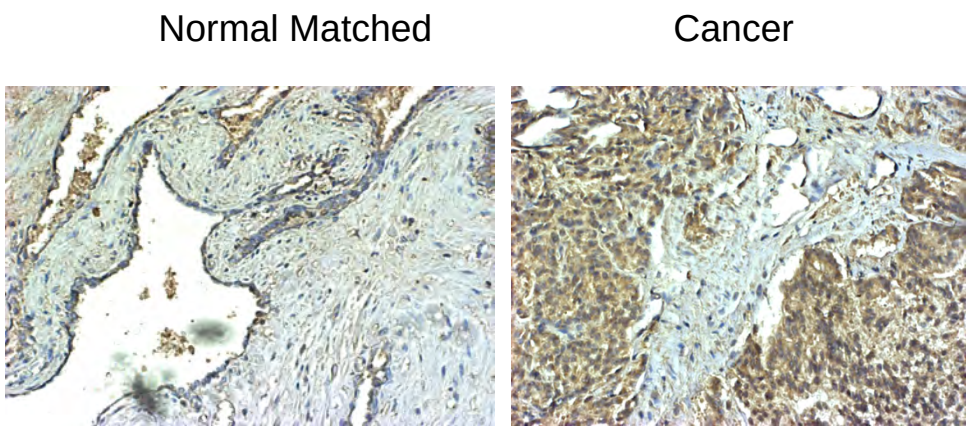
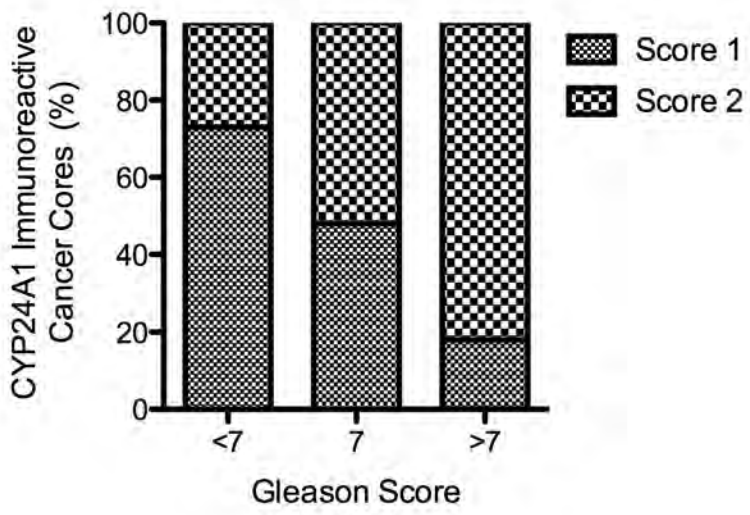
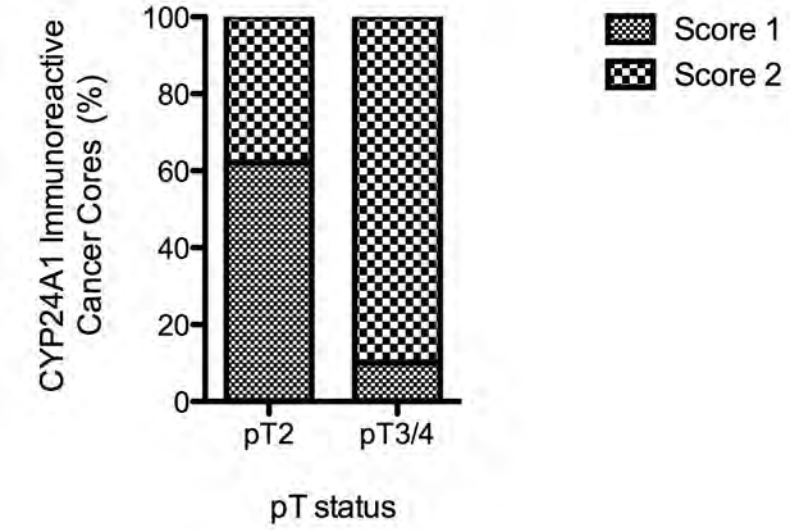

Normal Matched
Cancer

Figure 1

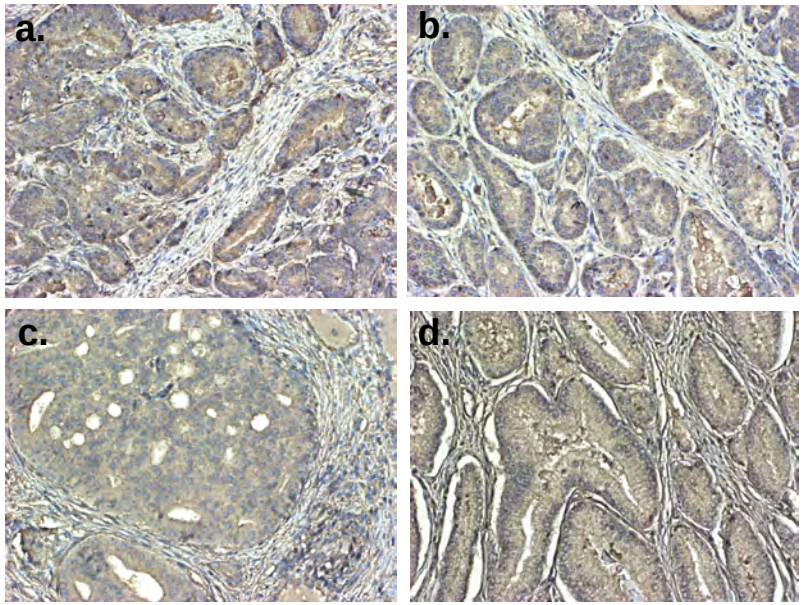
A.



B.



C.



D.

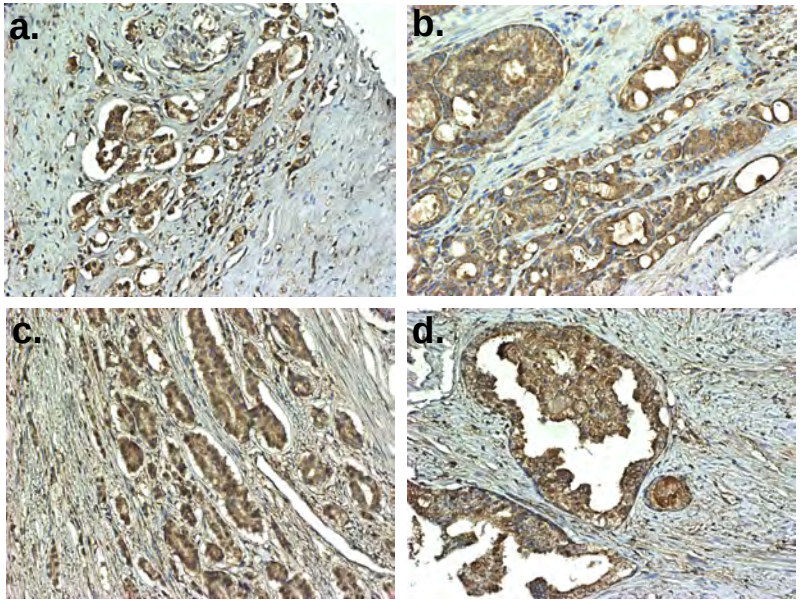
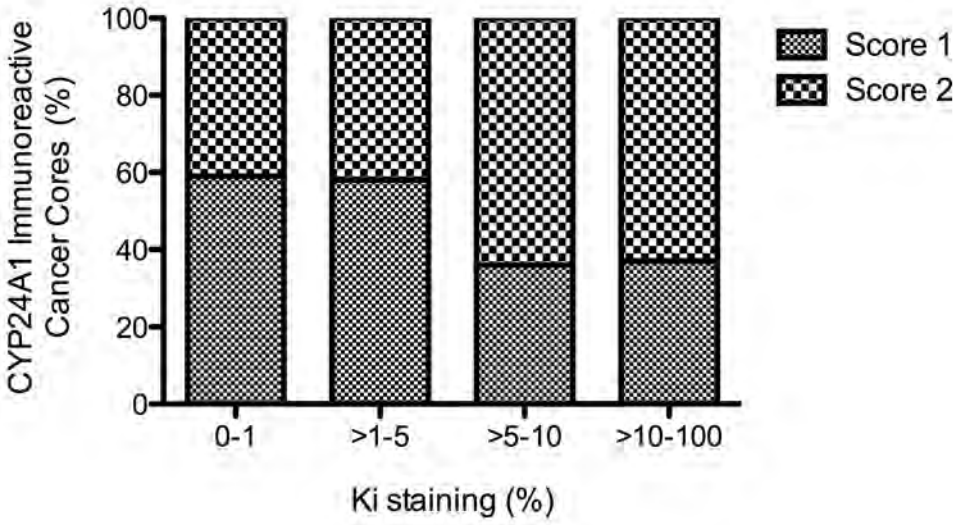


Figure 2

A.


CYP24A1 Immunoreactive Cancer Cores (%)
100
80
60
40
20
0
0-1
>1-5
>5-10
>10-100
Ki staining (%)
Score 1
Score 2

B.

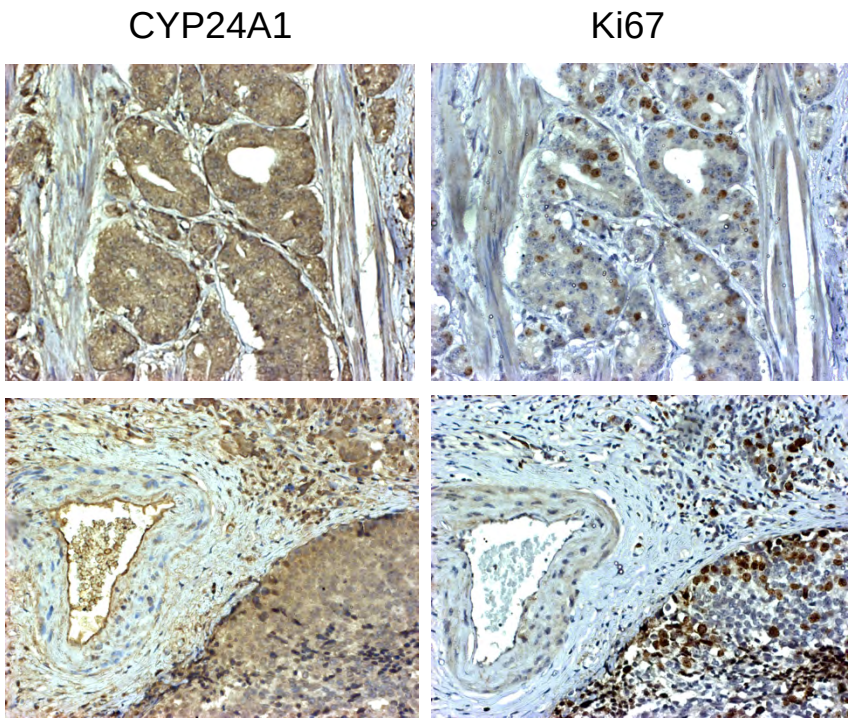

CYP24A1
Ki67

Figure 3

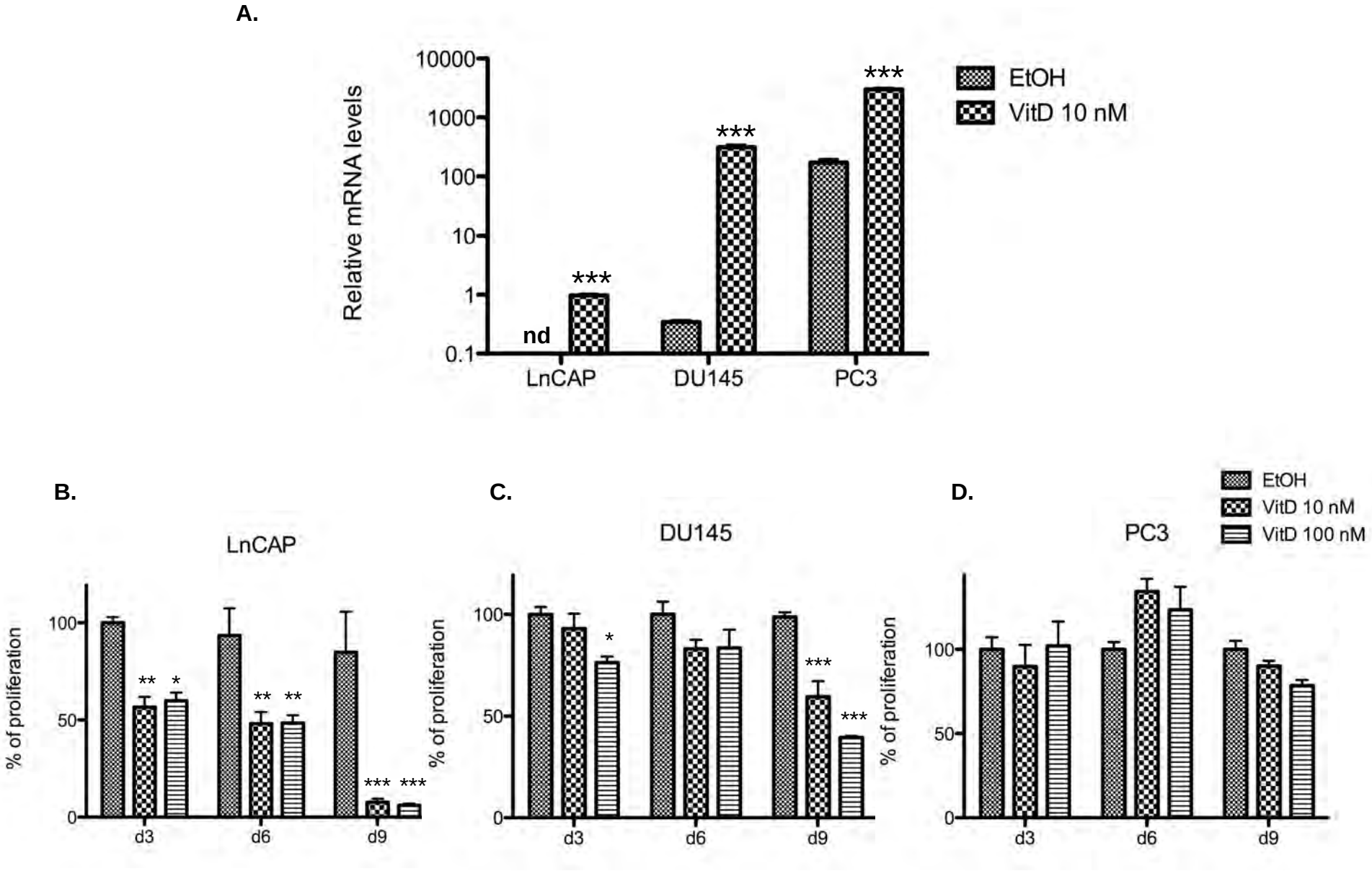

A.
Relative mRNA levels
10000
1000
100
10
1
0.1
nd
***
***
***
LnCAP
DU145
PC3
EtOH
VitD 10 nM
B.
LnCAP
% of proliferation
100
50
0
** *
** **
*** ***
d3
d6
d9
C.
DU145
% of proliferation
100
50
0
*
***
***
d3
d6
d9
D.
PC3
% of proliferation
100
50
0
d3
d6
d9
EtOH
VitD 10 nM
VitD 100 nM

Figure 4

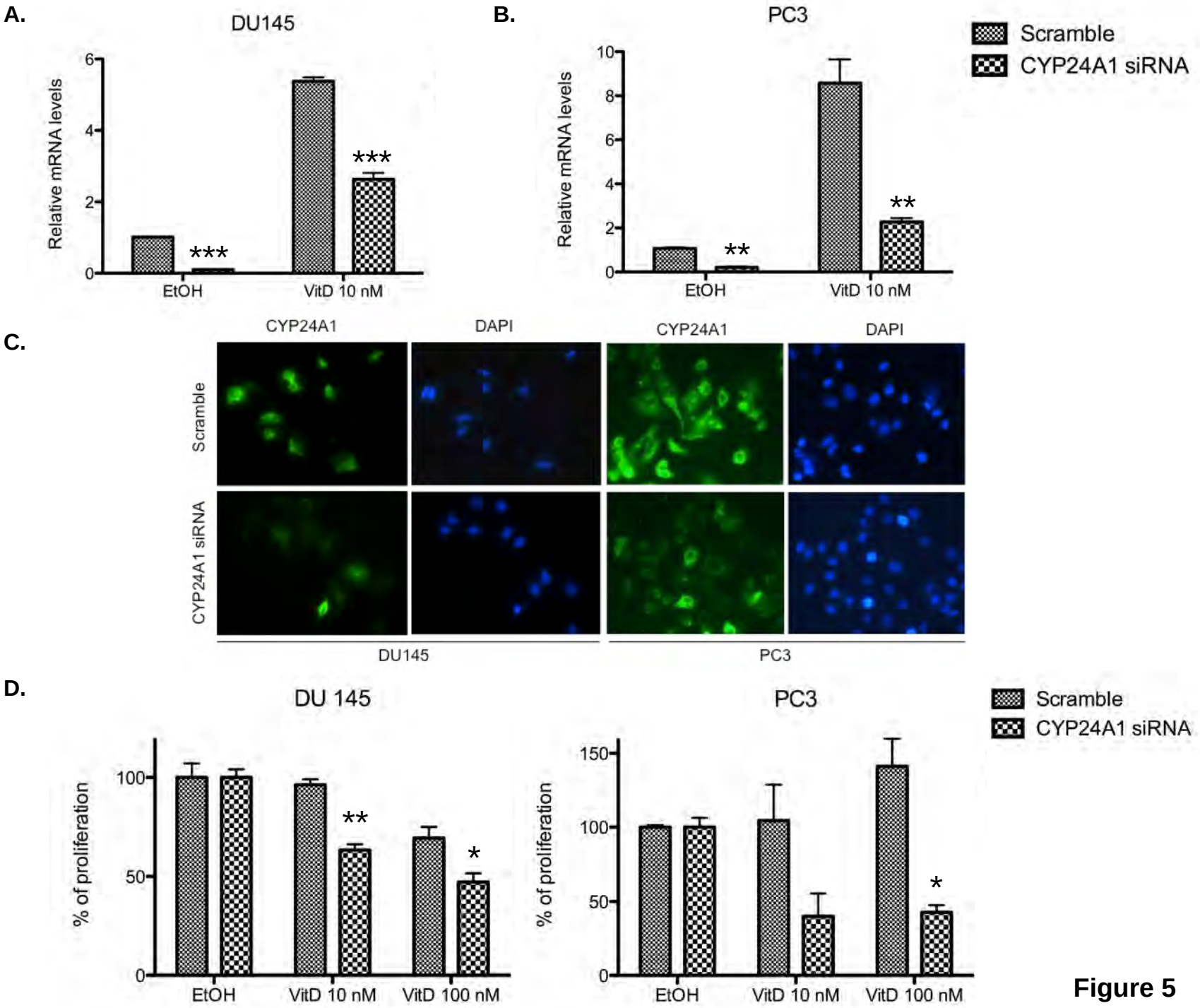

A.
DU145
Relative mRNA levels
6
4
2
0
***
***
EtOH
VitD 10 nM
B.
PC3
Relative mRNA levels
10
8
6
4
2
0
**
**
EtOH
VitD 10 nM
Scramble
CYP24A1 siRNA
C.
CYP24A1
DAPI
CYP24A1
DAPI
Scramble
CYP24A1 siRNA
DU145
PC3
D.
DU 145
% of proliferation
100
50
0
**
*
EtOH
VitD 10 nM
VitD 100 nM
PC3
% of proliferation
150
100
50
0
*
EtOH
VitD 10 nM
VitD 100 nM
Scramble
CYP24A1 siRNA

Figure 5

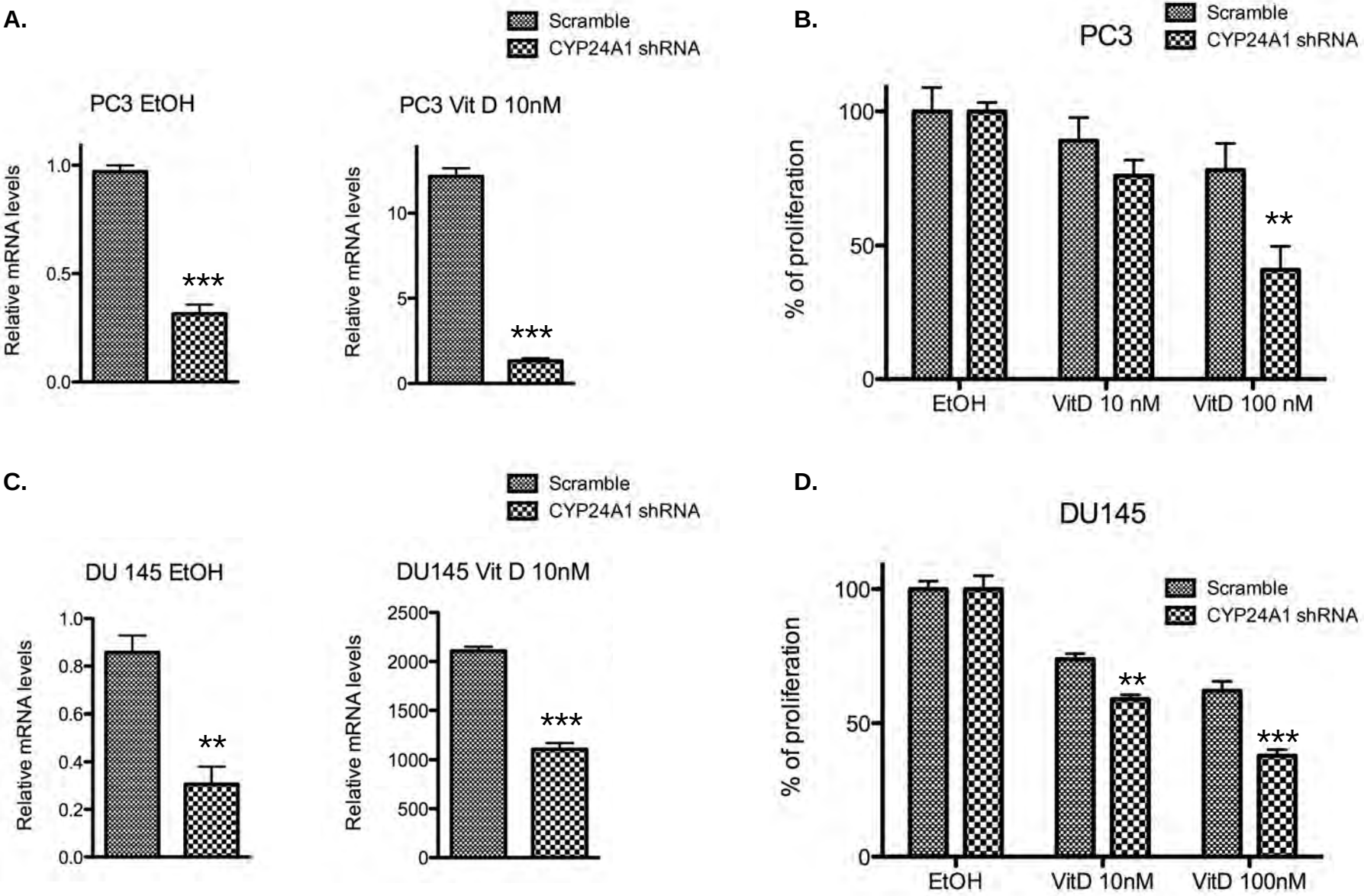

A.
Scramble
CYP24A1 shRNA
PC3 EtOH
Relative mRNA levels
1.0
0.5
0.0
***
PC3 Vit D 10nM
Relative mRNA levels
10
5
0
***
B.
PC3
Scramble
CYP24A1 shRNA
% of proliferation
100
50
0
**
EtOH
VitD 10 nM
VitD 100 nM
C.
Scramble
CYP24A1 shRNA
DU 145 EtOH
Relative mRNA levels
1.0
0.8
0.6
0.4
0.2
0.0
**
DU145 Vit D 10nM
Relative mRNA levels
2500
2000
1500
1000
500
0
***
D.
DU145
Scramble
CYP24A1 shRNA
% of proliferation
100
50
0
**
***
EtOH
VitD 10nM
VitD 100nM

Figure 6