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THE EFFECT OF VITAMIN D ON METASTATIC EPITHELIAL BREAST CANCER CELL CYCLE REGULATION

by

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Literature Review & Rationale

In the United States alone, approximately 1 in 8 women will develop invasive breast cancer in her lifetime making breast cancer the second most common and second most lethal cancer amongst women. According to American Cancer Society, 246,660 new cases of breast cancer will be diagnosed this year in 2016\(^1\). Not only is a breast cancer patient affected physically, but they will also be affected emotionally and psychologically. The effects are experienced by the family, friends, and colleagues and can cause shock, sadness, anxiety, and even anger\(^2\).

There are many risk factors which can increase a woman’s likelihood of developing breast cancer. Unfortunately, many of these factors are uncontrollable such as: being female, increased age, genetic factors, and family history. However, there are many factors that are controllable such as: exercise, weight, various environmental factors, and diet\(^1\).

The metastases of breast cancer are among one of the highest rates compared to other cancers and leads to a higher rate of mortality\(^3\). Metastasis is the development of secondary malignant growth that in sites distal from the primary tumor. It has been estimated that about 20-30% of breast cancer cases lead to metastasis\(^4\) and an estimated 40,450 women will die from breast cancer in 2016\(^1\). There are many different sites in the body where it can metastasize; each different site will have different behaviors and severity\(^3\). The most common sites of breast cancer metastasis is lungs, brain, liver, and bone. The variations in behavior and severity of metastasis support the seed and soil hypothesis. The seed and soil hypothesis explains that a primary tumor may metastasize (the seed) and begin growing at a distal site (the soil). These distant sites display differential characteristics in gene expression, growth factors, and receptors\(^3\).
Over the years, evidence has emerged suggesting vitamin D may reduce the risk of breast cancer although the relationship is not fully understood. Evidence shows that lower vitamin D is associated with an increased risk of metastasis, but the mechanistic basis for this is not well known. Studies have additionally identified an inverse association between serum 25 hydroxyvitamin D (25(OH)D) levels, a marker for vitamin D status, and the risk of breast cancer. The active form of vitamin D, 1,25dihydroxyvitamin D (1,25 (OH)₂D) has been shown to regulate a variety of components of cancer progression, including inhibiting cell proliferation and cell cycle arrest, as well as promoting cell differentiation and apoptosis in breast cancer cells in numerous studies.

There are two primary forms of vitamin D obtainable from the environment. Cholecalciferol, or vitamin D₃, is produced from cholesterol in the skin when exposed to sunlight or ultraviolet light. Ergocalciferol, or vitamin D₂, is obtained from the diet. Vitamin D₃ is hydroxylated in the liver to form its dominant circulating, inactive form, 25(OH)D. This inactive form 25(OH)D is converted to its active form1,25(OH)₂D by the enzyme 25-hydroxyvitamin D1α-hydroxylase. This process occurs in the proximal tubules of the kidney as well as in many other tissues including: immune system, skin, adrenal medulla, pancreatic islet cells, colon, and breast. The active form, 1,25(OH)₂D, is a secosteroid that is known for its role in regulating calcium and phosphate homeostasis in part through parathyroid hormone secretion via stimulating absorption from the gut, increasing calcium absorption and renal reabsorption. There are a range of other cellular functions associated with 1,25(OH)₂D including: antiproliferation, immunomodulatory properties, disease prevention, differentiation, and many others. The nuclear vitamin D receptor (VDR) binds to 1,25(OH)₂D functioning as a transcription factor in both normal and neoplastic cells. VDR binds to one of the retinoid X receptors (RXRs). The RXRs have been shown to have transcriptional activity on vitamin D-dependent genes.
receptors, forming a dimer that interacts with coactivators assisting in recruiting the RNA polymerase II complex. These coactivators are responsible for activating various gene expression\(^8\). **Figure 1** represents the metabolism of vitamin D.

**Figure 1. Vitamin D Metabolism.**
The metabolism of vitamin D\(_3\) into its active form, 1,25dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)) requires several steps. The active form functions as a hormone and transcription factor to its receptor vitamin D receptor (VDR). VDR forms a dimer with retinoid X receptor (RXR) and functions to recruit the RNA polymerase II transcription complex\(^8\).

In breast cancer cells, regulation of the cell cycle is transformed\(^{12}\). Previous findings from our lab have shown an association between 1,25(OH)\(_2\)D treatment with decreased viable cell number, induction of cell apoptosis, and cell cycle regulation by inducing G1/G0 cell cycle arrest. These results were modeled using MFC10A cells transfected with the Harvey *ras* oncogene (MCF10A-*ras*). This inhibition of cell cycle proliferation could contribute to the decrease in viable cell number via 1,25(OH)\(_2\)D and therefore preventing breast cancer progression and cell cycle regulation.

**Figure 1:** Vitamin D Metabolism. The metabolism of vitamin D\(_3\) into its active form, 1,25dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)) requires several steps. The active form functions as a hormone and transcription factor to its receptor vitamin D receptor (VDR). VDR forms a dimer with retinoid X receptor (RXR) and functions to recruit the RNA polymerase II transcription complex.
is a separate state in which cells are in a quiescent state. During proliferation the cell transitions through G1, a gap phase in which the cell increases in size as it grows and synthesizes mRNA and proteins. The cell then moves into the S phase, in which precise DNA replication occurs in the nucleus. Next, the cell enters another gap phase, G2, as the cell continues to grow. Finally, the cell will enter M phase, where mitosis occurs and the cell divides via cytokinesis. Figure 1 shows a summary of the steps of the cell cycle.

**Figure 1. The phases of the cell cycle.** The cell cycle progress from G1 gap phase (growth and protein synthesis) to S phase (synthesis) to G2 gap phase (growth) and finally through M phase (mitosis via cytokinesis).  

Throughout the cell cycle, there are various cell cycle check points that function to ensure accurate propagation. There are numerous molecules and proteins that are utilized as regulators of the cell cycle to ensure proper transition. Control of the G2/M transition has been found to be associated with cancer because it may result in chromosomal aberrations. The G1/S transition may be altered in breast cancer cells, where oncogenes may disrupt the normal function of genes and tumor suppressors. The arrest of cells in the G1/S phase prevents them from proliferating and further growth which is considered an essential component of cancer cells. During cancer development, several cell cycle regulator molecules have been found to be overexpressed, leading to loss of normal growth regulation and increased proliferation. The active form of vitamin D has been known to play a role in cell cycle arrest in breast cancer cells. It has been found that 1,25(OH)₂D effects the expression of specific cell cycle regulators which can initiate
G1/G0 cell cycle arrest and promote apoptosis\textsuperscript{14}. One type of cell cycle regulator, cyclin-dependent kinase inhibitors (CDI’s) negatively modulate the progression of the cell cycle. One CDI in particular, p27\textsuperscript{Kip1}, interacts with CDK2 and CDK4 by inhibiting cell cycle progression at the G1 phase when grown with 1,25(OH)\textsubscript{2}D\textsubscript{15}. Studies have shown that the addition of 1,25(OH)\textsubscript{2}D to neoplastic cells is associated with an increase in p27\textsuperscript{Kip1}, suggesting that this protein may be a principle mediator in 1,25(OH)\textsubscript{2}D G1 cell cycle arrest\textsuperscript{15}.

Another possible mechanism of dysregulation in cancer that affects cell cycle may be signal transduction pathways. One of these pathways includes the MAP kinase which is a part of signaling cascade pathway that regulates gene transcription\textsuperscript{16} important in cell cycle regulation. Recent studies have shown that Src, a non-receptor tyrosine kinase, becomes more phosphorylated with the addition of 1,25(OH)\textsubscript{2}D, inhibiting its kinase activity. This results in downstream MAPK (ERK-1 and ERK-2 MAP kinases) to be repressed\textsuperscript{16}, suggesting that 1,25(OH)\textsubscript{2}D may also regulate cell cycle through the Src/MAP kinase signaling pathway.

Previous findings from our lab have shown an association between 1,25(OH)\textsubscript{2}D treatment with decreased viable cell number, induction of cell apoptosis, and cell cycle regulation by inducing G1/G0 cell cycle arrest. These results were modeled using MFC10A cells transfected with the Harvey ras oncogene (MCF10A-\textit{ras}). This inhibition of cell cycle proliferation could contribute to the decrease in viable cell number via 1,25(OH)\textsubscript{2}D and therefore preventing breast cancer progression. The purpose of this study is to investigate the role of 1,25(OH)\textsubscript{2}D in cell cycle regulation and G1/G0 arrest in additional human breast epithelial cells in progression to cancer. Therefore, it is hypothesized that the addition of 1,25(OH)\textsubscript{2}D to the four cell lines used will induce G1/G0 cell cycle arrest by increasing the proportion of cells found in G1/G0 phase.
To study cancer progression and prevention, it is important to use appropriate models. The most commonly used and appropriate model for studying breast cancer progression are the MCF10A series (MCF10A, MCF10A-\textit{Ras}, MCF10A-\textit{ErbB2}, and MCF10CA1). MCF10A cells were derived from benign breast tissue that spontaneously immortalized and are proposedly the most common cell line used to model breast cells\textsuperscript{17}. These cells have proliferative properties, but do not express the estrogen receptor. They contain a deletion in the locus that contains genes important for regulation of senescence and overexpression of the Myc gene. However, the MCF10A cells express a basal-like phenotype, yet express some features resembling mesenchymal cancer cells. This cell line led to the establishment of a series of cell lines including more aggressive breast cancer lines\textsuperscript{17}. The MCF10A-\textit{Ras} cell line serves as a model for the Harvey-\textit{Ras} (Ha-\textit{Ras}) proto-oncogenic effector utilized for growth-dependent induction of several growth factors and cytokines\textsuperscript{18}. Ha-\textit{Ras} induced tumors contain activated mitogen-activated protein kinase (MAPK) signaling cascades. \textit{Ras} activation is also involved in overexpression of ErbB2 in breast tumors. Mutations in \textit{Ras} are rare in breast cancers, but \textit{Ras} is improperly activated in about 50\% of breast tumors and is associated with early neoplasia and poor prognosis\textsuperscript{18}.

The MCF10A-\textit{ErbB2} cell line contains a transformed \textit{ErbB2} gene. \textit{ErbB2} is an oncoprotein in the family of epidermal growth factor receptors (EFG) of receptor tyrosine kinases\textsuperscript{20}. However, \textit{ErbB2} does not directly bind growth factors because it does not contain a ligand binding domain. Instead, it forms a heterodimer with other EFG receptors that are bound to ligands. This interaction leads to increased ligand binding and increased kinase-mediated activation of downstream signaling pathways. Overexpression and amplification of \textit{ErbB2} is found in cancers\textsuperscript{20}, including about 1 in 4 cases of invasive breast cancer\textsuperscript{21}. The overexpression
of ErbB2 has also been shown to initiate breast cancer invasion and metastasis, leading to poor patient survival. The MCF10CA1a cell line is a metastatic line derived from the MCF10A-Ras cells. These cells are oncogenic, fully malignant, and are histologically undifferentiated, morphologically variable, and anchorage-independent. The MCF10CA1a cell line serves as a valuable model for analyzing the oncogenic potential of genes of interest.

There is evidence that a higher vitamin D status is associated with a decreased risk of metastasis, but the mechanistic basis for this is still under investigation. In numerous studies, 1,25(OH)₂D has been shown to inhibit cell differentiation, apoptosis promotion, cell proliferation, and cell cycle arrest in breast cancer cells. Cell proliferation is normally controlled by the cell cycle, which is composed of a series of events that control the replication of the genetic material and division of the cell. The cell cycle process is tightly controlled and divided into phases. In breast cancer cells, normal regulation of the cell cycle is typically altered in order to promote increased cell proliferation. Therefore, the regulation of the cell cycle is considered to be a potential target for cancer therapy. Previous research in our lab demonstrated that MCF10A-ras cells treated with 1,25(OH)₂D induced G1 cell cycle arrest. These results led to the interest in further investigating the effects of 1,25(OH)₂D in human breast epithelial cells during cancer progression.

**Purpose**

The purpose of this study is to investigate the role of 1,25(OH)₂D in cell cycle regulation and G1/G0 arrest in human breast epithelial cells in progression to cancer.
Study Design & Methods

Cell Lines

The cell lines used for experiments were MCF10A, MCF10A-ras, MCF10A-ErbB2, and MCF10CA1a. MCF10A cells are benign human breast epithelial cells\textsuperscript{17}. MCF10A-Ras are Harvey-Ras oncogene transfected MCF10A cells\textsuperscript{18}. MCF10A-ErbB2 are ErbB2/HER2/Neu oncogene transfected MCF10A cells\textsuperscript{20}. MCF10CA1a cells are tumorigenic derivative from MCF10A-ras cells\textsuperscript{22}.

Cell Culture

Cells were grown and maintained in culture Dulbecco’s Modified Eagle Medium/F12 (DMEM/F12) (1:1) nutrient mix containing 5% horse serum, 1% penicillin/streptomycin (Life Technologies, Gibco-BRL, Rockville, MD). For MCF10A, MCF10A-ErbB2, and MC10A-Ras: 10mg/L insulin, 20ug/L epidermal growth factor, 50mg/L hydrocortisone (Sigma-Aldrich, St. Louis, MO), and 50ug/L cholera toxin (Calbiochem, Darmstadt, Germany) were supplemented to the media. Cells were plated at 300,000 cells per 60mm culture dish and allowed to attach overnight. Then 10nM 1,25(OH)\textsubscript{2}D (Biomol, Plymouth Meeting, PA) treatment was delivered to cells in 100% ethanol at a final ethanol concentration of <1% for 72 hours. Media supplemented with 1,25(OH)\textsubscript{2}D was replaced every 24 hours during the treatment period. Plates were kept in a humidified environment at 37\degree C.

Cell Cycle Measurement

Flow cytometry was used for cell cycle measurements. From each sample, 1x10\textsuperscript{6} cells
were harvested using phosphate buffered saline in a single cell suspension. Cells were fixed with ice cold ethanol then pretreated with 0.2 mg/mL Rnase A, and stained with 10 ug/mL propidium iodide (Sigma-Aldrich, St. Louis, MO). Cell cycle quantifications were achieved using flow cytometry analysis with a Beckman Coulter FC500 flow cytometer equipped with a 488 nm laser. The results were analyzed with FloJo (Tree Star, Inc., Ashland, OR). Results were expressed as percentage of total cells arrested in each phase in the cell cycle.

**Figure 2. Cytomics FC 500 Flow Cytometer.** Cells were stained with propidium iodide (PI) which binds to double stranded DNA by intercalating between base pairs. The flow cytometer emits a laser light that passes through one cell at a time. A detection apparatus absorbs the refracted light at FL3 (670LP, red). The fluorescence intensity is proportional to the DNA concentration within the cell and this is indicative of its cell cycle phase.  

Statistical Analysis

Results were compared using the Student’s t-test including the means +/- SD. P-values <
0.05 are considered significant.

**Results**

*Impact of 1,25(OH)$_2$D on cell cycle regulation in MCF10A, MFC10A-Ras, MCF10A-ErbB2, and MCF10CA1a cells.*

In order to determine if 1,25(OH)$_2$D regulates the cell cycle in breast epithelial cells, analysis of cell cycle phases was determined in MCF10A, MFC10A-Ras, MCF10A-ErbB2, and MCF10CA1a cells. In MCF10A cells, the addition of 1,25(OH)$_2$D (10 nM) significantly increased the number of cells arrested in the G1/G0 phase (p=0.03). Treatment with 1,25(OH)$_2$D did not significantly change the number of cells in G2 relative to the vehicle (p=0.21).

![Figure 3. 1,25(OH)$_2$D Regulation of MCF10A Cell Cycle.](image)

Cells were treated with either vehicle or 10 nM 1,25(OH)$_2$D for 72 hours, and harvested for cell
cycle analysis and quantification by flow cytometry. Bars with asterisks (*) indicate significance (p < 0.05) by Student’s t-test including the means +/- SD.

In MCF10A-Ras cells, the addition of 1,25(OH)$_2$D (10 nM) significantly increased the number of cells arrested in the G1/G0 phase (p=0.002). The number of cells in G2 significantly decreased relative to the vehicle with 1,25(OH)$_2$D treatment (p=0.0006).

**Figure 4. 1,25(OH)$_2$D Regulation of MCF10A-Ras Cell Cycle.** Cells were treated with either vehicle or 10 nM 1,25(OH)$_2$D for 72 hours, and harvested for cell cycle analysis and quantification by flow cytometry. Bars with asterisks (*) indicate significance (p < 0.05) by Student’s t-test including the means +/- SD.
In MCF10A-\textit{ErbB2} cells, the addition of 1,25(OH)\textsubscript{2}D (10 nM) significantly increased the number of cells arrested in the G1/G0 phase (p=0.01). With 1,25(OH)\textsubscript{2}D treatment, the number of cells in G2 did not show a significant change relative to the vehicle (p=0.83).

\textbf{Figure 5. 1,25(OH)\textsubscript{2}D Regulation of MCF10A-\textit{ErbB2} Cell Cycle.} Cells were treated with either vehicle or 10nM 1,25(OH)\textsubscript{2}D for 72 hours, and harvested for cell cycle analysis and quantification by flow cytometry. Bars with asterisks(*) indicate significance (p < 0.05) by Student’s t-test including the means +/- SD.
In MCF10CA1a cells, the addition of 1,25(OH)$_2$D (10 nM) significantly increased the number of cells arrested in the G1/G0 phase (p=0.00005). The number of cells in G2 did not significantly change relative to the vehicle with 1,25(OH)$_2$D treatment (p=0.23).

![Graph showing the regulation of cell cycle phases by 1,25(OH)$_2$D.](image)

**Figure 6. 1,25(OH)$_2$D Regulation of MCF10CA1a Cell Cycle.** Cells were treated with either vehicle or 10nM 1,25(OH)$_2$D for 72 hours, and harvested for cell cycle analysis and quantification by flow cytometry. Bars with asterisks (*) indicate significance (p < 0.05) by Student’s t-test including the means +/- SD.

**Discussion**

There is evidence that higher vitamin D status is associated with a decreased risk of metastasis, but the mechanistic basis has not been determined. In numerous studies, 1,25(OH)$_2$D has been shown to inhibit cell proliferation, cell cycle arrest, cell differentiation, and promote
apoptosis in breast cancer cells. Previous studies in our lab have shown that 1,25(OH)\(_2\)D arrests cells in the cell cycle and induces apoptosis in MCF10A and MCF10A-\(Ras\) cell lines. In the current study, it is demonstrated that 1,25(OH)\(_2\)D regulates the cell cycle by increasing the percentage of cells arrested in the G1/G0 phase of the cell cycle. All four cell lines modeled for breast cancer progression showed significant increases when treated for 72 hours: MCF10A (\(P=0.03\)), MCF10A-\(ras\) (\(P=0.002\)), MCF10A-\(ErbB2\) (\(P=0.01\)), and MCF10CA1a (\(P=0.00005\)).

The results from this study are consistent with previous results from our lab using MCF10A and MCF10A-\(Ras\) cells in which 1,25(OH)\(_2\)D arrested cells in the G1/G0 phase of the cell cycle. This study re-analyzed the MCF10A and MCF10A-\(Ras\) cells as well as two other cell lines, MCF10A-\(ErbB2\) and MCF10CA1a and the increased number of cells arrest in the G1/G0 phase further supports the hypothesis and previous findings.

There have been several possible mechanisms proposed to demonstrate the role of vitamin D on the regulation of the cell cycle. Studies have shown that 1,25(OH)\(_2\)D plays a role in the expression of specific cell cycle regulators such as cyclin-dependent kinase inhibitors (CDI’s). In particular the addition of 1,25(OH)\(_2\)D to the CDI, \(p27^{\text{Kip1}}\), inhibits cell cycle progression at the G1 phase through the interactions with CDK2 and CDK4\(^{15}\). Another proposed mechanism of 1,25(OH)\(_2\)D regulation is via signal transduction pathways such as Src and the MAP kinase cascade which regulate gene transcription. An upstream regulator of MAP kinase, Src, may be inhibited by phosphorylation due to the addition of 1,25(OH)\(_2\)D, resulting in downstream repression of MAPK\(^{16}\). There is evidence that 1,25(OH)\(_2\)D regulates various pathways in gene transcription and cell cycle regulation that may lead to the arrest in G1/G0 phase and therefore reduction in cell proliferation and apoptosis.
These results indicate that 1,25(OH)₂D is associated with regulating the cell cycle, although the exact mechanism is still under consideration. Inhibition of the cell cycle may explain the mechanism of vitamin D to inhibit breast cancer progression. Vitamin D may be an essential molecule for inhibiting cell growth and proliferation which may contribute to its role in breast cancer progression.

Understandings on the mechanisms by which vitamin D inhibits breast cancer development and metastasis is important in order to design clinical trials to test the effect in humans. Thus, the information from this study will potentially contribute to developing recommendations for vitamin D intake that will prevent breast cancer development and metastasis.

**Conclusion**

In MCF10A, MCF10A-\textit{Ras}, MFC10A-\textit{ErbB2}, and MCF10CA1a breast epithelial cell lines, 1,25(OH)₂D treatment for 72 hours increased the percent of cells arrested in G1/G0 phase of the cell cycle. This indicates that these cells may be inhibited from progressing through the cell cycle due to arrest in the G1/G0 phase. Therefore, inhibition of the cell cycle may explain the effect of vitamin D to inhibit breast cancer progression. These results support the evidence that vitamin D may be a factor in breast cancer prevention and inhibitor for proliferation.
References:


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