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College of Health and Human Sciences

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1,25-dihydroxyvitamin D Regulation of von Hippel-Lindau (VHL) and Prolyl
Hydroxylase 2 (PHD2) in MCF10A Breast Epithelial Cells containing the Harvey-*Ras*-
Oncogene

by

Alle Barnard

A Thesis Submitted in Partial Fulfillment
Of the Requirements for a Degree with Honors
(Didactic Dietetics)

The College of Health and Human Sciences

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Approved by

Advisor: Dorothy Teegarden

Purpose

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) is shown to have preventative effects against breast cancer progression by inhibiting angiogenesis, inducing apoptosis, reducing proliferation, and promoting differentiation (Ben-Shoshan, 2007). The purpose of this study is to determine the impact of $1,25(\text{OH})_2\text{D}$ on VHL and PHD2 protein expression and mRNA expression in untransformed MCF10A (NeoN) and oncogene (Harvey-*ras*) transfected MCF10A breast epithelial cells.

Literature Review & Rationale

Breast cancer is the second most common cancer affecting women in the United States with 202,964 women diagnosed and 40,589 women deceased from breast cancer in 2007, according to the Centers for Disease Control and Prevention (US Cancer Stats). Breast cancer tumors go through four stages of development called, carcinogenesis. Carcinogenesis begins with tumor initiation and then tumor promotion, malignant conversion, and finally tumor progression (Weston, 2000). Tumor initiation begins with irreversible genetic mutations, often resulting in oncogene activation.

Oncogenes are normal cell genes that control cell proliferation, differentiation, and apoptosis but when altered or mutated, can cause uncontrolled growth, therefore contributing to breast cell tumorigenesis (Weston, 2000). The Harvey-*ras* oncogene is present in many cancer tumors and when can lead to increased cell proliferation and decreased apoptosis. A study by Moon et. al. determined that the H-*ras* oncogene was the mediator of invasive activity in MCF10A breast cancer epithelial cells (Moon, 2000). Additionally, the H-*ras* oncogene has been linked to reduced transcription regulation of the nuclear vitamin D receptor (nVDR) in transfected cells (Taber, 2009). The ligand bound nVDR translocates and binds to vitamin D response elements (VDREs), which regulate gene transcription (Chun, 2002). The biologically active form of vitamin D, $1,25(\text{OH})_2\text{D}$, has a high affinity for nVDR, and has two pathways of action: transcriptional regulation and transcriptionally independent regulation. Studies to determine the role of

these elements in cancer progression are vital to the development of vitamin D recommendations for cancer prevention and therapy.

Vitamin D can be obtained from ingestion of foodstuffs or conversion in the skin via sunlight. Foods naturally rich in vitamin D include fatty fish (salmon, tuna, etc), egg yokes, and cod liver oil (NCM, 2011). Milk (cows and soy), yogurts, and some juices are among the vitamin D-fortified foodstuffs (NCM, 2011). However, the majority of vitamin D generated in the human body is derived from the skin through sunlight. When UV form sunlight is absorbed in the skin, it converts 7-dehydrocholesterol, the vitamin D precursor, to previtamin D₃, which is quickly isomerized into vitamin D₃ (cholecalciferol) in the dermis and epidermis. Cholecalciferol, or vitamin D, from both sunlight and foodstuffs, then flows through the bloodstream and enters the liver where it is metabolized to 25-hydroxyvitamin D (25(OH)D₃) by 25-hydroxylase (Jiang, 2010; DeLuca, 2004). Because this conversion is uncontrolled, serum 25(OH)D₃ is a valid biomarker of vitamin D status. However, this metabolite does not readily bind to nVDR and so it is not biologically active. The 1 α -hydroxylase in the kidney further hydroxylates 25(OH)D₃ to its bioactive form, 1,25(OH)₂D, which has a high affinity for nVDRs. Additionally, this active metabolite regulates serum concentrations of calcium and phosphorus, regulation of certain hormones, and reduces the risk of many diseases (DeLuca, 2004).

One role of vitamin D in breast cancer prevention is via inhibition of angiogenesis (Jiang, 2010). Angiogenesis is the growth and differentiation of blood vessels to feed an organ or tumor, and can be regulated by low oxygen status (hypoxia). Solid tumors in a hypoxic state survive by inducing angiogenesis to bring nutrients and oxygen to the tumor site. Hypoxia induces angiogenesis, in part, through transcriptional factors, one such being hypoxia-inducible factor 1 (HIF-1). Increased levels of HIF-1 have been associated with tumor aggressiveness, vascularization, therapeutic resistance, and mortality (Jiang, 2010; Ben-Shoshan, 2007). HIF-1 is composed of two subunits, HIF-1 β and HIF-1 α , which heterodimerize to form the HIF-1 transcription factor. HIF-1 β is constitutively expressed and HIF-1 α is the regulatory

subunit and is oxygen regulated. HIF-1 α can promote angiogenesis through activation of gene products, such as vascular endothelial growth factor (VEGF) (Chun, 2002).

HIF-1 α protein expression is regulated by protein stability, mRNA stability, and transcription (Chun, 2002). In normoxia, one of the prolyl hydroxylases (PHD 1- 3) hydroxylates HIF-1 α , promoting recognition and binding of the von Hippel-Lindau (VHL) tumor suppressor protein to form an E3 ubiquitin-protein ligase complex that mediates degradation of HIF-1 α (See Fig. 1) (Chun, 2002; Demidenko, 2005; Jiang , 2010). In a hypoxic state, PHDs cannot hydroxylate HIF-1 α since dioxygen is not present, therefore no VHL binding occurs and HIF-1 α rapidly accumulates, causing ubiquination with HIF-1 β and activate target transcription factors. Hence, an increase in PHDs down regulates HIF-1 α protein via decreasing protein stability.

It is suggested that PHD2 is the major oxygen sensor and when reduced can cause an increase in HIF-1 α levels (Chan, 2002). Results suggest that levels of PHD2 in clinical breast cancer cases are significantly lower compared to that of normal breast tissue and are correlated with an increase in tumor growth (Chan, 2002). Increasing expression of PHD2 mRNA correlates to an increase in PHD2 protein expression which decreases HIF-1 α expression, and is therefore a target of cancer prevention.

Tumor suppressor protein VHL, as part of the E3 ubiquitin-protein ligase complex, functions primarily to promote the degradation of ubiquitinated proteins (Zia, 2007). A decrease in VHL protein may contribute to tumorigenesis since VHL protein expression is required for the down-regulation of HIF-1 α . In clear cell renal carcinoma, alterations in VHL cause upregulation of HIF-1 α which regulates tumorigenesis (Barrisford, 2011). Patients with partial VHL gene deletion are noted to have an increased incidence of renal cell carcinoma compared to those with full deletions (Barrisford, 2011). A study by Zia et al. suggests that the lowest VHL levels were found in tumors which developed local recurrence and that breast cancer survivors (with no recurrence) had the highest levels of VHL (Zia, 2007). The same study

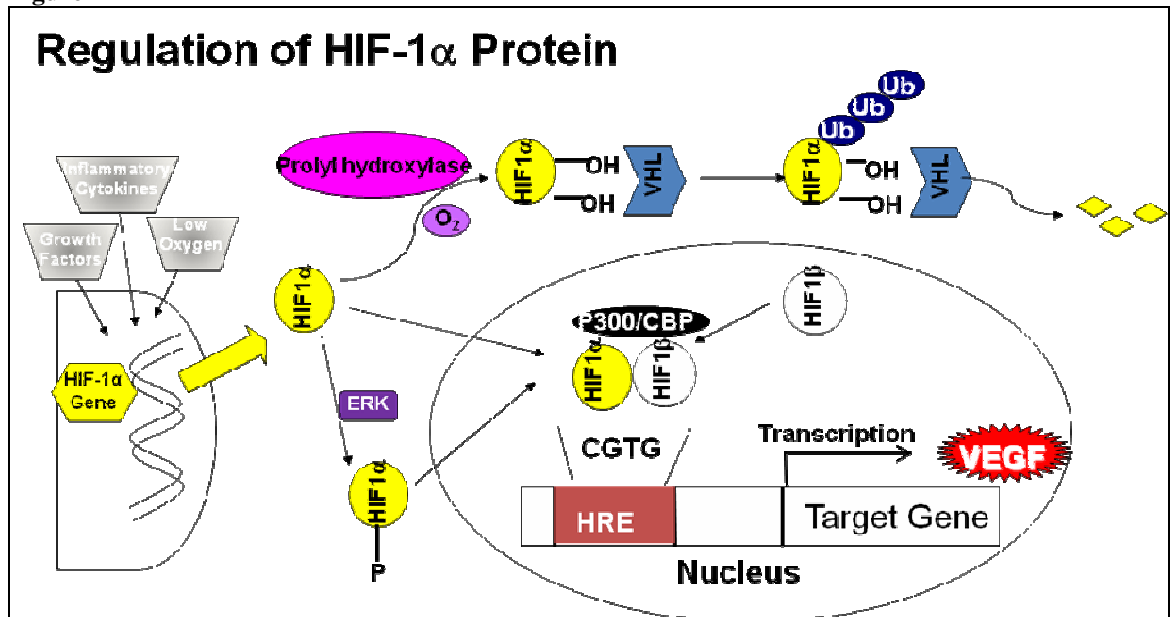
also stated that cancer cells had much weaker stains for VHL protein compared to normal cells and specifically breast cancer cells had low to moderate staining (Zia, 2007).

The link between breast cancer and vitamin D are being thoroughly researched (Welsh, 2004; Bertone-Johnson, 2009). The NHANES I study conducted an epidemiologic follow-up of vitamin D and breast cancer risk in 8596 women and found that those with high sunlight exposure had a 25-65% decrease in breast cancer risk (John, 1999). A review of vitamin D and breast cancer compiled by Welsh, J. cited several animal studies in which increased vitamin D intake (from either food or sunlight) was associated with a reduced risk in breast cancer (Welsh, 2004). A population-based case control study of pre-menopausal women found a significant association with cutaneous vitamin D, produced from sunlight, and a reduced risk of breast cancer (Anderson, 2011).

It is known that $1,25(\text{OH})_2\text{D}$ regulates multiple cell signaling pathways that in turn regulate proliferation, differentiation, apoptosis, and most likely angiogenesis, characteristics of cancer cells, in several cell types (Levine, 2004). Specifically, vitamin D receptors (VDRs) are mediators of cell proliferation, differentiation, and apoptosis when activated by $1,25(\text{OH})_2\text{D}$ (Welsh, 2004). Results of studies by Ben-Shoshan et al. suggest that under hypoxic conditions, $1,25(\text{OH})_2\text{D}$ caused a reduction in HIF-1 α expression thus decreased VEGF levels, in CL-1, MCF-7, and SW-480 breast epithelial cell lines. Results from the same study indicate that $1,25(\text{OH})_2\text{D}$ did not alter HIF-1 α on a transcriptional level, therefore a study on the affect of $1,25(\text{OH})_2\text{D}$ on HIF-1 α protein expression is needed to determine where $1,25(\text{OH})_2\text{D}$ is working (Ben-Shoshan, 2007). Since both $1,25(\text{OH})_2\text{D}$ and VDRs are found in cancerous tissues (Ben-Shoshan, 2007), it is possible that a higher $1,25(\text{OH})_2\text{D}$ status may act as an inhibitor to breast cancer tumors. In our laboratory we show that 12-hour and 24-hour $1,25(\text{OH})_2\text{D}$ treatment induced an increase in stability of HIF-1 α (See Fig.2). Transcriptional and protein degradation inhibitors were used to test the mechanism of $1,25(\text{OH})_2\text{D}$ induction of HIF-1 α (See Fig. 3). The results suggest that protein expression is regulated by different mechanisms between the MCF10A and the *ras*-transfected cell

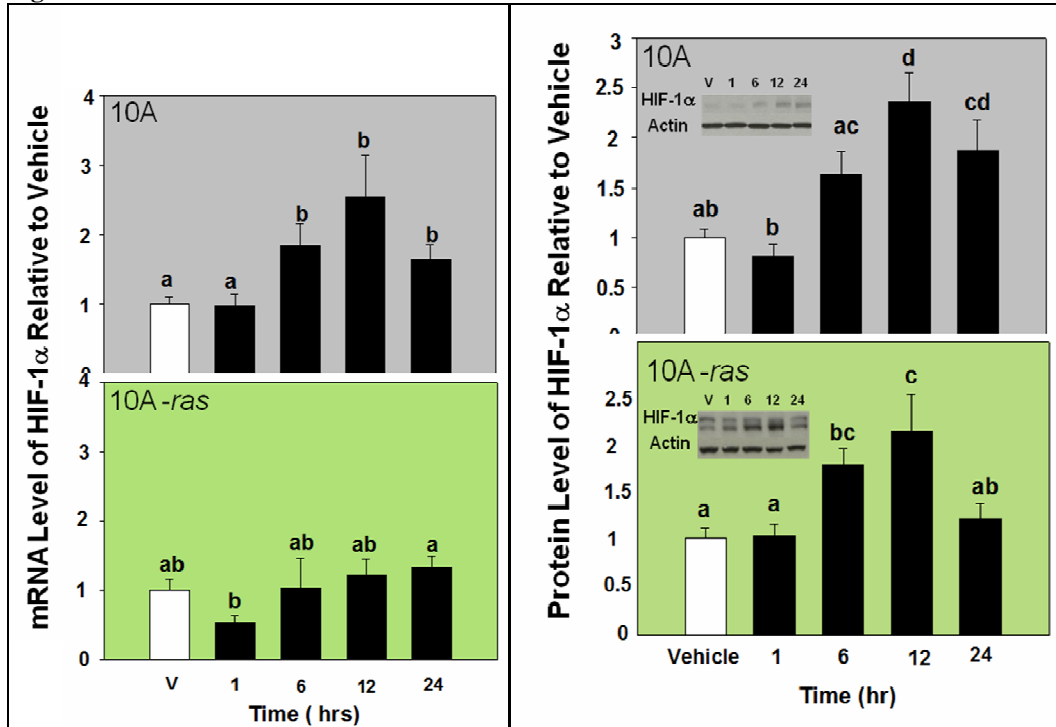
lines(Jiang, 2010). In MCF10A cells, it was shown that 1,25(OH)₂D likely mediates transcriptional regulation of HIF-1 α protein by VDR (Jiang, 2010). In MCF10A-*ras* cells, the studies suggest 1,25(OH)₂D regulates HIF-1 α through protein stability (Jiang, 2010). Lastly, the study suggests that the *Ras*-oncogene may down-regulate HIF-1 α transcription, since the transcriptional inhibitor had no effect on HIF-1 α protein levels of MCF10A cells but increased HIF-1 α protein levels in MCF10A-*ras* cells (Jiang, 2010). Determining the role of 1,25(OH)₂D in PHD2 and VHL expression, and therefore progression of cells to tumors, is vital to understanding 1,25(OH)₂D's role in breast cancer progression and the development of vitamin D recommendations for prevention and therapy. The hypothesis of this study is that 1,25(OH)₂D regulates PHD2 independent of transcription and has no impact on the VHL tumor suppressor protein in Harvey-*ras* transfected MCF10A breast epithelial cells.

Figure 1



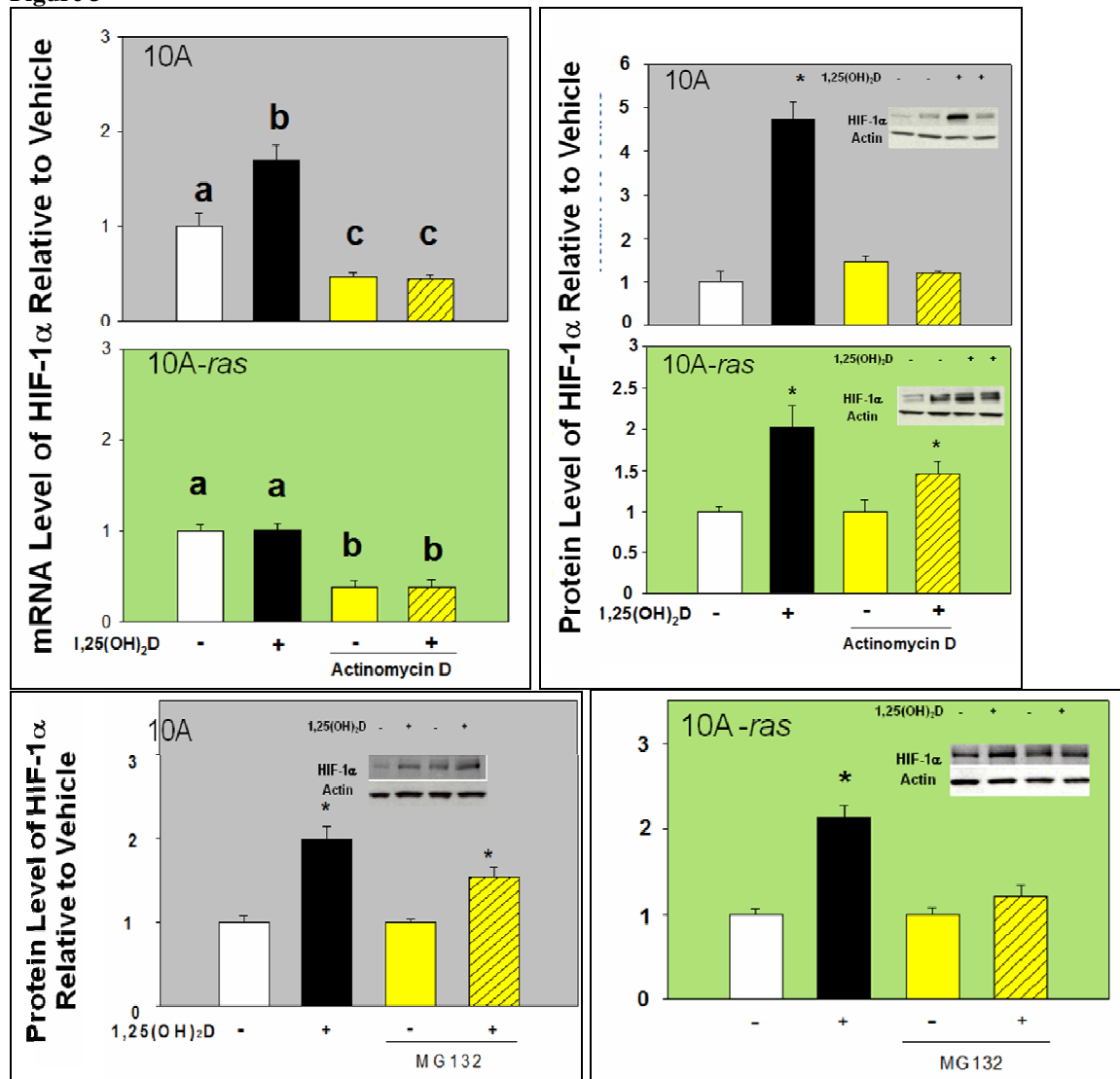
The Effect of 1,25(OH)₂D on Protein and Gene Expression of HIF-1 α in 10A and 10A-ras Cells

Figure 2



**Transcriptional Inhibition and Inhibition of Protein
Degradation Influenced 1,25(OH)₂D Induced Protein
Expression of HIF-1 α in 10A and 10A-*ras* Cells**

Figure 3



Study Design & Methods

Cell Culture

The untransformed (MCF10A) and the Harvey-*ras* oncogene transfected MFC10A-*ras*) human breast epithelial cells were used in the experiments. Cells were maintained in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F12(1x)), containing 5% horse serum and supplemented with 10mg/L insulin, 20µg/L of epidermal growth factor, 50µg/L cholera toxin, 50mg/L hydrocortisone, 100units/ml of penicillin, and 0.1mg/mL streptomycin in a humidified environment at 37 C with 5% CO₂. Cells were plated at a density of 345,000 cells per 60mm culture dish and treated with vehicle (ethanol) or 10nM of 1,25(OH)₂D for 24 hours.

Methods

The total level of protein was determined by bicinchoninic acid protein (BCA) assay for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE). Kaleidoscope (Bio-Rad, Hercules, CA) and Biotin (Cell Signaling Technology, Beverly, MA) markers were used per manufacturer instructions. The electrophoresis was run with a 10% Tris-HCL gel transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). A 1:1000 dilution for the VHL primary antibody (anti-mouse) (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:2000 dilution for the PHD2 primary antibody (Abcam, Cambridge, MA) were employed. Secondary antibodies were diluted to 1:2000 (anti-Rb/anti-Mouse) and 1:4000 (anti-biotin) for VHL and PHD2. Actin protein (42 kDa) was used as a standard marker with a 1:2000 dilution for the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and a 1:2000 (anti-Rb) and 1:4000 (anti-biotin) dilution for the secondary antibody. Lumiglo Reagent A and Peroxide Reagent B (Cell Signaling Technology, Beverly, MA) were used for blotting and diluted per manufacturer instructions. Quantifications of the westerns were performed using UN-SCAN-IT Program.

Total RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH) following manufacturer's instructions. Reverse transcription of total RNA was performed using MMLV reverse transcriptionase (Promega, Madison, WI). Quantifications of cDNA were performed using the Brilliant II SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA). The mRNA abundances of VHL and PHD2 were determined from the threshold cycle (Ct) value. The mRNA expression was then compared to the 18S expression and results were expressed as arbitrary units.

18S primer: Forward: 5'-TTAGAGTGTTCAAAGCAGGCCCGA-3'

Reverse: 5'-TCTTGGCAAATGCTTTCGCTCTGG-3'

VHL primer: Forward: 5'-AGAGATGCAGGGACACACGATGGG-3'

Reverse: 5'-AGGCTTGACTAGGCTCCGGACA-3'

PHD2 primer: Forward: 5'-ATGAACAAGCACGGCATCTGTGTG-3'

Reverse: 5'-ATAACCCGTTCCATTGCCCCGATA-3'

Statistical Analysis

Results were compared using student's t-tests or by analysis of variance (ANOVA). A p-value of less than 0.05 is defined as statistically significant.

Results

Impact of 1,25(OH)₂D on VHL protein expression in MCF10A and MCF10A-ras breast epithelial cells.

In order to examine the mechanism of regulation of 1,25(OH)₂D on VHL, the protein expression of VHL following treatment was determined in untransformed MCF10A and MCF10A-ras cells. In the

untransformed breast epithelial cells, the MCF10A (NeoN) cell line, the addition of 10nM of 1,25(OH)₂D did not significantly impact the expression of VHL protein (p=0.36) relative to the vehicle (EtOH). (Fig. 2). In the breast epithelial cells transfected with the Harvey-*ras* oncogene, the MCF10A-*ras* (NeoT) cell line, the addition of 10nM of 1,25(OH)₂D did not significantly impact the expression of VHL protein (p=0.24) relative to the vehicle (EtOH). (Fig. 2)

*Impact of 1,25(OH)₂D on PHD2 protein expression in MCF10A and MCF10A-*ras*, breast epithelial cells.*

In order to examine the mechanism of regulation of 1,25(OH)₂D on PHD2, the protein expression of PHD2 following treatment was determined in untransformed MCF10A and MCF10A-*ras* cells. In the untransformed breast epithelial cells, the MCF10A (NeoN) cell line, the addition of 10nM of 1,25(OH)₂D significantly decreased the expression of PHD2 protein (p=0.049) relative to the vehicle (EtOH). (Fig. 3). In the breast epithelial cells transfected with the Harvey-*ras* oncogene, the MCF10A-*ras* (NeoT) cell line, the addition of 10nM of 1,25(OH)₂D did not significantly impact the expression of PHD2 protein (p=0.18) relative to the vehicle (EtOH). (Fig. 3)

*Impact of 1,25(OH)₂D on VHL mRNA expression in MCF10A and MCF10A-*ras*, breast epithelial cells.*

In order to examine the mechanism of regulation of 1,25(OH)₂D on VHL, the mRNA expression of VHL following treatment was determined in untransformed MCF10A and MCF10A-*ras* cells. In the untransformed breast epithelial cells, the MCF10A (NeoN) cell line, the addition of 10nM of 1,25(OH)₂D significantly decreased the expression of VHL mRNA (p=0.0056) relative to the vehicle (EtOH). (Fig.4). In the breast epithelial cells transfected with the Harvey *ras* oncogene, the MCF10A-*ras* (NeoT) cell line, the addition of 10nM of 1,25(OH)₂D did not significantly affect the expression of VHL mRNA (p=0.061) relative to the vehicle (EtOH). (Fig 4)

Impact of 1,25(OH)₂D on PHD2 mRNA expression in MCF10A and MCF10A-ras, breast epithelial cells.

In order to examine the mechanism of regulation of 1,25(OH)₂D on PHD2, the mRNA expression of PHD2 following treatment was determined in untransformed MCF10A and MCF10A-*ras* cells. In the untransformed breast epithelial cells, the MCF10A (NeoN) cell line, the addition of 10nM of 1,25(OH)₂D did not significantly affect the expression of PHD2 mRNA ($p=0.28$) relative to the vehicle (EtOH). (Fig. 5). In the breast epithelial cells transfected with the Harvey *ras* oncogene, the MCF10A-*ras* (NeoT) cell line, the addition of 10nM of 1,25(OH)₂D significantly decreased the expression of PHD2 mRNA ($p=0.012$) relative to the vehicle (EtOH). (Fig 5)

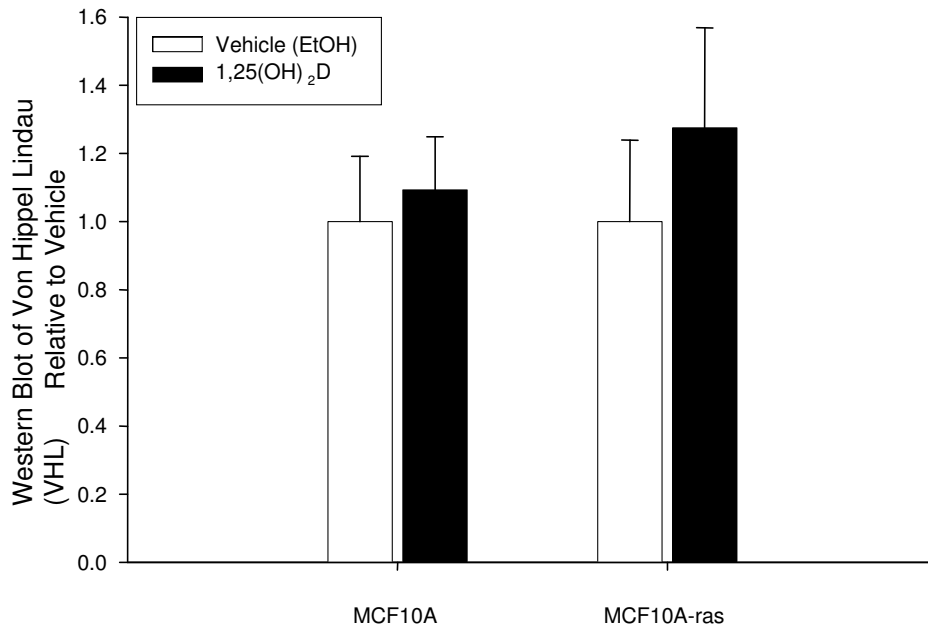


Figure 2: 1,25(OH)₂D Regulation of VHL Protein, Untransformed (MCF10A) and transformed (MCF10A-*ras*) cells were treated with either vehicle or 10nM of 1,25(OH)₂D for 24 hours, and harvested for determination of VHL protein expression by Western Blots. Bars with asterisks(*) indicate significance (p -value <0.05) as assessed by student's t -tests.

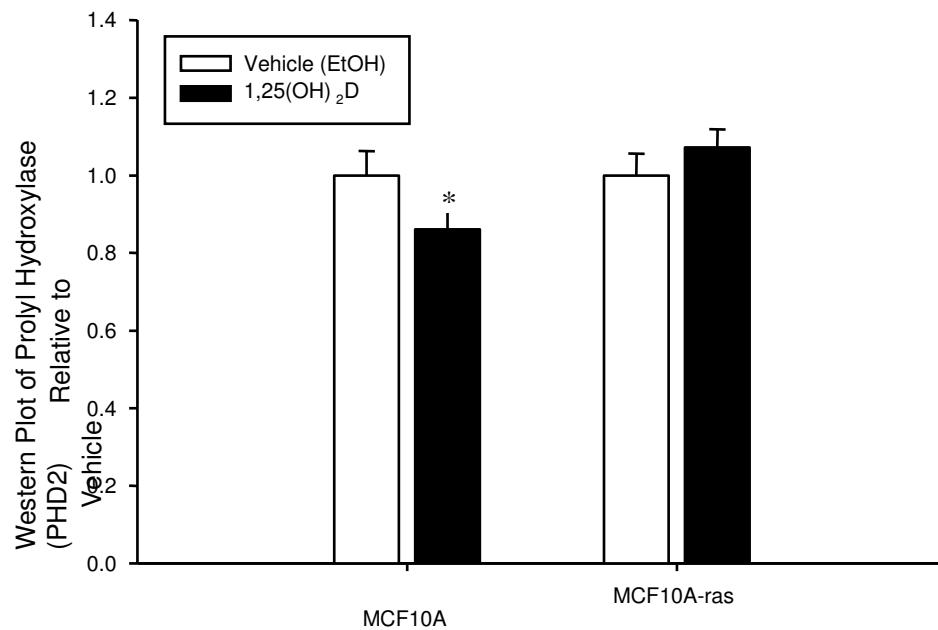


Figure 3: 1,25(OH)₂D Regulation of PHD2 Protein. Untransformed (MCF10A) and transformed (MCF10A-ras) cells were treated with either vehicle or 10nM of 1,25(OH)₂D for 24 hours, and harvested for determination of PHD2 protein expression by Western Blots. Bars with asterisks(*) indicate significance (p-value <0.05) as assessed by student's t-tests.

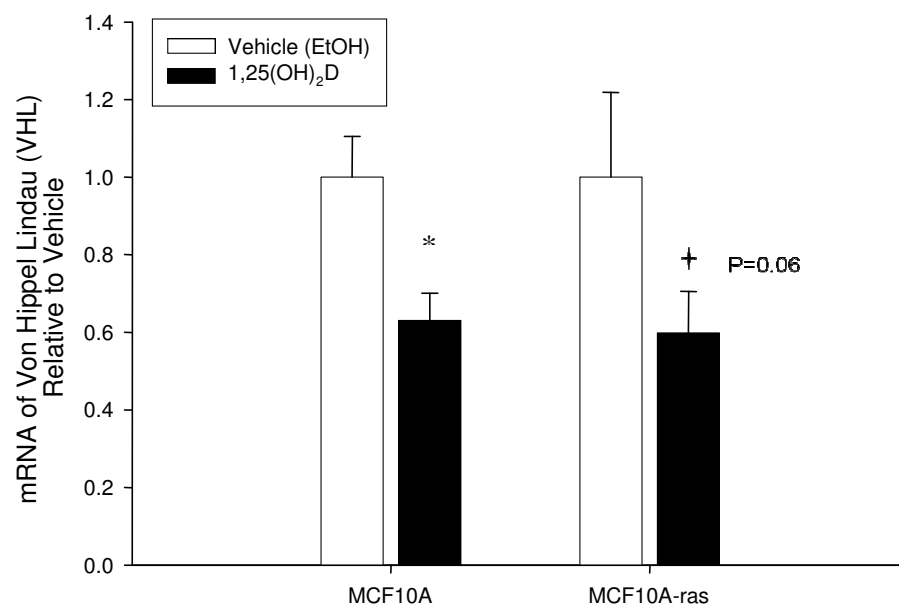


Figure 4: 1,25(OH)₂D Regulation of VHL mRNA, Untransformed (MCF10A) and transformed (MCF10A-*ras*) cells were treated with either vehicle or 10nM of 1,25(OH)₂D for 24 hours, and harvested for determination of VHL mRNA expression by RT-PCR. Bars with asterisks(*) indicate significance (p-value <0.05) as assessed by student's t-tests.

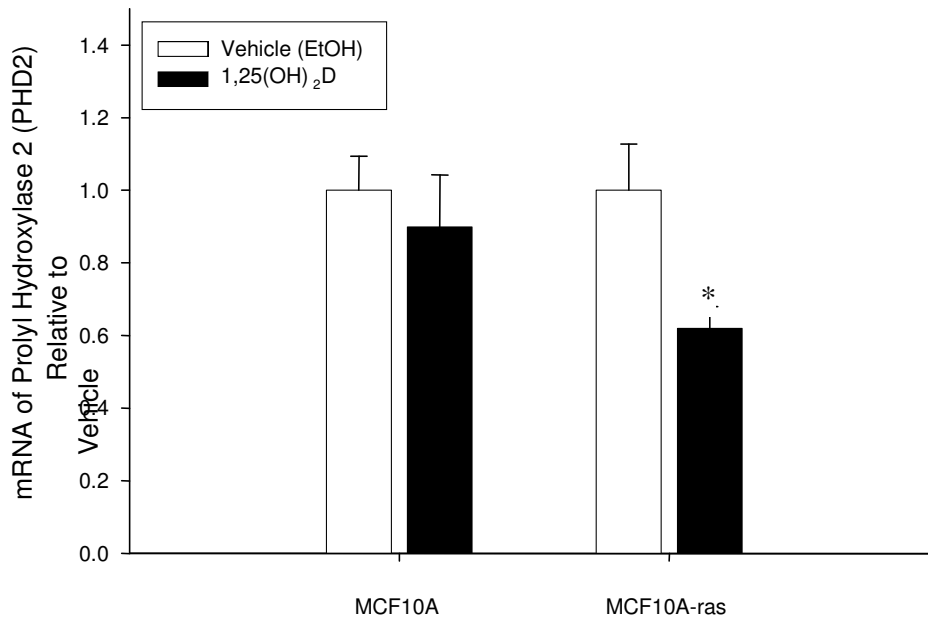


Figure 5: 1,25(OH)₂D Regulation of PHD2 Protein, Untransformed (MCF10A) and transformed (MCF10A-*ras*) cells were treated with either vehicle or 10nM of 1,25(OH)₂D for 24 hours, and harvested for determination of PHD2 mRNA expression by RT-PCR. Bars with asterisks(*) indicate significance (p-value <0.05) as assessed by student's t-tests.

Discussion

Vitamin D is associated with decreased risk of breast cancer, but the mechanism remains unclear. Our previous studies show that the active metabolite of vitamin D, 1,25(OH)₂D, differentially up-regulates HIF-1 α by transcriptional and protein stability, in MCF10A and MCF10A-*ras* cells, respectively. In this study, we show that VHL protein targets ubiquitinated proteins to degradation, thus a decrease in VHL may

lead to an increase in the accumulation of proteins. The mRNA levels of VHL when treated with $1,25(\text{OH})_2\text{D}$ significantly decreased relative to the vehicle in the MCF10A line ($p<0.05$) and also tended towards a decrease in MCF10A-*ras*. However, the protein expression showed an insignificant trend towards an increase in both MCF10A and MCF10-*ras* cell lines. This suggests that $1,25(\text{OH})_2\text{D}$ may affect the mRNA level but VHL levels are likely controlled through the translation process or by protein stability mechanisms. It is also possible that the number of samples ($n=6$ from two experiments) may limit the ability to detect a difference. These results suggest that VHL does not play a role in $1,25(\text{OH})_2\text{D}$ regulation of VHL.

Prolyl hydroxylase 2 (PHD2) plays an active role in HIF-1 α expression as it hydroxylates HIF-1 α , promoting recognition and binding of the VHL tumor suppressor protein to form an E3 ubiquitin-protein ligase complex that causes mediation and degradation of HIF-1 α (Fig. 1) (Jiang, 2010; Chun, 2002; Demidenko, 2005). Thus an increase in PHD2 or VHL activity may lead to a reduced level of HIF-1 α or conversely, a decrease in either PHD2 or VHL may lead to an increase in HIF-1 α and promote progression of cells to a tumorigenic phenotype. Determining the effect of $1,25(\text{OH})_2\text{D}$ on the regulation of PHD2 will lead to better understanding the mechanism by which $1,25(\text{OH})_2\text{D}$ regulates HIF-1 α . In our study, the mRNA levels of PHD2 when treated with $1,25(\text{OH})_2\text{D}$ had a decreasing trend in the MCF10A line and significantly decreased ($p<0.05$) in the MCF10A-*ras* line, relative to the vehicle. The protein expression was significantly decreased ($p<0.05$) in MCF10A cells, yet was unchanged in the MCF10A-*ras* line. The downward trend of PHD2 in both mRNA and protein expression in the MCF10A line treated with $1,25(\text{OH})_2\text{D}$, suggests the possible mechanism of action through transcription. In MCF10A-*ras* cells the mRNA of PHD2 was significantly decreased but the protein expression was unchanged, suggesting that there is a different mechanism of inhibition between the two cell lines that can be further explored via inhibitor trials.

Studies by Chan et al. and Henze et al. demonstrate that when PHD2 is decreased, there is a subsequent increase in HIF-1 α , a major promoter of tumorigenesis. The study by Chan et al. using knockdown PHD2 in cells showed that lack of PHD2 conferred a significant increase in tumor growth compared to wild-type PHD2 control cells (Chan, 2002). There is a noted decrease of PHD2 in many human cancers, particularly colon and breast, which may be caused by mutations or epigenetic silencing by methylation (Chan, 2002). Similarly, Henze et al found that in gliomablastoma cells when HIF-1 α was decreased an increase in PHD2 and PHD3 correspondingly occurred (Henze, 2010). Due to this effect, PHD2 may be a potential target for anti-cancer therapy or treatment in the future. In our study, the level of mRNA was significantly decreased in the MCF10A-*ras* line, when treated with 1,25(OH) $_2$ D, suggesting an increase in HIF-1 α , as previously stated. However, when quantifying the amount of PHD2 protein expression, the level of PHD2 was unchanged in MCF10A-*ras* cells treated with 1,25(OH) $_2$ D. This lack of change suggests that HIF-1 α may be unchanged after all. In the control MCF10A cells, PHD2 was significantly decreased ($p < 0.05$) in protein expression and tended toward a decrease in mRNA expression, marking a trend that indicates the possibility of PHD2 mediating an increase in HIF-1 α . The difference between the PHD2 protein expression in the MCF10A cells and the MCF10A-*ras* cells is likely due to a difference in the mechanism of action of 1,25(OH) $_2$ D. A study by Jiang, Zheng, and Teegarden showed that 1,25(OH) $_2$ D treatment increased both mRNA and protein expression of HIF-1 α in MCF10A control cells yet the same treatment had no effect in mRNA and little effect on HIF-1 α expression in the MCF10A-*ras* lines (Jiang, 2010). Further, inhibitor studies with transcription inhibitor, actinomycin D and proteasome inhibitor, MG132, concluded that 1,25(OH) $_2$ D regulates HIF-1 α protein expression through transcriptional control in the MCF10A cells but effects proteosomal stability in the MCF10A-*ras* cells (Jiang, 2010). It is likely that this sort of differentiation of control between cell lines is occurring with this study on PHD2. Further testing of PHD2 mRNA and protein levels with the same inhibitors (Actinomycin D, MG132) should be done in both cell lines to assess the mechanism of regulation by 1,25(OH) $_2$ D.

Our VHL results were less conclusive with decreased levels of mRNA yet unchanged levels of protein in both cell lines treated with 1,25(OH)₂D. Decreased VHL mRNA has been suggested as a marker of poor prognosis in cancer patients yet high VHL protein is known to reduce tumor invasion (Zia, 2007). But, studies by Zia et al note that VHL is less specific to HIF-1 α as VHL is known to have various distinct functions (Zia, 2007). The same study confirmed this fact by showing that VHL protein is present in both normal and tumor cells when immunohistochemically stained (Zia, 2007). Studies by Demidenko et al and Blancher et al suggest that there is an alternative pathway of HIF-1 α regulation independent from VHL (Demidenko, 2005; Blancher, 2001). Similarly, 1,25(OH)₂D seems to have a completely different effect between mRNA expression and protein expression that is likely due to other undiscovered protein stability mechanisms and/or effects on translation.

In combination, our study suggests that cells treated for 24 hours with 1,25(OH)₂D may mediate an increase in HIF-1 α , therefore potentially promoting tumor growth. The VHL results did not show a trend when treated with 1,25(OH)₂D and is likely too general to HIF-1 α to conjecture a direct regulation at this point. The results of the western blots may need more trials (>n=6) to confirm the differing trend of the VHL mRNA and protein expression. To further explore the role of 1,25(OH)₂D on PHD2 in MCF10A and MCF10A-*ras* cells, inhibitor trials utilizing a transcription inhibitor and then a proteasome inhibitor should be done to conclude the mechanism that is causing the differences in the MCF10A-*ras* results and the difference between the PHD2 protein levels of the two cell lines when treated with 1,25(OH)₂D.

If the regulatory control of HIF-1 α is discovered, this pathway would be an attractive target for pharmaceutical agents to decrease or even prevent cancer cell proliferation, angiogenesis, and metastasis of breast tumors. Similar studies and pharmaceuticals could be translated into other forms of cancer also expressing an increased level of activated HIF-1 α . Discovering the direct controls of HIF-1 α potentially has a serious impact on the ability to formulate agents that fight against breast cancer tumorigenesis.

Conclusion

In both breast epithelial cell lines, treated with 1,25(OH)₂D for 24 hours, the mRNA levels of VHL were significantly decreased but the protein expression was unchanged. This suggests that 1,25(OH)₂D does not regulate HIF-1 α protein expression via VHL protein levels and that VHL is unlikely to play a role in 1,25(OH)₂D regulation of HIF-1 α protein expression. In untransformed cells, 1,25(OH)₂D decreased PHD2 protein but not mRNA expression which suggests that PHD2 protein is not regulated by 1,25(OH)₂D mRNA abundance. It also suggests that PHD2 protein level may play a role in 1,25(OH)₂D regulation of HIF-1 α protein levels in untransformed cells. Lastly, in the *ras*-transfected cells, 1,25(OH)₂D decreased PHD2 mRNA, but not protein, expression. This suggests that PHD2 protein is not regulated by 1,25(OH)₂D mRNA expression and that PHD2 protein levels likely do not play a role in 1,25(OH)₂ regulation of HIF-1 α protein levels.

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