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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

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1) Introduction: The goal of this study is to enhance the antiproliferative activity of Vitamin D in prostate. Our long-term goal is to use this approach for both the chemoprevention of disease initiation and treatment of advanced disease. Epidemiologic studies revealed that Vitamin D ($1\alpha,25(OH)_2D_3$) is important for prostate cancer, however, it does not work as well as one would predict to inhibit growth of prostate cancer. Our preliminary studies showed that CYP24, an enzyme that catalyzes the initial stages of degradation of $1\alpha,25(OH)_2D_3$ is over-expressed in prostate cancer and this pathway represents a key target for therapeutic intervention.

Table 1: General Design--- Assays for CYP24 siRNA and Vitamin D Action

Treatment	*Cells Incubated in Triplicate with Combinations of Reagents Below*																	
Control (Silencer N)	-	+		+	+	+							+	+			+	+
siRNA193+560 other siRNAs	-		+							+	+	+			+			+
1α,25(OH)$_2$D$_3$ (0.1-100 nM)	-			+			+					+		+	+	+	+	+
DHT or R1881 (0.1-100 nM)	-				+			+		+			+	+	+	+	+	+
Ketoconazole (0.1-100µg/ml)	-					+			+		+		+			+		+

2) Body

a) Aim #1 focuses on the optimization of approaches to inhibit CYP24 expression. We began by optimizing our experimental conditions. Three cell lines were utilized: PC3, LNCaP and DU145. Experimental treatments were exactly as proposed in the initial application and are summarized below. Cell growth assays were performed on days 6, 9, and 12 with media changes every three days and siRNA added on day 3. We are currently testing adding the siRNA at each media change.

i) Results:

(1) LNCaP were growth inhibited over the 12 day culture period (as expected) by increasing concentrations of Vitamin D from 0.1-100 nM (Figure 1). Maximal inhibition was approximately 70% by day 12. The addition of 10 :g/ml ketoconazole (a known inhibitor of CYP24 which serves as a positive control) in combination with Vitamin D enhanced the action of vitamin D and this effect was more marked at the lower concentrations of Vitamin D (0.1 nM and 1 nM). Not surprisingly, ketoconazole (frequently used as a topical treatment for fungus and yeast), affects a number of cellular enzymes including those that are involved in steroid biosynthesis. Others have shown that ketoconazole is growth inhibitory when given alone to both DU145 and PC3 cells and our results confirm this finding.

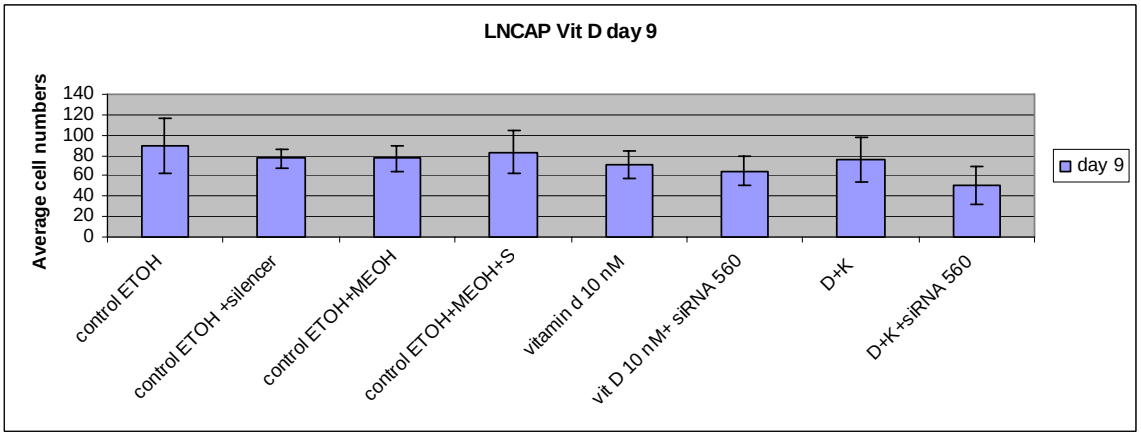

Figure 1 Growth Inhibition of LNCaP Cells by Vitamin D is Enhanced with CYP24 siRNA (560) and Ketoconazole (K). LNCaP cells were plated in 6 well plates with 4000 cells/well in triplicate. On day 3 of culture, the cells were treated with either vehicle control (ethanol, ,methanol alone or in combination), 10 nM Vitamin D alone or in combination with CYP24 siRNA (560, 40 ng/ml) or ketoconazole (1 ug/ml). Media was changed on day 6 and cells counted on day 9 in the Coulter Counter. Results represent the mean x 10^3 $\pm$ standard deviation of triplicate determinations. .

(2) The effect of ketoconazole together with Vitamin D was less marked in DU145 cells. Using the same strategy as described above, ketoconazole increased the growth inhibition seen with increasing concentrations of Vitamin D. DU145 cells are less sensitive to

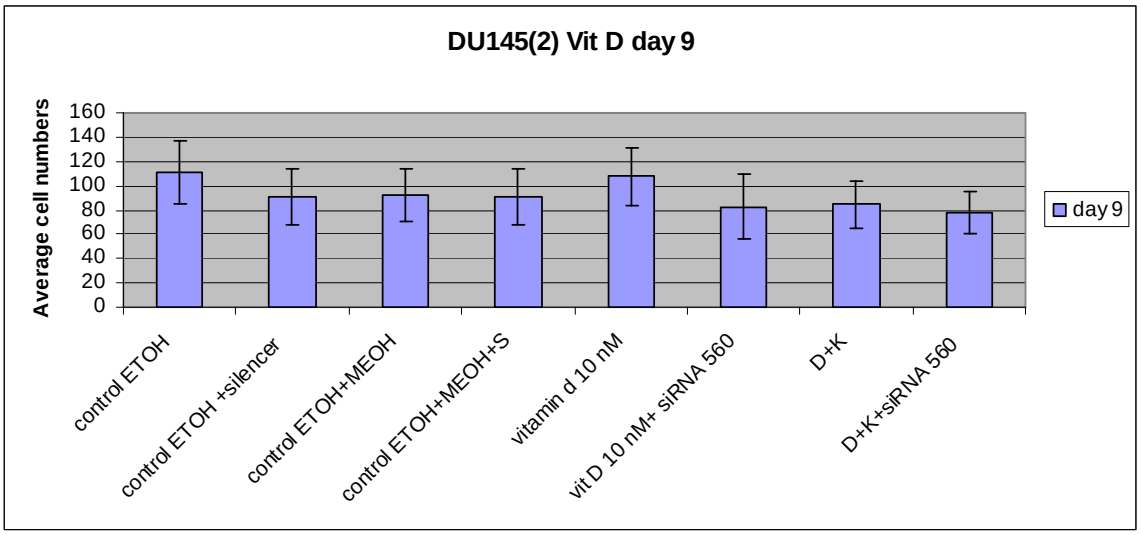

Figure 2 Growth Inhibition of DU145 Cells by Vitamin D is Moderately Enhanced with CYP24 siRNA (560) and Ketoconazole (K). DU145 cells were plated in 6 well plates with 4000 cells/well in triplicate. On day 3 of culture, the cells were treated with either vehicle control (ethanol, ,methanol alone or in combination), 10 nM Vitamin D alone or in combination with CYP24 siRNA (560, 40 ng/ml) or ketoconazole (1 ug/ml). Media was changed on day 6 and cells counted on day 9 in the Coulter Counter. Results represent the mean x 10^3 $\pm$ standard deviation of triplicate determinations. .

the growth inhibiting actions of $1\alpha,25(OH)_2D_3$ and our preliminary studies showed a high level of CYP24 expression (Figure 2).

(3) Vitamin D was growth inhibitory to PC3 cells, but the inhibitory action of ketoconazole together with $1\alpha,25(OH)_2D_3$ and CYP24 siRNA was more marked (Figure 3).

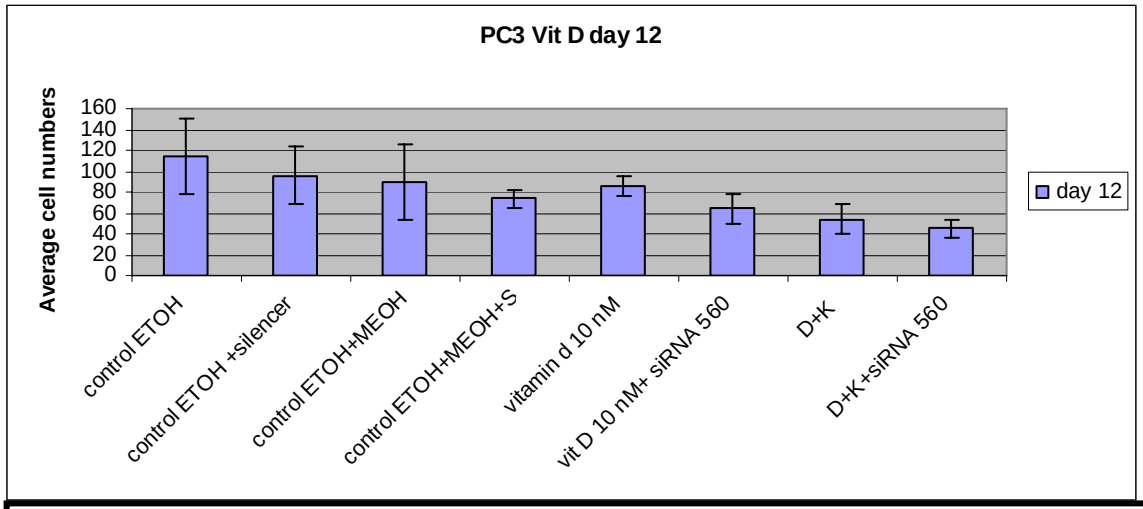

Figure 3 Growth Inhibition of PC3 Cells by Vitamin D is Moderately Enhanced with CYP24 siRNA (560) and Ketoconazole (K). PC3 cells were plated in 6 well plates with 4000 cells/well in triplicate. On day 3 of culture, the cells were treated with either vehicle control (ethanol, ,methanol alone or in combination), 10 nM Vitamin D alone or in combination with CYP24 siRNA (560, 40 ng/ml) or ketoconazole (1 ug/ml). Media was changed on day 6, 9 and cells counted on day 12 in the Coulter Counter. Results represent the mean x 10^3 $\pm$ standard deviation of triplicate determinations. .

(4) CYP24 siRNA action was transient, with maximal effect seen at 3 days after siRNA administration. This was true for PC3 cells, as well as DU145 cells and PC3 cells. This points to the need for enhancing transfection/viral transduction and development of stable cell lines as proposed in the original application. In addition, ongoing studies compare transfection using lipofectamine (as we used in our preliminary studies),

(a) Analysis of CYP24 mRNA Concentrations by Q-PCR: We compared the effect of CYP24 siRNA (various constructs) on CYP24 mRNA. QPCR studies are underway with analysis of both the full length and variant mRNA transcripts, and downstream genes expressed that reflect cell proliferation (p27, p21, cdk2) and apoptosis (Blc2, Bcl-X). These studies are performed in parallel with a cell growth assay under identical conditions.

(b) Analysis of CYP24 Protein Expression: Effect of siRNA:

(i) Western Blot Analysis: We realized that when using a slightly different gel electrophoresis protocol that the predominant CYP24 protein migrated with a slightly lower molecular weight then expected (Figure 4). This resulted from the existence of a 24-OHase splice variant that results in a truncated protein that maintains the substrate binding domains but

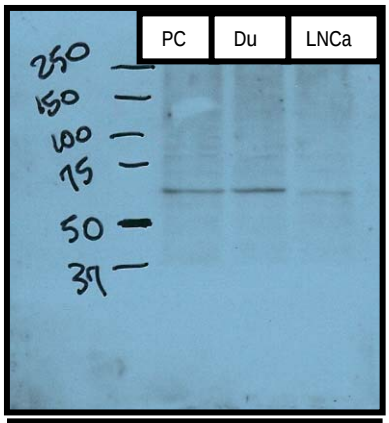

Figure 4 CYP Expression in Prostate Cancer Cell Lines: Western Blot (MW positions are denoted on the side x 10^3)

lacks the mitochondrial targeting sequences causing them to be catalytically inactive (1). In addition, Trump and colleagues (2) showed that single nucleotide polymorphisms (SNPs) and splicing variants differed between LNCaP, DU145 and PC3 cells. As we had reported in our Preliminary studies, CYP24 expression is low in LNCaP, higher in PC3 and highest in DU145 cells. After calcitriol treatment (a synthetic vitamin D), the pattern of CYP24 expression varied by cell line with different splice variants present (Figure 5). This complicates our analysis as both the antibody we generated using synthetic peptides and the antibody we purchased from Santa-Cruz against the C-terminal region of CYP24 will not distinguish between the variant and the full length protein using immunocytochemistry. We have purchased the antibody directed against the n-terminal region of CYP24 that is expected to discriminate between the two variants. The use of these two antibodies will allow us to distinguish between a knock-down of the active protein and a

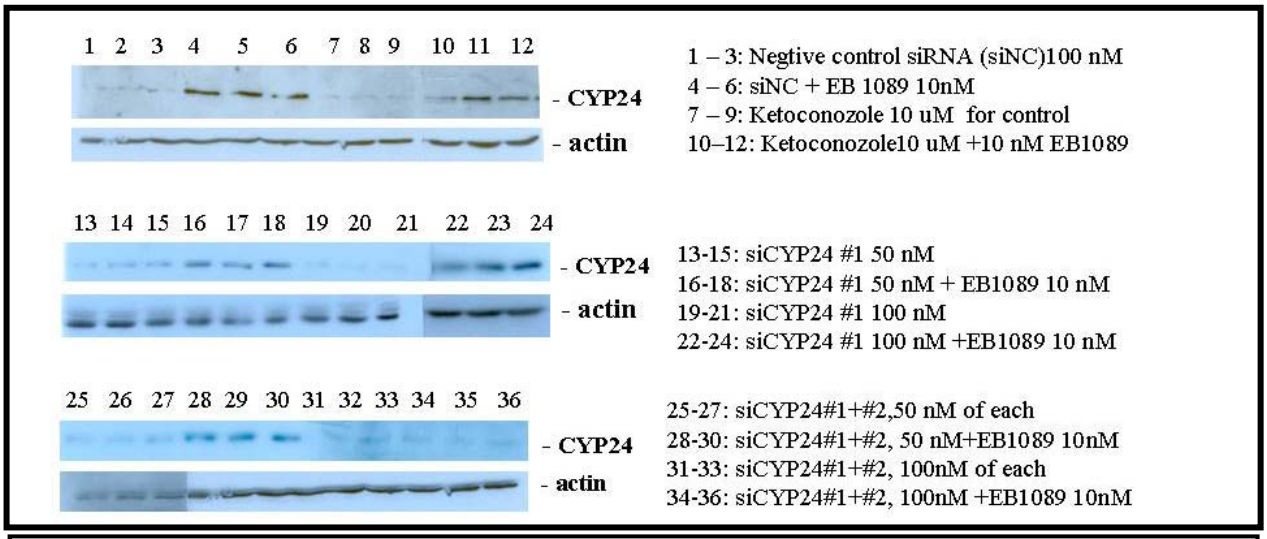

Figure 5 A Combination of Two CYP24 siRNA Knocks Down CYP24 Expression Induced by the Synthetic Vitamin D (EB1089) (note: Data not final and gels cut and spliced above for preliminary analysis only)

knockdown of the inactive splice variant. Figure 6 shows the induction of CYP24 by the administration of the synthetic vitamin D analogue, EB1089. In addition, although neither siRNA alone was sufficient to totally block the CYP24 protein, together knockdown was achieved. This is in contrast to the results with the treatment with the chemical inhibitor, ketoconazole which did not block the protein induction.

(ii) Immunohistochemical Analysis of CYP24 In Prostate Cancer Cell Lines: As described above, the discovery of splice variants of CYP24 has complicated our

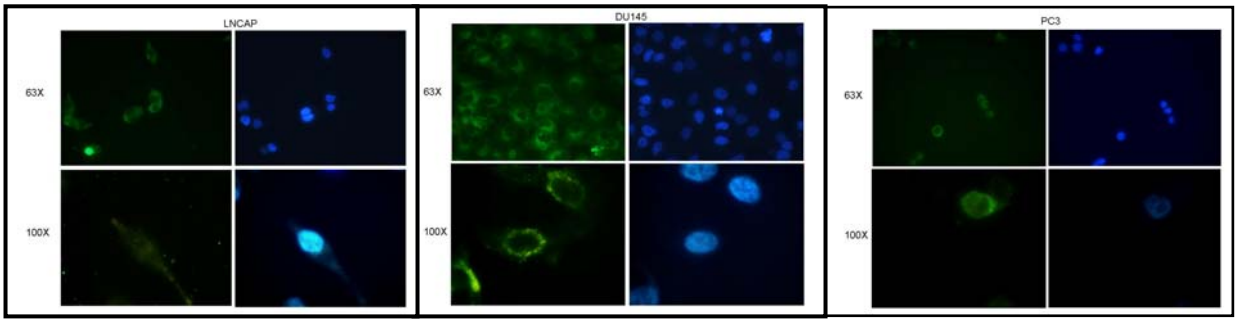

Figure 6 Immunocytochemistry of CYP24 In Prostate Cancer Cell Lines: LNCaP (left panel), DU145 (center panel) and PC3 (right panel) cells were fixed and incubated with the C-terminal CYP24 antibody obtained from Santa-Cruz at 1:100 dilution. The cells were counterstained with DAPI. Several observations were noteworthy: LNCaP again showed the lowest and DU145 the highest levels of CYP24 protein with PC3 cells intermediate. In the DU145 a distinct punctate pattern was observed and studies are underway to determine if this staining is mitochondrial as expected. This was evident in the other cell lines to a lesser degree. Not shown are the negative controls: lacking first antibody and pre-immune serum where staining was not observed.

analysis of the protein and knockdown with siRNA. We must distinguish the non-active variants from the full length protein. To accomplish this required that we obtain the antibodies now commercially available to CYP24 to the N- and COOH termini (when we began this project, no

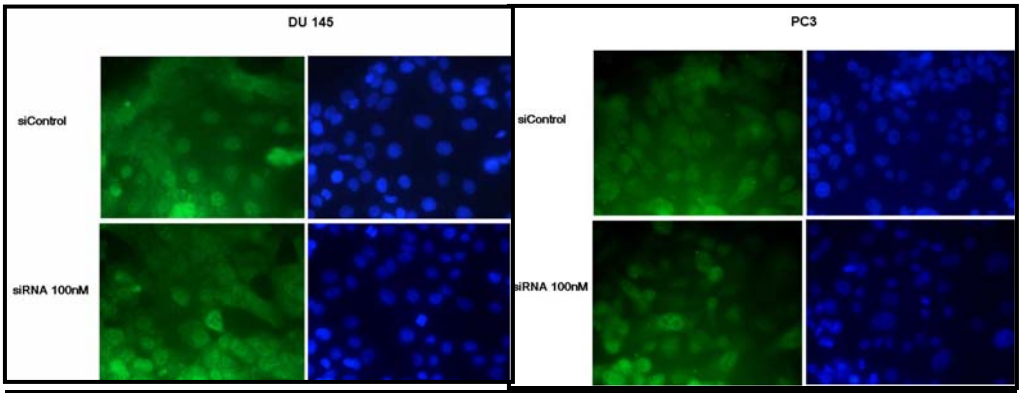

Figure 7 siRNA Knockdown of CYP24 Protein in DU145 and PC3 Cells is Partially Effective In this series of experiments testing the 100 nM 560 CYP24 siRNA , we observed only diminished expression of CYP24 protein. In addition, using the antibody we generated using synthetic peptides it appeared that some of the staining was nuclear which remained fairly constant with lesser staining of the cytoplasm after knockdown. These studies are being repeated with the Cter and Nter antibodies obtained from Santa-Cruz.

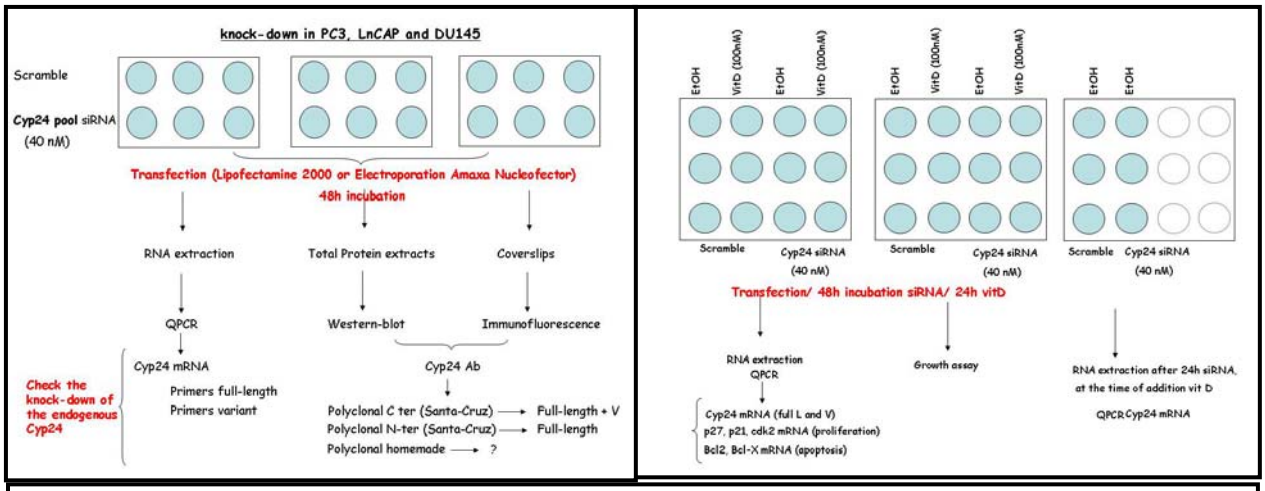

Figure 8 Current Experimental Strategy to Improve CYP24 Knockdown In Vitro

antibodies were available and we made our own which can not distinguish the splicing variant from the native form). We have used the C-terminal antibody first to define subcellular localization of the CYP24 protein as described in Figure 7. The strategy of our current analysis is detailed in Figure 8 above. Figure 7 shows the results of siRNA knockdown using the 560 siRNA alone. More recently we have purchased a pool of CYP24 siRNA constructs from Dharmacon, as well as additional custom siRNA to knock down the full length active form of siRNA, as well as separate targeting of the splicing variant to distinguish these two proteins in the cells. The use of the CYP24 siRNA pool has markedly improved our ability to knock down the CYP24 mRNA levels. As shown in Figure 9, 40 ng CYP24 siRNA pool results in an 80% decrease of mRNA levels in PC3 and DU145 cells. In LNCaP, CYP24 levels are nearly

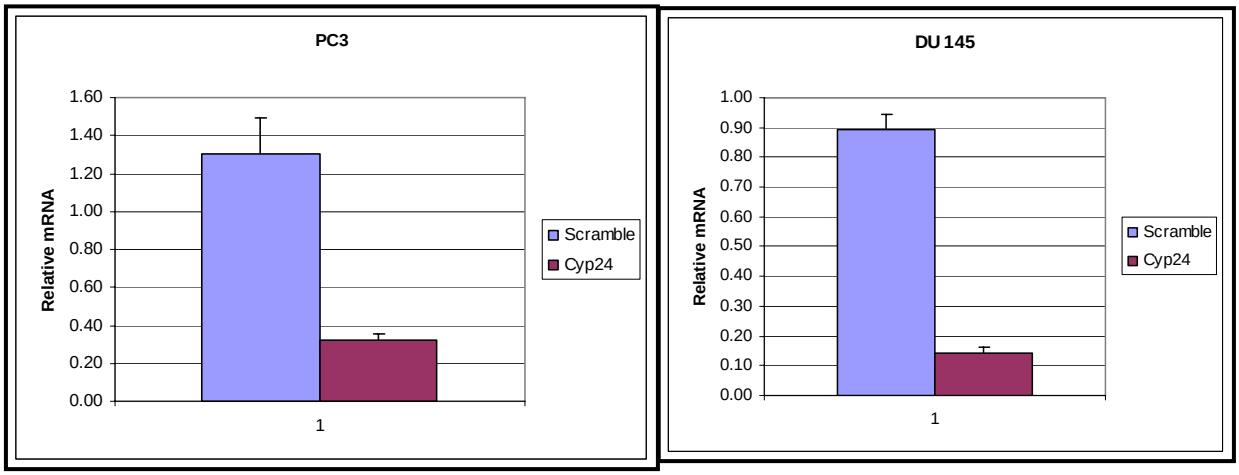

Figure 9 Pooled CYP24 siRNA effectively knocks down CYP24 mRNA in PC3 and DU145 cells measured by Real-Time RT-PCR (LNCaP done but not shown—CYP24)

undetectable and induced by Vitamin D administration. A very significant knockdown of the protein was observed as well with the pooled CYP24 siRNA administration. When tested in the presence or absence of 10 nM Vitamin D, Figure 10 shows the diminished CYP24 protein levels

48 hr after pooled SYP24 siRNA administration using immunocytochemistry for detection. It has become obvious to us that in addition to our need to distinguish the full length protein from the variant, we also need to improve our delivery of the siRNA. We have utilized cell electroporation Amaxa Nucleofactor with limited success and are currently optimizing the system. Our design of the lentiviral constructs for the production of the stable transfectants is underway.

3) Key Research Accomplishments

a) Optimization of the CYP24 Knockdown

i) Lipofectamine

ii) Cell electroporation

iii) Lentiviral Constructs

b) Enhancement of Vitamin D inhibition of prostate cancer cell growth with CYP24 siRNA in combination with ketoconazole

c) Distinguishing the full length active protein from the variant by immunocytochemistry

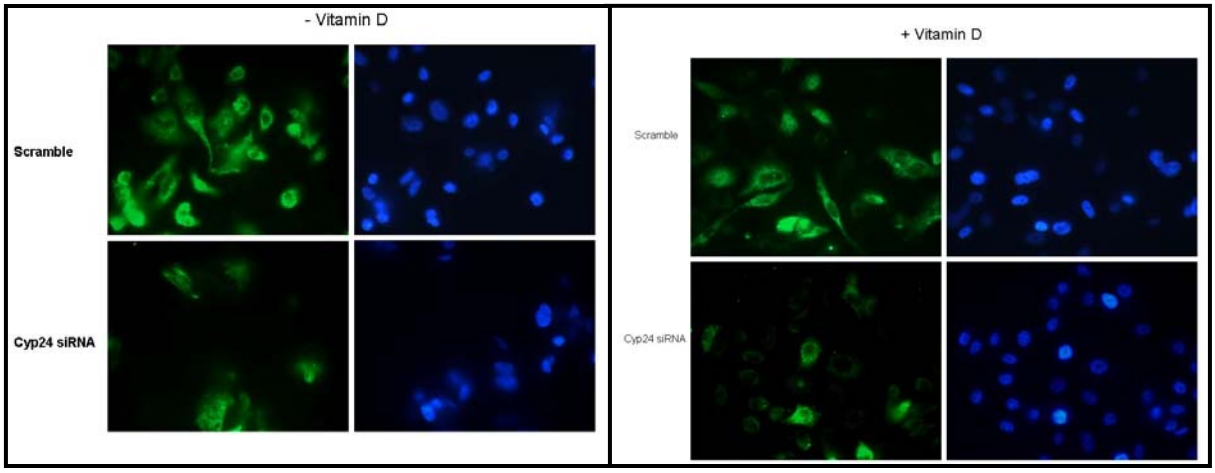

Figure 10 Administration of Pooled CYP24 siRNA to DU145 cells results in protein knockdown 48 hr after administration

4) Reportable Outcomes:

a) Zhang L, Smith RG and Lamb DJ. Enhancement of Vitamin D Action in Prostate Cancer Through Silencing of CYP24. Session of Effects of Hormones on Cancers: Translational. The 89th Annual Meeting of the Endocrine Society, 2007.

5) Conclusion: In conclusion, this has been a productive year for this project. Personnel were hired to replace Dr. Lixin Zhang who moved to accept a junior faculty position at MD Anderson Cancer Center. We have completed about 2/3 of aim #1 which seeks to further develop knockdown strategies for CYP24 in vitro. We have almost completed our studies of CYP siRNA knockdown of CYP24 mRNA levels. Using immunocytochemistry and Western blot analysis we

have assessed the CYP24 protein in three different cell lines with varying expression levels of CYP24. The existence of the splicing variant that is inactive has somewhat complicated this portion of the aim but we now have site specific antibodies that should allow us to distinguish these two proteins. We have also analyzed the effect of CYP24 knockdown on prostate cancer cell apoptosis in LNCaP cells using a TUNEL assay and DNA fragmentation by comet assay.

6) References

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Appendices……

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Title: Enhancement of Vitamin D Action in Prostate Cancer Through Silencing of CYP24

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Vitamin D deficiency and diminished exposure to sunlight are implicated in the development and progression of prostate cancer in humans. Prostate cancer cells express the receptor for Vitamin D and its action in prostate cancer cell lines results in growth inhibition and apoptosis when tested in vitro and a slower tumor growth and diminished metastasis in animal models. Vitamin D administration also induces the expression of CYP24, a P450 enzyme that catalyzes the first step in the degradation of Vitamin D, effectively blunting the growth inhibitory action of the Vitamin D signaling system. RNA expression of CYP24 increases markedly with increasing TMN (Tumor Size, Lymph Node Involvement, and Metastasis) stage of prostate cancer averaging 250-fold relative to control in TMN stage IV. There is an inverse relationship between the degree of Vitamin D growth inhibition and CYP24 expression. We developed an antibody to human CYP24. CYP24 is expressed in TMV II prostate cancer with little or no expression in the histologically normal adjacent prostate tissue. Expression is prominent in the highest stage prostate cancer (TMV IV). Thus, CYP24 is expressed at all stages of prostate cancer (as well as in LNCaP, DU145 and PC-3 cells) and the level of expression increases with increasing disease progression. Thus, prostate cancers become refractory to the inhibitory actions of Vitamin. We tested the hypothesis that inhibition of CYP24 would enhance Vitamin D inhibition of prostate cancer growth. Inhibitors of CYP24 are have limited application because of their low selectivity and side effects that prohibit long term use. CYP24 siRNA enhances the Antiproliferative Action of Vitamin D in LNCaP, DU145 and PC-3 cells. CYP24 siRNA blocks CYP24 protein expression. 100 nM siCYP24 (8% inhibition) or 1nM 1α,25(OH)2D3 (16% inhibition) alone did not significantly effect LNCaP cell growth. However, 1 nM 1α,25(OH)2D3 combined with 100 nM siCYP24 inhibited the growth by 43 % ($p < 0.001$) compared to the control. 10 nM 1α,25 (OH)2D3 alone inhibited the cell growth by 62 %, but combination of 10 nM 1α,25(OH)2D3 and 100 nM siRNA inhibited growth by 83 %. When CYP24 is inhibited, a lower dose of Vitamin D blocks cell growth. 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.

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