THE IMPACT OF VITAMIN D ON INFLAMMATORY BREAST CANCER CELLS

A Senior Thesis Submitted to the

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for University and Departmental Honors.

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Glossary of Abbreviations and Terms

IBC: Inflammatory Breast Cancer

PBS: Phosphate Buffered Saline

VDREs: Vitamin D Responsive Elements

VDR: Vitamin D Receptor

HER2: Human Epidermal Growth Factor Receptor 2

ER: Estrogen Receptor

PR: Progesterone Receptor

NFkB: Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells

FBS: Fetal Bovine Serum

DMSO: Dimethyl sulfoxide

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Abstract

Inflammatory breast cancer (IBC) is the most aggressive and lethal manifestation of local advanced breast cancer with the inherent ability to rapidly metastasize/spread throughout the body. IBC carries an extremely poor prognosis, and the low survival rate of IBC is due to the highly invasive nature of the disease. Specifically, IBC has a proclivity to invade and block the dermal lymphatics of the skin overlying the breast, allowing the disease to spread rapidly, and making it the most lethal form of breast cancer. Because of the aggressive, invasive, and lethal nature of IBC, therapeutics are desperately needed. Vitamin D is a known regulator of cancer cell apoptosis, proliferation, migration, and invasion. Recent studies in various cancer types have suggested vitamin D may be able to inhibit the ability of cancer cells to move. The goal of this study was to determine the impact of Vitamin D on inflammatory breast cancer cell phenotype, proliferation, apoptosis, and migration capabilities. The results from this study suggest that Vitamin D modifies cellular phenotype, and decreases proliferation and migration of IBC cells, with no significant impact on cell death.

Introduction and Background

Inflammatory Breast Cancer

When an individual thinks of a typical presentation of breast cancer, oftentimes a lump is the first symptom that comes to mind. Women consistently check for those lumps and equate that symptom with a problem and seek immediate attention. However, there is a subset of breast cancer that has flown under the radar for quite some time because it does not present this way. Inflammatory breast cancer (IBC) is a rare and aggressive form of locally advanced breast cancer that represents 2.0-4.0% of all breast cancers (1). Despite this low incidence rate, IBC accounts for 7% of total breast cancer mortalities (1). In fact, the aggressive tumor behavior has a 2.9-4,2-year median survival period, which is significantly less than non-IBC cancers (2). Another aspect of IBC that stands out against non-IBC patients, is that it affects younger women, often occurring in women under 40 (3). The concern with cancers such as this with atypical symptoms is in the misdiagnosis and mistreatment of symptoms by individuals and health care professionals.

Symptoms/Diagnosis

Inflammatory breast cancer hides behind a mask of probable mastitis. Meaning, it presents with very similar clinical signs to mastitis. Mastitis is inflammation of the breast tissue, usually caused by an infection and seen most frequently in nursing mothers. With IBC, the cancer cells infiltrate and block the lymph vessels present in the breast. Unable to drain fluid away due to the blockage, the lymph vessels have a buildup of fluid which leads to symptoms similar to mastitis (3). These symptoms include edema (Figure 1-A), skin changes like peau d' orange (Figure 1-B) or nipple inversion (Figure 1-C), erythema (Figure 1-D), and breasts that are warm to the touch (1). While all telltale signs of infection, they do not point directly to cancer. Thus, IBC is misdiagnosed and mistreated so that upon diagnosis it is only ever present as stage

3 or stage 4 (4). Stage 3 would indicate the breast cancer cells have grown into the skin while stage 4, which is the diagnosis for 1/3 of the women treated, has already spread to nearby organs (4). Currently the best methods of diagnosis are breast and skin biopsies as a mammogram or physical exam may not result in much without a palpable lump (5).

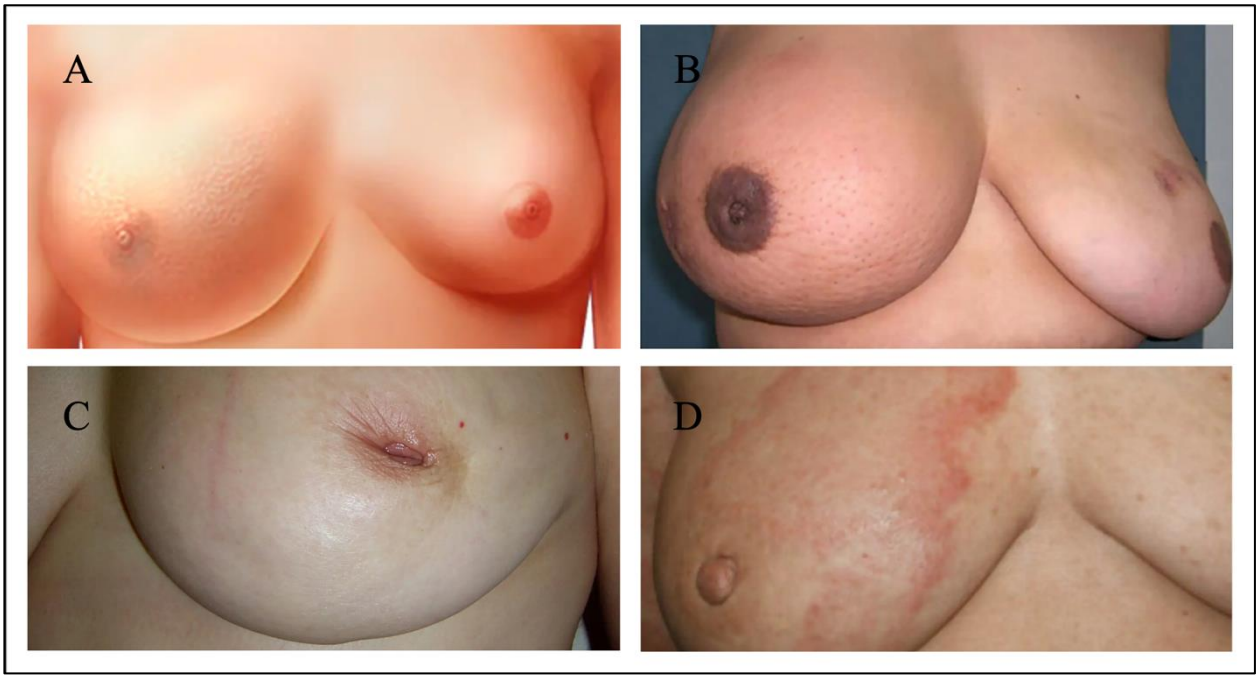

Figure 1. Representative images of symptoms of IBC including edema (A), peau d'orange (B), nipple inversion (C), and erythema (D). Images obtained from https://www.healthgrades.com/right-care/breast-cancer/pictures-of-breast-cancer-lumps

Molecular Subtypes

Breast cancers have a variety of subtypes that are relevant to the treatment plan for the individual. These subtypes are typically grouped into four categories, and they are grouped by their immunohistochemical expression of hormone receptors (6). Estrogen receptor positive (ER) breast cancer is characterized by high ER expression and is present in 70-75% of breast cancers.

Progesterone receptor positive (PR) breast cancer with a high PR expression is present in more than 50% of those who are also ER positive, but rarely in those who are not. HER2 (Human epidermal growth factor receptor 2) expression accounts for 15-25% of breast cancers and is also relevant when determining treatment. Breast cancers with higher than normal levels of HER2 on the surface of the cancer cells are considered HER2-positive. As two of these proteins bond they create a dimer which signals to the cell that promotes both growth and multiplication. Triple-negative breast cancer covers most breast cancers that do not have one of the molecular receptors discussed and represents about 20% of all breast cancers (6). Triple negative tends to be the most difficult to treat and has the worst prognosis due to the fact it is unresponsive to hormone therapies that could be effective in other subtypes. The important thing to note is that inflammatory breast cancer patients could also get any of these subtypes, making the prognosis worse and the treatment more difficult.

Treatments/Prognosis

If IBC is diagnosed at stage 3, the treatment would include chemotherapy to shrink the tumor and then surgery for removal of the tumor. Surgery may be followed by chemotherapy or targeted drug therapy. Stage 4 diagnosis lends itself to similar treatments, though depending on the distance of spread of the cancer, surgery may not be effective. Additional treatment for those that are HER2-positive involves anti-HER2 therapy that targets the HER2 protein. Endocrine therapy targets receptor/hormone recognition which stops or slows the growth of the cancer (6). Other varieties of breast cancer typically try to conserve breast tissue but the aggressive nature of IBC leaves this as less of a priority (7). The current overall 5-year survival rates range from 30-70% and can be impacted by the stage at diagnosis and other clinical indicators (2).

Biological Mechanisms

There are many physiologic factors which create the dangerous environment of IBC. Previous gene expression studies report that IBC tumors are characterized by amplification/over-expression of growth factor receptor HER2 and down-regulation of hormone receptors ER/PR (8). Anti-apoptotic signaling, through hyperactivation of NF-κB and its target genes have been identified both in the biology of IBC and its resistance to treatment. Cytokines and chemokines are both in abundance for the tumor microenvironment which could also enhance the aggressiveness of the tumor as well as enhance the angiogenic processes and evasion of immune surveillance (8).

Vitamin D

Vitamin D is an essential micronutrient that our bodies need. Typically, there are a wide number of uses for this vitamin that would come to mind ahead of being used as a cancer preventative or even cancer treatment. Even so, Vitamin D is one of the most studied supplements for both the prevention and treatment of cancer. Many studies have shown that deficiencies in Vitamin D have been linked to an increased risk of developing malignancies (9). Vitamin D is delivered to the body through sun exposure, foods, and supplements. It must then undergo two hydroxylation reactions before activation. The liver houses the first hydroxylation and converts Vitamin D to 25-hydroxyvitamin D [25(OH)D] or calcidiol. Next, primarily the kidney is responsible for the second hydroxylation which forms the physiologically active 1,25-dihydroxyvitamin D [1,25(OH)2D], or calcitriol (10). Once in this state the body uses vitamin D for many functions.

Vitamin D functions

The biological actions of calcitriol include modulation of gene expression at the transcriptional level and are mediated through the binding to a Vitamin D receptor (VDR) which is found most commonly in the nuclei of target cells (11). Specific Vitamin D-responsive elements (VDREs) are present in many human genes involved in a variety of both classical and non-classical roles. Classical roles would include promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations. These concentrations are what enable bone mineralization. Vitamin D is also needed for bone growth and remodeling (10). Some non-classical roles of Vitamin D include the regulation of cell proliferation, cell differentiation, and apoptosis. Suggestions have been made that it may also have immunomodulatory and anti-proliferative effects through paracrine and autocrine pathways. The immunomodulatory suggestions come from the findings that deficiencies in Vitamin D can be prevalent in those with autoimmune diseases (12). Vitamin D takes on many roles at a cellular level that most do not consider.

Levels in body

While Vitamin D is a great micronutrient to ensure one consumes, there are points where too much of a particular vitamin can be harmful. That is why when supplementing there are always warning labels and recommended dosages. That recommendation can vary based on age, gender, and stage in life (pregnancy, lactation). Vitamin D deficiency has been linked to hypocalcemia which can be harmful for bone mineralization but on the opposite side, too much vitamin D can cause hypercalcemia and eventually become Vitamin D toxicity (VDT). This is incredibly rare through dietary intake, but can occur with medical doses of Vitamin D without proper medical monitoring. There is concern both in the acute treatment with high dose Vitamin

D but also in chronic administration over years. The levels that warrant concern for acute toxicity would be caused by doses of Vitamin D more than 10,000 IU/day, creating serum 25 (OH)D concentrations above 150ng/ml (above 375nmol/1). Potential chronic toxicity would result from administration of doses above 4,000 IU/day for long periods of time, including years, which would lead to serum 25(OH)D concentrations between 50–150 ng/ml (125–375 nmol/l) (11).

Uses in other cancers

As Vitamin D can be incredibly beneficial as well as harmful, it is important a medical provider is dosing an individual and providing guidance. As far as applications in a clinical sense, perhaps the most interesting is its ability to help mediate in cancerous environments. While many studies have been conducted, the results are not entirely consistent. Some instances have found that Vitamin D in combination with anti-cancer drugs exhibits strong antitumor behavior (9). It is believed that by binding to VDR, $1,25(OH)_2D_3$ produced antitumor efficacy by regulating target gene expression or nongenomic actions related to different signaling pathways in normal cancer cells as well as cancer stem cells. The anticancer effects of $1,25(OH)_2D_3$ encompass the induction of cell cycle arrest, cell differentiation, cell apoptosis, autophagic cell death, and the inhibition of metastasis tumor angiogenesis (9).

As Vitamin D has been shown to have some promising anti-cancer properties it is a worthwhile path to examine for IBC. Given inflammatory breast cancer's aggressive and metastatic nature, any impact on the success of the cancer cells to reproduce and migrate would be impactful. This study grew SUM149 human inflammatory breast cancer cells in media and treated with calcitriol to investigate its impact on cell function. We hypothesize that Vitamin D will slow the spread of inflammatory breast cancer cells.

Methodology

Cell culture

Cell lines were maintained under defined, well-tailored culture conditions optimal for growth. The SUM149 human IBC cell line was established from a primary IBC and grown in Ham's F12 medium (Gibco) supplemented with 10% FBS (Gibco), 1% Penicillin/Streptomycin (Gibco), and an insulin/transferrin/selenium cocktail (Gibco). Cells were passaged every three to four days using 0.25% Trypsin-EDTA (Gibco). The cell line was grown at 37 degrees Celsius in 5.0% CO_2.

Vitamin D Treatment

Cells were treated with Vitamin D at various concentrations. 1a, 25-Dihydryoxyvitamin D3 (Santa Cruz Biotechnology) was stored at -20 degrees Celsius. It was resuspended in 1x Phosphate Buffered Saline (PBS) to a stock concentration of 10μM. Working concentrations for treatment of IBC cells were made by diluting the stock reagent in cell culture media. Cells were treated at concentrations of 10nM, 30nM, 50nM, and 100nM. All treatment groups were compared to untreated control cells.

Cell Phenotype

Cells were examined with a Zeiss Axiovert 10 Inverted Microscope. Phenotype of Vitamin D-treated cells was assessed in comparison to untreated cells to determine alterations in the physical appearance of the cells. This was conducted during multiple experiments at multiple time points. Total magnification was varied (200X or 400X) depending on the intention of the particular experiment. Cells for this study were imaged using this microscope.

Cell Counting

A Trypan Blue dye exclusion method was used to determine the number of viable cells present in a cell suspension. IBC cells were trypsinized and centrifuged for 5 minutes at roughly 1,300 rpm. 100 μL of cell suspension was added to 400 μL of Trypan Blue dye (0.4%) (Gibco). Using a pipette, 100 μL of the Trypan Blue cell suspension was applied to a hemocytometer. A microscope was then used to focus on the grid lines of the hemocytometer at 100X magnification and a hand tally counter was used to count the number of live, unstained cells in a set of 16 squares. The hemocytometer was moved to the next set of 16 corner squares until the four sets of corner squares were counted. The number of viable cells per mL was calculated by averaging the cell counts between the four squares and multiplying by a factor of 10^4 and then a factor of 5 to account for the 1:5 dilution from the Trypan Blue addition. The number of viable cells per mL was used to plate consistent cells numbers for experiments. The hemocytometer was also used to count dead cells.

Cell Viability/Proliferation Assay

The reduction of tetrazolium salts was used to examine cell proliferation. SUM149 cells were plated at a density of 2,000 cells per well of a 96 well plate. Cells were allowed to attach to the wells, reach approximately 60-70% confluency and then either left untreated or treated with Vitamin D at concentrations of 10nm or 100nm. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltatrazolium bromide) (Abcam) solution was prepared fresh for each trial experiment at a concentration of 5 mg/ml in PBS. At the end of each Vitamin D treatment time point being evaluated (24,48 hours), twenty microliters of MTT solution was added directly to the media of each well and thoroughly resuspended. The plate was incubated at 37 degrees Celsius in 5.0% CO_2 for 3 hours to allow the MTT to be metabolized. The media was dumped off and 200

microliters of dimethyl sulfoxide (DMSO) was added to each well to resuspend the MTT metabolic product formazan. The plate was shaken for 3 minutes at 150rpm to thoroughly mix the formazan into the DMSO. The absorbance was read at an optical density of 562nm, and any background was subtracted.

Cell Migration

IBC cells were trypsinized and plated to grow in 37 degrees Celsius and 5% CO_2 until the plates reached 100% confluency. The media was removed from the plates and a 20-microliter pipette tip was used to scratch the monolayer of cells present on the plate. Following the scratch, cells were either left untreated or treated with Vitamin D at concentrations of 10nM, 30nM, 50nM, and 100nM. All wells had a volume of 1mL between media and Vitamin D treatment. All conditions were imaged at 0 hours, 24 hours, and 48 hours.

Data analysis

Image J was used to calculate the scratch length. Equation used to calculate percentage of scratch closure to day 0 was ((Day 0 wound - day X wound)/Day 0 wound)*100).

All experiments had three technical replicates as well as 3 biological replicates.

Results

Investigation of changes in IBC cell phenotype with Vitamin D treatment

Human inflammatory breast cancer cells when grown in normal culture media only were elongated in shape with many processes and extensions. The cells formed a tight network as they became more confluent but maintained their extensions (Figure 2D-G). When treated with varying concentrations of Vitamin D, the phenotype of the cells appeared altered. Cells went from elongated to cuboidal and lost most of their processes and extensions (Figure

2B,C,E,F,H,I). The cells went from tight networks to individual cells. The increase in confluency present in the control was not observed in the experimental groups. This is observed more so in 100 nM in comparison to even the 10 nM as time went on (Figure 2I).

Examination of proliferation/viability changes in IBC cells with Vitamin D treatment

When studying cell proliferation, the lower the absorbance, the less cellular activity and replication that is occurring. This is because the absorbance is dependent on the metabolic activity of the cells. It was found that absorbance decreased with Vitamin D treatment. Absorbance has a negative correlation to amount of Vitamin D treatment as well as time since treatment. As time went on, excluding the control, the absorbance levels dropped between 24 and 48 hours (Figure 3). As increasing concentration of Vitamin D treatment was used, the absorbance levels decreased as well.

Alteration in IBC cell migration with Vitamin D treatment

In comparison to cells grown in normal culture media, cells treated with various doses of Vitamin D migrated less. By 24 hours there was less migration at 10nM than the control (Figure 4D-E). The 100nM treatment may have been too strong of a concentration as cell confluency decreased more substantially than in the control and 10 nM treatment groups (Figure 4G-I). The 100 nM treatment therefore lacked a visible scratch to compare. The scratch widths of the control were narrower than those at 10 nM (Table 1) for both 24 and 48 hours, as cells migrated to close the scratch.

Seeing potential toxicity at 100 nM led to the addition of varying other doses of Vitamin D treatment including 30 nM and 50 nM. By 24 hours there was less migration in all three treatment groups (10 nM, 30 nM, and 50 nM) than the control (Figure 5E-H). By 48 hours, the control was almost indistinguishable from the rest of the culture while there was a visible scratch

remaining for the rest of the treatment groups. The control scratch was roughly 3x smaller than the highest treatment group (50 nM) by 48 hours (Figure 5I,L). With increasing concentrations of Vitamin D, there was a decrease in migration for all time frames after 0 hours.

Cell count per mL observations in IBC cells treated with Vitamin D

Both live and dead cell counts per mL were examined at 0-, 24-, and 48-hour time frames with Vitamin D concentrations of 0 nM (untreated control), 10 nM, 30 nM, and 50 nM. Live cell counts per mL all increased between time frames excluding 30 nM which had a decrease of 38,000 cells per mL between 0 hours and 24 hours before increasing by 1,346,000 cells per mL between 24 and 48 hours (Table 3). The greater the concentration of Vitamin D treatment, the less the cell count increased. The greatest live cell increase was at 0nM and was 1,887,500 cells per mL and the smallest live cell increase was at 50 nM at 816,500 cells per mL. By 48 hours there was a consistent correlation between amount of Vitamin D treatment and number of live cells (Figure 6).

Dead cell counts showed less consistent data. While 0 hours was consistently the lowest number of dead cells for all treatment groups, that was the only similarity between groups and time frames. The largest jump between 0 and 24 hours occurred in the control group with an increase from 12,500 cells per mL to 166,500 cells per mL. The largest jump between 0 and 48 hours was also the control group from 12,500 cells per mL to 129,000 cells per mL (Table 4). There was inconsistency between an increase at 24 hours and then a decrease at 48 hours. No correlational information could be drawn from the data (Figure 7). No correlational information is good information because that indicates there was no increase in cell death per mL overall.

Discussion

Being that inflammatory breast cancer is so rapidly progressing, the aim of treatments would be to lessen its metastasis and progression. In the context of this study, the results were promising. The result of the alteration in phenotype was a much less mobile cell. With cells that have extensions, the cells can move and infect other areas quickly. In contrast, the cuboidal independent cells represent a group of cells more likely to be sedentary. Upon completion of the viability assay, the lower levels of replication and activity found is promising as the goal would be to lessen the sheer amount of replication cancer cells undergo, as this hinders spread. The scratch assay further went on to show that cells were migrating less with treatments of Vitamin D. Given that cancer often changes the mechanics of cells to enable cancer cells to migrate and invade, the migration of cells is a necessary piece of the invasive potential cells carry (14). Less migratory cells mean a slower spread of cancer. Another indicator of slower spreading cancer would be the decrease in the number of live cells with treatment of Vitamin D. This in combination with the fact that there were no more dead cells in the treatment groups would lead to the assumption that the lack of replication also seen in the viability assay is supported. These data collectively suggest that Inflammatory Breast Cancer cells replicate and move less when treated with Vitamin D. This could be incredibly important in creating a longer survival rate for patients when diagnosis of this cancer is done when the cancer itself is already at stage III or stage IV.

Vitamin D would never be the sole treatment for a cancer. Assumedly it would be used to supplement other treatment options previously discussed. Similar supplements would be the hormone therapy already offered. The biologically active form of vitamin D, calcitriol, was used in this study and has a half-life of about 15 hours. In comparison, calcidiol has a half-life of

about 15 days (15). Vitamin D is a fat-soluble vitamin, stored in adipose tissue. This means that as previously discussed, it can build up to levels of toxicity. The Upper Tolerable Intake levels for adults for Vitamin D is 4000IU (16) which is comparable to 100 micrograms. The amount of vitamin D used in this study, at its highest concentration of 100nM, was only 6.4 micrograms. While the number should certainly be smaller as the number of cells examined were nowhere near the number of cells present in the entire human body, the fact there were results with such a small concentration is certainly significant.

Despite the levels for this study being so much lower than the upper tolerable intake, it is still important to examine how these experiments and other similar studies would impact non-cancerous breast cell lines. The anti-proliferative and anti-migratory effects of Vitamin D seen in this study are only beneficial to the cancerous cells and it would be important to ensure that healthy cells would not be damaged by prolonged exposure to the Vitamin D treatments.

While the results of this study are promising, these experiments are truly only scratching the surface for work that could be done. There is no other work examining the specific relationship between Vitamin D and inflammatory breast cancer specifically and the impact of Vitamin D on other cancers is variable. Vitamin D has been used more frequently as preventative measure than as a treatment. This was initially examined due to epidemiological studies where cancer death and progression was lesser in areas of Southern latitude with more natural exposure to Vitamin D (17). Further epidemiological studies have shown those with higher blood levels of Vitamin D had less of a prevalence of developing cancers such as colorectal cancer (17).

In vitro studies are important as they show an initial look at the potential reaction of cells to different reagents. However, there could be questions to what the clinical application of Vitamin D treatment or prevention would look like. Given that Vitamin D is safe to take and

there are guidelines already for recommended daily amounts, the safety of taking a certain amount of Vitamin D is widely accepted. There are multiple oral forms of Vitamin D available as well as injections that patients could receive. Understanding the way that Vitamin D would metabolize in these various carriers in relation to the spread of cancer would be helpful in creating treatment plans with patients and their families.

Tables and Figures

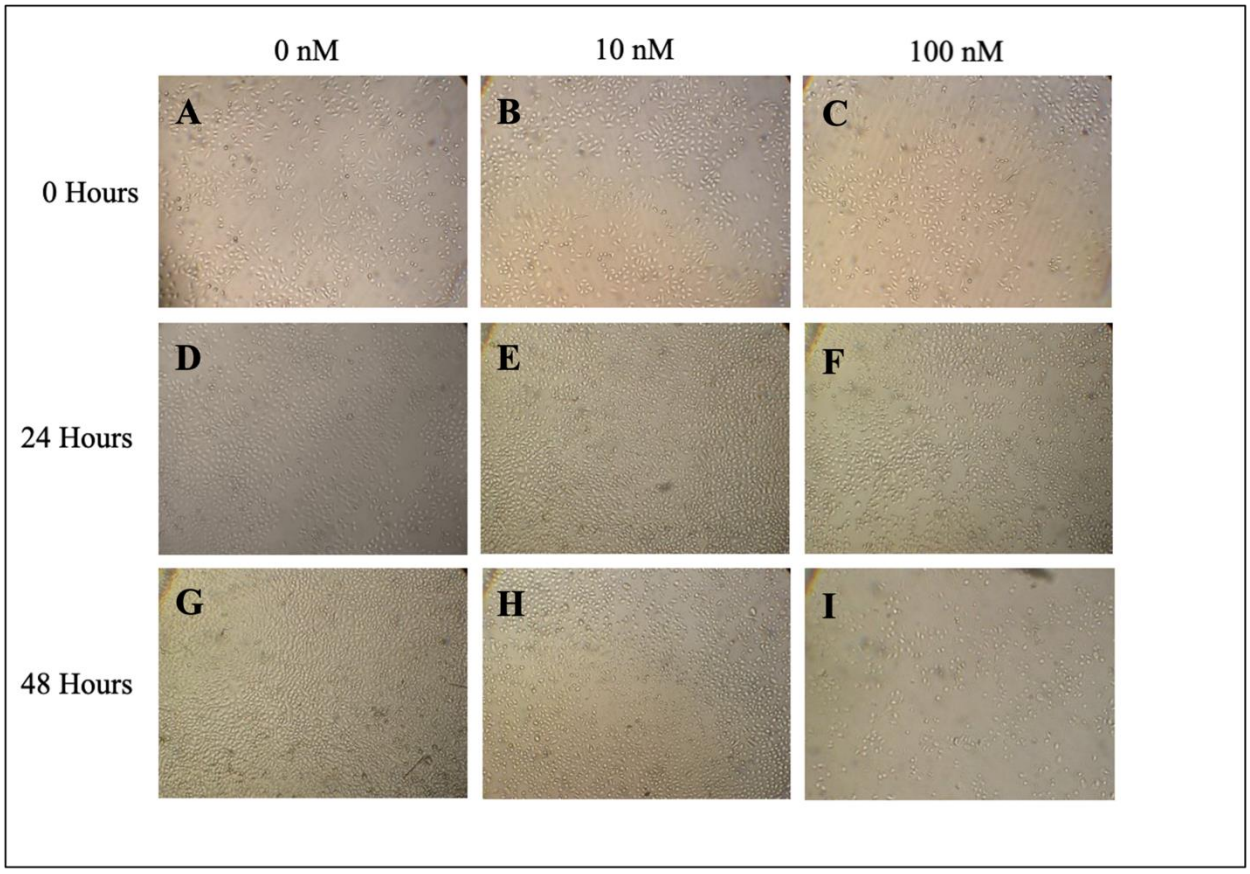

Figure 2. Investigation of changes in IBC cell phenotype with Vitamin D treatment. Cells were treated with 10 nM or 100cnM concentrations of Vitamin D and compared to untreated control cells. Photographs were taken at 0-, 24-, and 48- hours post treatment. The phenotype was altered in cells treated with Vitamin D as compared to the control with control cells appearing more cuboidal and isolated (n=3).

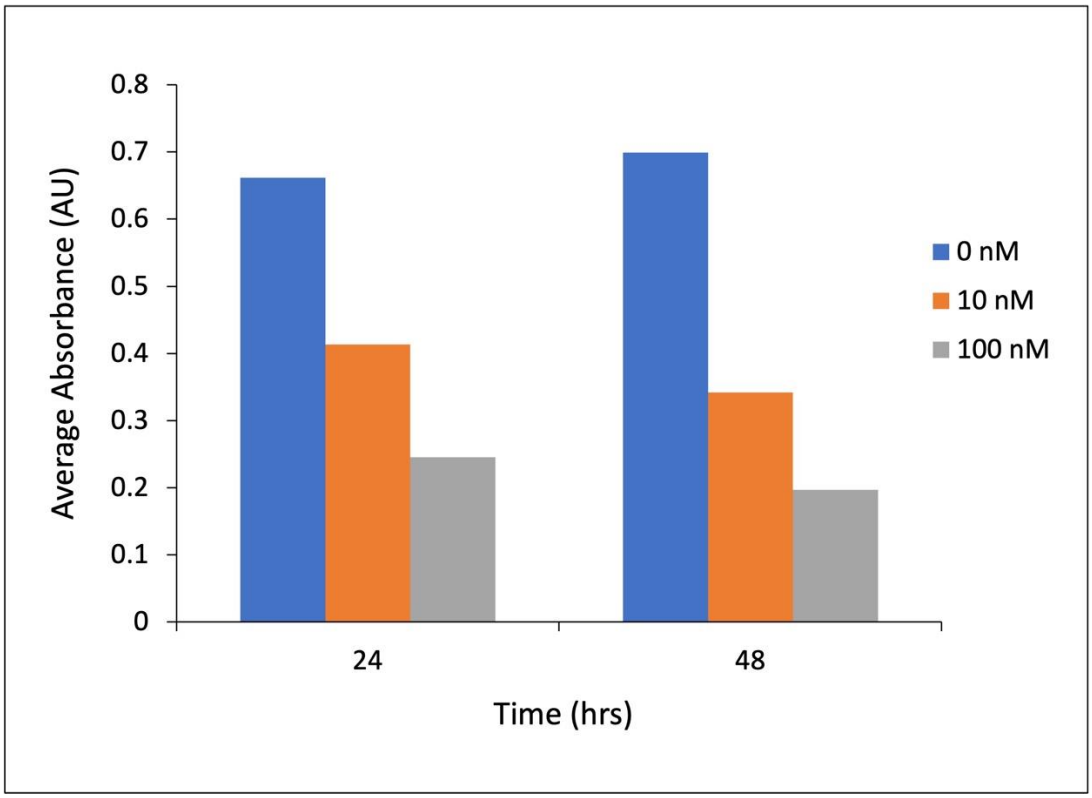

Figure 3. Examination of Proliferation/Viability changes in IBC cells with Vitamin D treatment**.** Cells were treated with 10 nM or 100 nM concentrations of Vitamin D and compared to a control. Absorbance was read at 562nm at 24- and 48-hours post-treatment (n=3).

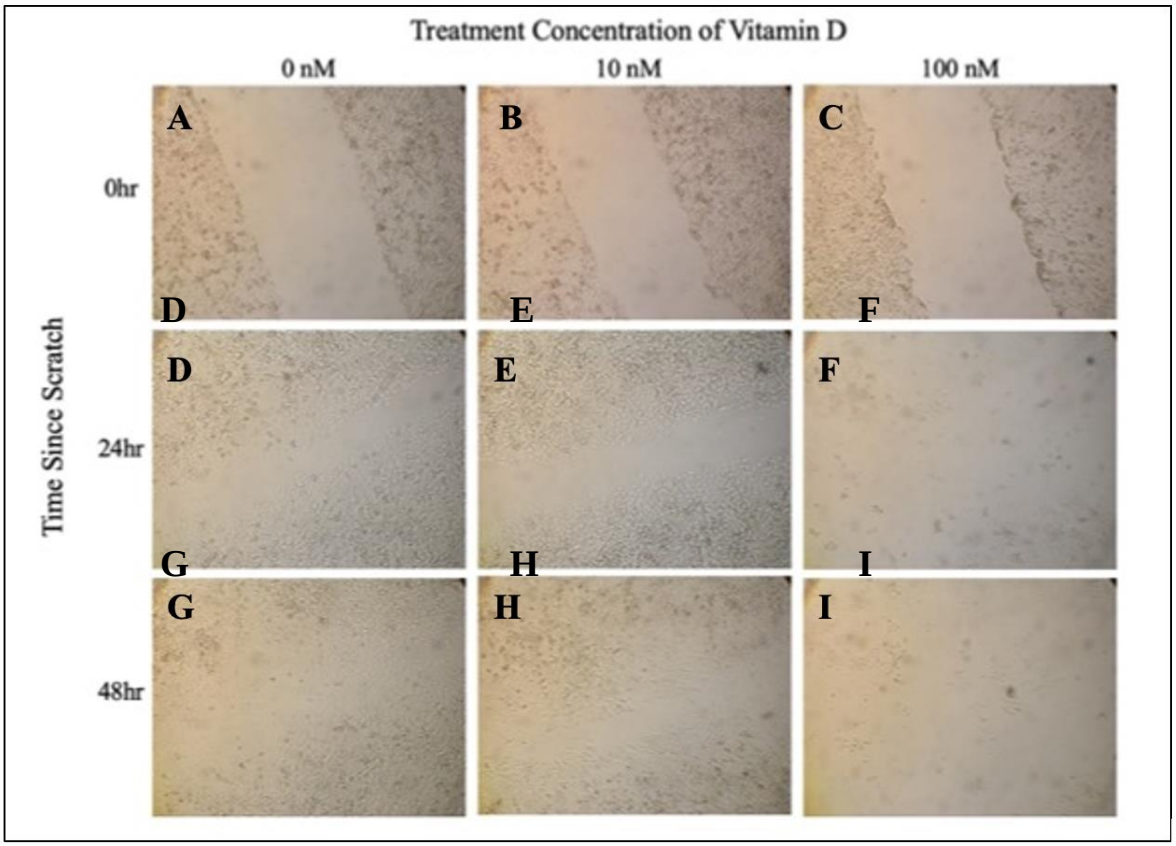

Figure 4. Alteration in IBC cell migration with Vitamin D treatment. Cells were treated with concentrations of 10nM or 100nM of Vitamin D in comparison to a control. Photographs were taken at 0-, 24-, and 48-hours post-treatment. Migration was less at 10nM treatment than in the control (n=3).

Table 1. Alteration in IBC cell migration with Vitamin D treatment. Images taken at 0, 24, and 48 hours with a control and two treatment groups (10nM, 100 nM). Image J was used to measure scratches in relative units. Measurements were converted to represent percentage of scratch closure compared to day 0 using the equation: ((Day 0 wound - day X wound)/Day 0 wound)*100). Vitamin D treatment increased scratch length. N/A* are scratches that are unidentifiable (n=3).

	0 nM	**10 nM**	**100 nM**
0 Hours	0%	0%	0%
24 Hours	74.39%	57.38%	N/A*
48 Hours	84.23%	71.50%	N/A*

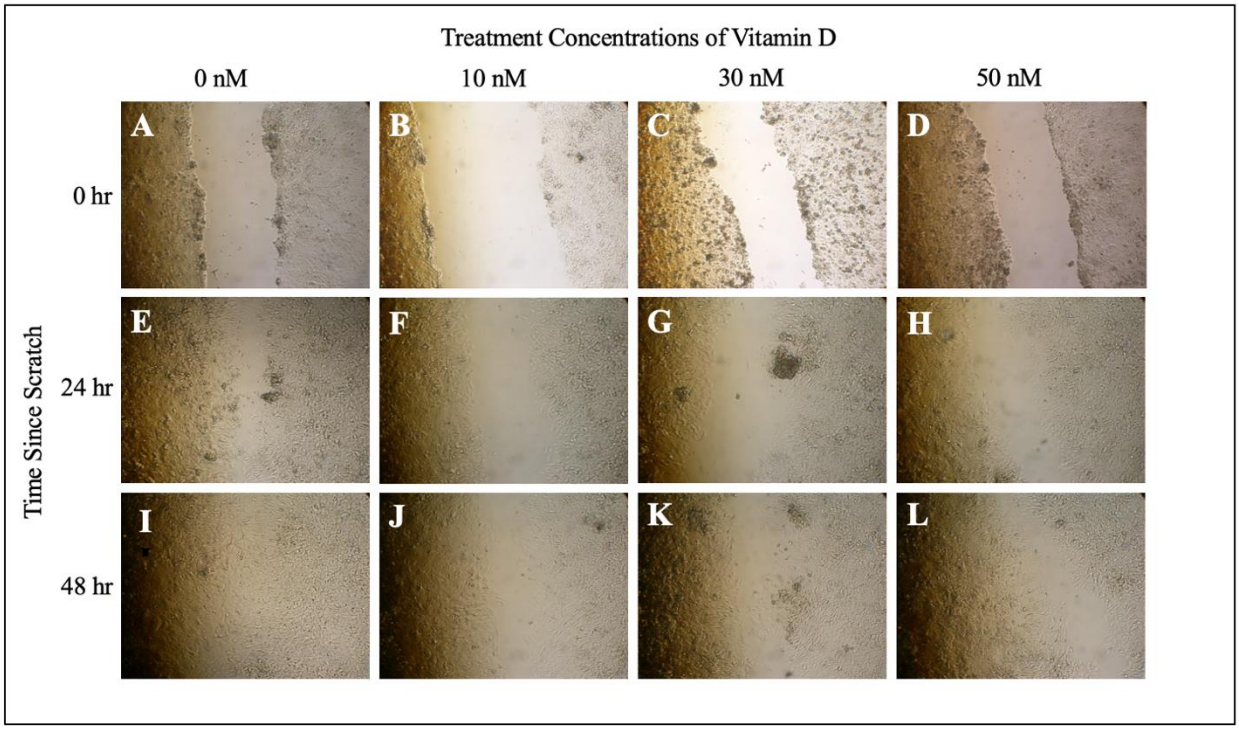

Figure 5. Alteration in IBC cell migration with Vitamin D treatment. Cells were treated with 10 nM, 30 nM, and 50 nM of Vitamin D and compared to an untreated control. Photographs were taken at 0-, 24-, and 48- hours post-treatment. Migration decreased as concentration of Vitamin D increased (n=3).

Table 2. Alteration in IBC cell migration with Vitamin D treatment. Images taken at 0, 24, and 48 hours with a control and three treatment groups (10 nM, 30 nM, 50 nM). Image J was used to measure scratches in relative units. The equation used to calculate the percentage of scratch closure compared to day 0 was: ((Day 0 wound - day X wound)/Day 0 wound)*100). Vitamin D treatment increased scratch width (n=3).

	0 nM	**10 nM**	**30 nM**	**50 nM**
0 Hours	0%	0%	0%	0%
24 Hours	74.11%	61.64%	54.98%	40.13%
48 Hours	93.55%	88.09%	76.43%	74.24%

Table 3. Live cell count per mL observations in IBC cells treated with Vitamin. Cells treated with 10 nM, 30 nM, or 50 nM concentrations of Vitamin D and compared to an untreated control. Cell counts were obtained at 0, 24, and 48 hours post-treatment through Trypan Blue staining and examination under a microscope with a hemocytometer. Cell counts per mL decreased with increasing Vitamin D concentrations (n=3).

	0nm	**10nm**	**30nm**	**50nm**
0 hours	1829000	2125000	1775000	1508500
24 hours	1821000	2304000	1737500	1375000
48 hours	3716500	3437500	3083500	2325000

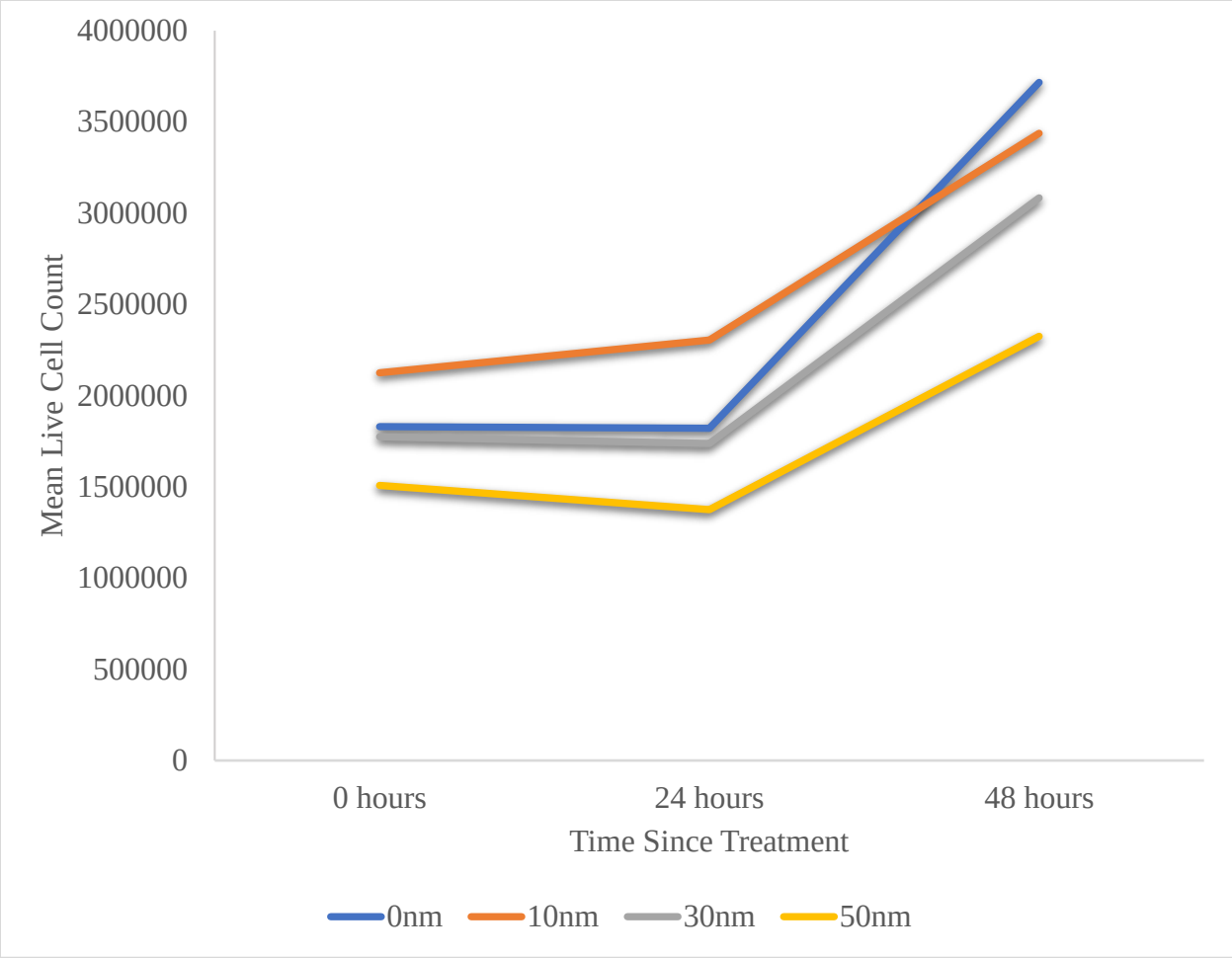

Figure 6. Live cell count per mL observations in IBC cells treated with Vitamin. Cells treated with 10nM, 30nM, or 50nM concentrations of Vitamin D and compared to a control. Cell counts per mL were obtained at 0, 24, and 48 hours post-treatment through Trypan Blue staining and examination under a microscope with a hemocytometer. Cell counts per mL decreased with increasing Vitamin D concentrations (n=3).

Table 4. Dead cell count per mL observations in IBC cells treated with Vitamin. Cells treated with concentrations of vitamin D of 10 nM, 30 nM, or 50 nM and compared to an untreated control. Cell counts per mL were obtained at 0, 24, and 48 hours post-treatment through Trypan Blue staining and examination under a microscope with a hemocytometer. Cell counts per mL varied little between treatment groups and control (n=3).

	0nm	**10nm**	**30nm**	**50nm**
0 hours	12500	25000	25000	25000
24 hours	166500	129000	83500	108500
48 hours	129000	133500	108500	129000

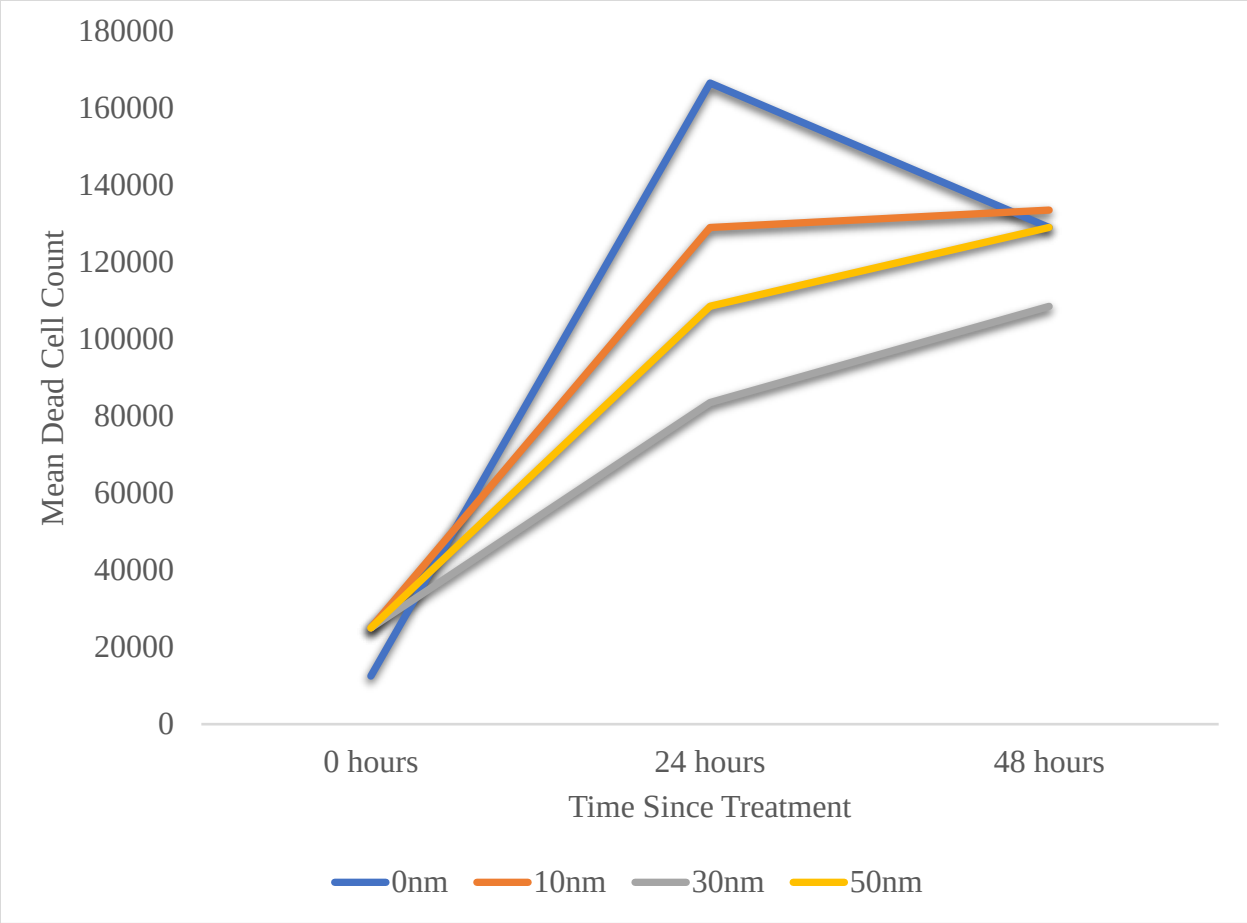

Figure 7. Dead cell count per mL observations in IBC cells treated with Vitamin. Cells treated with concentrations of vitamin D of 10 nM, 30 nM, or 50 nM and compared to an untreated control. Cell counts per mL were obtained at 0, 24, and 48 hours post-treatment through Trypan Blue staining and examination under a microscope with a hemocytometer. Cell counts per mL varied little between treatment groups and control (n=3).

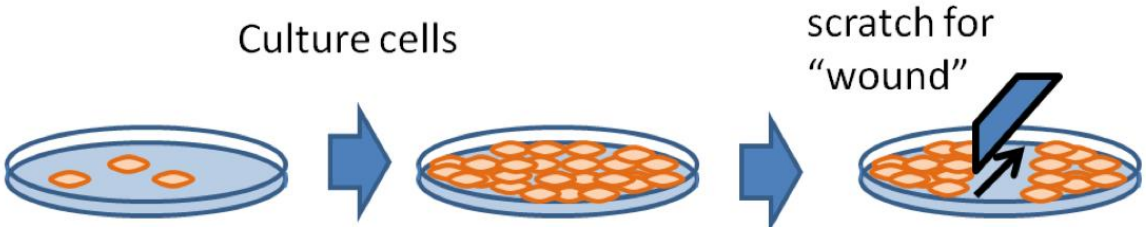

Figure 8. Diagram depiction of scratch/wound assay. Cells were grown to confluency before being scratched with a pipette tip to imitate a wound. Cells were monitored and imaged at 0, 24, and 48 hours post-treatment for wound closure.

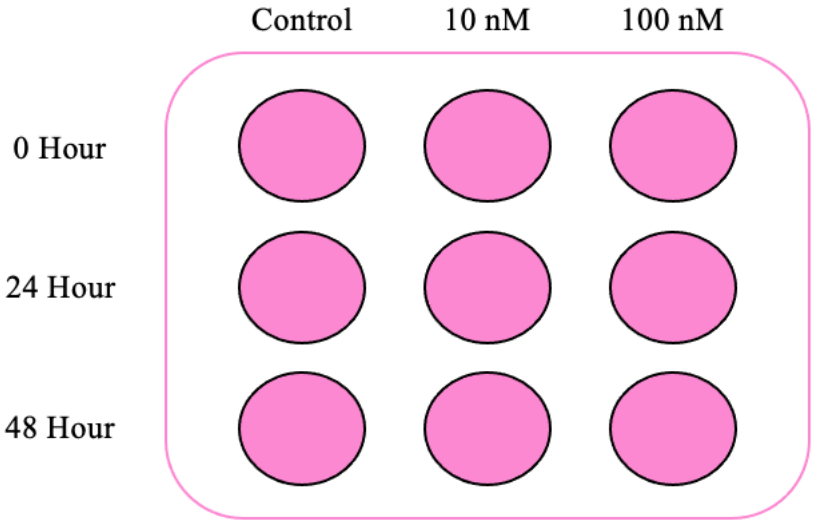

Figure 9. Schematic of 9 wells of a plate with treatment concentrations and time frame set ups for phenotype and scratch assays with 10 nM and 100 nM Vitamin D treatments along with a control.

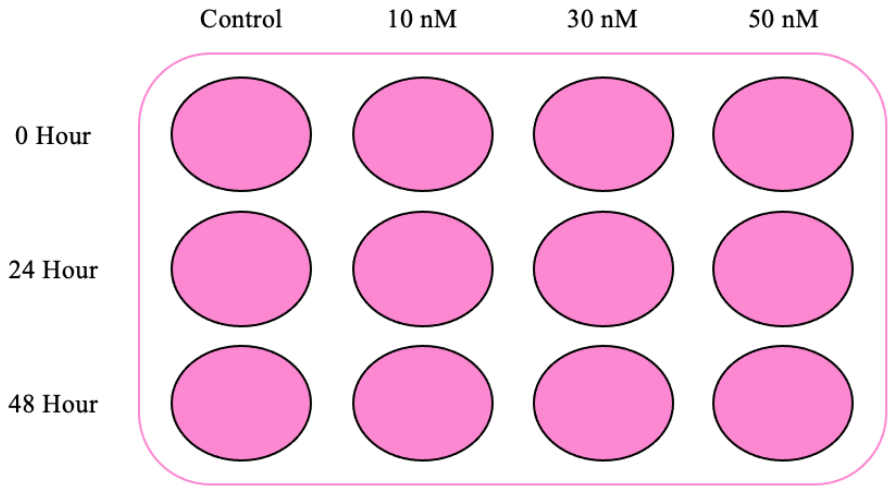

Figure 10. Schematic of a 12-well plate with treatment concentrations and time frame set ups for scratch assays and cell counts with 10 nM, 30 nM, and 50 nM Vitamin D treatments along with a control.

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