Vitamin D Effect on Growth and Vitamin D Metabolizing Enzymes in Triple-negative Breast Cancer

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Abstract. *Background: Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer that disproportionately affects women with darker skin. Epidemiological studies indicate that higher vitamin D levels prevent incidence of TNBC and translate to higher survival rates in those that have TNBC. Materials and Methods: The growth inhibition effects of two forms of vitamin D were assessed in MCF-7 and three TNBC lines using CellTiter-Glo. Expression of vitamin D-metabolizing enzymes was measured after vitamin D treatment by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Results: MCF-7 was growth inhibited by vitamin D at high concentrations but the TNBC lines were not. All cell lines demonstrated large increases in CYP24A1 mRNA levels under vitamin D treatment but there was little change in CYP27B1 or VDR mRNA levels. Conclusion: These TNBC cell lines are resistant to growth inhibition by vitamin D. This could be due to large inactivation of vitamin D by CYP24A1 or by another mechanism.*

The term triple-negative breast cancer (TNBC) describes breast cancers that do not express the estrogen receptor, the progesterone receptor or excessive amounts of the human epidermal growth factor receptor 2 (Her2), as other breast cancers commonly do (1). These breast cancers disproportionately affect African-American women over Caucasian women and African-American women have much higher mortality rates for all breast cancers than Caucasians, even after correction for other factors (1-3). This disparity in diagnosis of aggressive cancer and health outcomes mirrors the difference in vitamin D levels between Caucasians and non-Caucasians (4, 5).

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Some studies suggest a connection between vitamin D physiology and the progression of breast cancer. Expression of vitamin D receptor in breast cancer cells has been shown to correlate with an increased period of relapse-free survival (6). In other studies, low serum levels of 25-OH have been shown to correlate to higher grade cancers, estrogen receptor negative cancers and later tumor stages (7, 8). TNBC have been reported to express large amounts of both epidermal growth factor receptor and insulin-like growth factor-1 receptor (9-12). Preliminary studies show vitamin D to be involved in the regulation of both pathways. Vitamin D has also been shown to regulate insulin growth factor binding protein (13, 14) and the amount of EGFR in ovarian cancer cells (15). With numerous studies hypothesizing a link between vitamin D deficiency and increased incidence or increased morbidity from cancer (6, 7, 16-21), it is possible that vitamin D deficiency and/or differences in vitamin D metabolism may contribute to the aggressiveness of TNBC.

In order to study vitamin D metabolism in TNBC, we used four breast cancer cell lines, three of which were triple-negative (MDA-MB-157, MDA-MB-231 and MDA-MB-468) and one which expresses the typical hormone receptors (MCF-7). We first determined the effect of both 25-hydroxyvitamin D3 (25-OH) and 1,25-dihydroxyvitamin D3 (1,25D3) on the *in vitro* growth of the cell lines. We were curious to see the effect of 25OH on these cell lines as most studies focus on 1,25D3. We then determined the mRNA expression of *CYP27B1, CYP24A1* and the vitamin D receptor (*VDR*) under the same conditions. CYP27B1 converts 25-OH into the active 1,25D3 form and CYP24A1 is the enzyme responsible for degradation of both forms of the vitamin. Together, these two enzymes modulate the amount of active vitamin D available to the tissue while expression of VDR allows cells to respond to vitamin D.

Materials and Methods

Materials. The four cell lines used in this study were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). All cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA), except for charcoal/dextran stripped serum, which was purchased from Atlanta Biologicals

(Flowery Branch, GA, USA). 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 were purchased from Sigma (St. Louis, MO, USA) and diluted to 1 mM with ethanol. The CellTiter-Glo, ReliaPrep RNA Cell Miniprep System and GoTaq 1-step RT-qPCR systems were purchased from Promega (Madison, WI, USA). QuantiTect Primer Assays were purchased from Qiagen (Germantown, MD, USA).

Cell lines and culture conditions. MDA-MB-231 cells were maintained in minimal essential media with GlutaMAX (MEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml-1 penicillin, 100 μg ml-1 streptomycin, 0.25 μg ml-1 Fungizone® and 1 mM sodium pyruvate. MDA-MB-157 and MDA-MB-468 cells were maintained in Dulbecco's modified Eagle's media with GlutaMAX (DMEM) with 10% heat-inactivated FBS, 100 U ml-1 penicillin, 100 μg ml-1 streptomycin, 0.25 μg ml-1 Fungizone® and 1 mM sodium pyruvate. MCF-7 cells were cultured in the same media as MDA-MB-157 supplemented with 10 μg ml-1 insulin. Cells were passaged twice per week by washing once with phosphate-buffered saline (PBS) and rinsing with 0.25% trypsin-EDTA solution. After dissociation from the cell culture flask, the cells were re-suspended in the appropriate media and moved to the appropriate vessel.

Effect of vitamin D on cell growth. MDA-MB-231 cells were plated at a density of 1,000 cells per well in a volume of 70 μl media the day before cytotoxicity treatments in clear, cell culture-treated 96-well plates. Cells were allowed to attach overnight at 37˚C in a 5% CO_2 incubator. The next day, 10 μl of vitamin D in media was added to each well. The final concentration of vitamin D in the plate ranged from 0-1,000 nM. The concentration of vehicle (ethanol) was 0.1% in all wells of each plate. The plates were returned to the incubator for 72 h. At that time, the number of live cells in each well was determined as a function of ATP content using the CellTiter-Glo luminescent assay as directed by the manufacturer. Briefly, 80 μl reconstituted assay reagent was added to each well. The plate was agitated for 2 min on a plate shaker and incubated at room temperature for at least 15 min. Eighty μl of the mixture were then moved to a white luminometer plate and the luminescence was measured on a GloMax® Multi Detection System luminometer. Measurements were determined in quadruplicate on each plate and three biological replicates were run for each cell line.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cells were plated at a density of 32,000 cells per well in 6-well plates in a total of 5 ml of media with charcoal/dextran stripped serum the day before treatment. Cells were allowed to attach overnight at 37˚C in a 5% CO_2 incubator and then 25-OH or 1,25D3 was added to a final concentration of 200 nM. Vehicle control wells contained 0.02% ethanol. Cells were returned to the incubator for 72 h. Total RNA was then isolated from the cell cultures with the ReliaPrep RNA Cell Miniprep System following the manufacturer's instructions. Pre-designed and validated gene-specific primers (QuantiTect Primer assay) were used with the GoTaq One-Step qRT-PCR System assay. PCR efficiency was verified to be between 90-105% under our conditions and products generated gave a single melt peak in post-run analysis. The following PCR protocol was used: a 15 min cycle at 37˚C for reverse transcription followed by a 10 min denaturation at 95˚C and 35 cycles of 10 sec denaturation at 95˚C, followed by a 30 sec annealing at 60˚C and an extension of 30 sec at 72˚C. The PCR reaction volume was 20 μl. *GAPDH* was used as a housekeeping gene to normalize data and wells without reverse transcriptase were used as a control for genomic DNA. Fold change data was calculated by the $2^{-\Delta\Delta Ct}$ method. Samples were run in triplicate and three biological replicates were obtained. Samples were analyzed using a Mastercycler ep Realplex (Eppendorf, Hauppauge, NY, USA) using the Realplex 1.5 software (Eppendorf, Hauppauge, NY, USA).

Statistical analysis. The data were graphed using the GraphPad Prism 5 software and are presented as the mean±standard error of the mean (SEM). The data were analyzed using the SPSS for Windows, release 20 (IBM, Armonk, NY, USA). Statistical analysis was performed using the paired Student's *t*-test. Statistical analysis for RT-PCR data was performed using ΔCt values. Results with a *p*-value of 0.05 or less were considered statistically significant.

Results

Effect of 25-OH and 1,25D3 treatment on breast cancer cell growth. The effects of increasing concentrations of both 25-OH and 1,25D3 on the four cell lines are shown in Figure 1. 25-OH caused increase in the amount of viable MDA-MB-157 cells after 72 hours, with viabilities after treatment ranging from 106-114% of the vehicle control (Figure 1A). 1,25D3 had a smaller effect, with cell viabilities ranging between 100-105%. MDA-MB-231 cells were relatively resistant to the effects of either 25-OH or 1,25D3. Only two concentrations of 25-OH resulted in statistically significant decreases in cell viability and all MDA-MB-231 cells treated with 25-OH were between 95-100% viability (Figure 1B). While MDA-MB-157 cells showed viability increases with 25-OH, MDA-MB-468 cells showed increases with both vitamin D species and the increases with 1,25D3 were greater than those with 25-OH (Figure 1C). Cell viabilities for all 25-OH concentrations were found to be 108-110%. Cell viabilities with 1,25D3 start at around 108% between 0.1-10 nM and increase to 120-122% at 100-1000 nM.

Unlike the three triple-negative cell lines described above, MCF-7 cells did not exhibit an increase in cell viability with either form of vitamin D and exhibited a marked decrease in cell viability at higher levels of vitamin D, especially 1,25D3 (Figure 1D). At 100 nM, with either form of vitamin D, MCF-7 viability decreases to 92-93%. At 1,000 nM, cell viability decreases to 86% with 25-OH and 73% with 1,25D3. This represents a potentially important difference in response to vitamin D between triple-negative breast cancer cells and those that express hormone receptors.

Effect of 25-OH and 1,25D3 on CYP24A1, CYP27B1 and VDR mRNA levels. Cells were treated with 200 nM of either 25-OH or 1,25D3 for 72 h before RNA isolation. This concentration was chosen because it is in the upper part of the normal blood range for 25-OH, which is 75-250 nM or 30-100 ng/μl (5). After quantitation by RT-qPCR, the largest

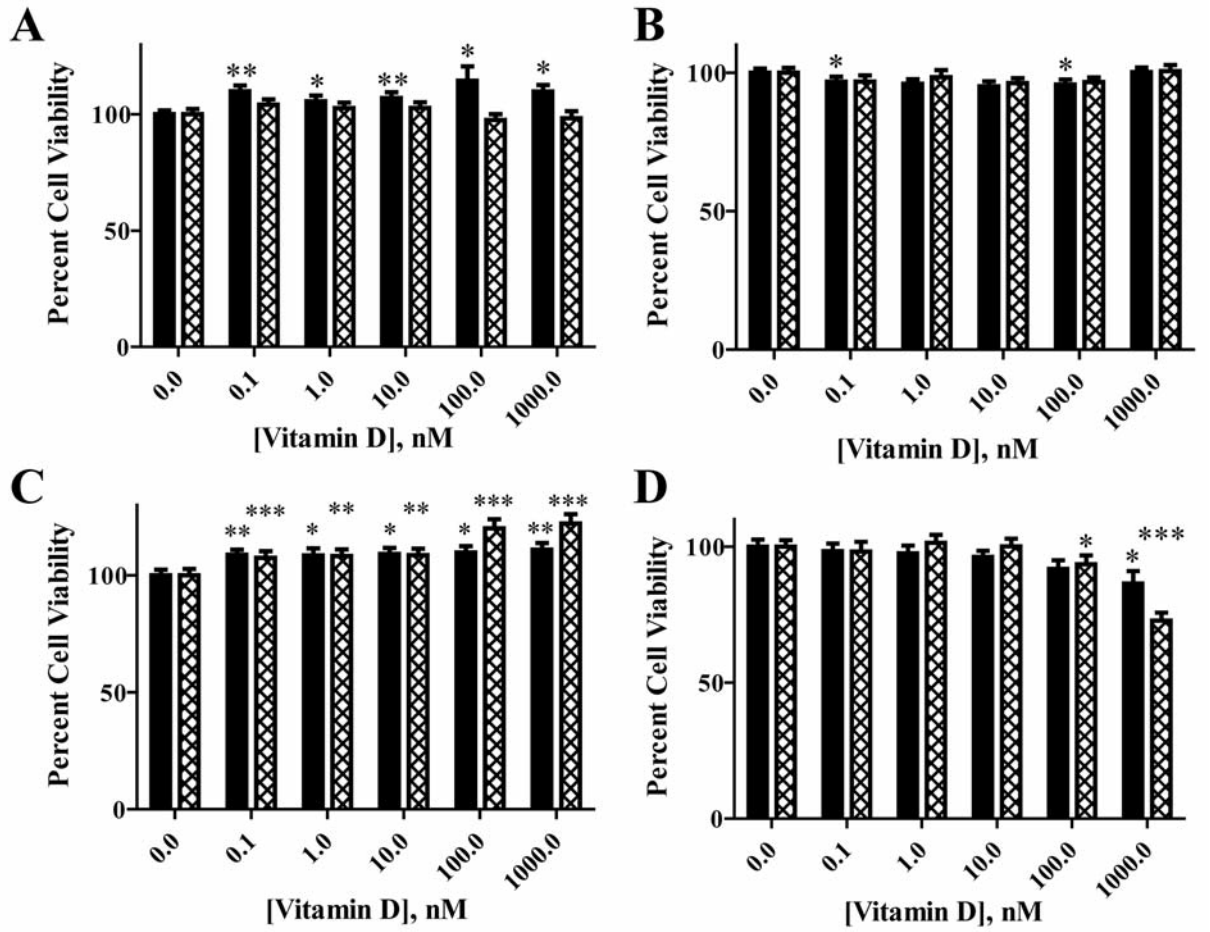

Figure 1. *Effect of Vitamin D treatment on growth of A) MDA-MB-157, B) MDA-MB-231, C) MDA-MB-468 and D) MCF-7 cells. Black-filled bars are 25-OH and cross-hatched bars are 1,25D3. Cells were treated with the indicated amounts of vitamin D for 72 h and cell numbers were measured using CellTiter-Glo. Data were normalized as a percentage of the vehicle control. Data are presented as mean±SEM. p-Values have been determined by the Students t-test against the vehicle control. *p<0.05, **p<0.01, ***p<0.001.*

changes in mRNA levels were found with *CYP24A1* (Figure 2A). The effect was small with 25-OH, with levels ranging from no statistical change from control for MDA-MB-468 to 2.7-fold higher levels with MDA-MB-157. All four cell lines had greatly increased levels after treatment with 1,25D3 ranging from 500-fold more with MDA-MB-231 to 28,000-fold more with MDA-MB-468. All values with 1,25D3 were statistically significant compared to both vehicle and the 25-OH treatments ($p<0.001$).

There was very little change in the mRNA levels for *CYP27B1* and *VDR* in response to vitamin D treatments in any of the cell lines (Figure 2B-C). Changes in *CYP27B1* mRNA levels reached statistical significance only with MDA-MB-468 but the change was small; a 1.2-fold decrease with 25-OH and a 1.4-fold decrease with 1,25D3. For *VDR*, while most cell lines showed a statistical difference between the two treatments, most fold-change values were not statistically different from the vehicle control. The only statistically significant difference from vehicle control was with 25-OH in MDA-MB-231, resulting in 1.4-fold less *VDR* mRNA.

Discussion

Breast cancer is one of the top diseases affecting women and TNBC is one of the most aggressive varieties with one of the lowest 5-year survival rates. Due to the lack of estrogen receptor in these breast cancers, they cannot be treated with hormone-targeted treatments like tamoxifen. Because they lack Her2 over-expression, they cannot be treated with treatments targeting Her2 like Herceptin. This makes TNBC inherently more difficult to treat than other breast cancers. Epidemiological data indicate that women with sufficient vitamin D have lower incidence of TNBC and greater survival rates if suffering from TNBC (6, 7, 16-21). This has helped

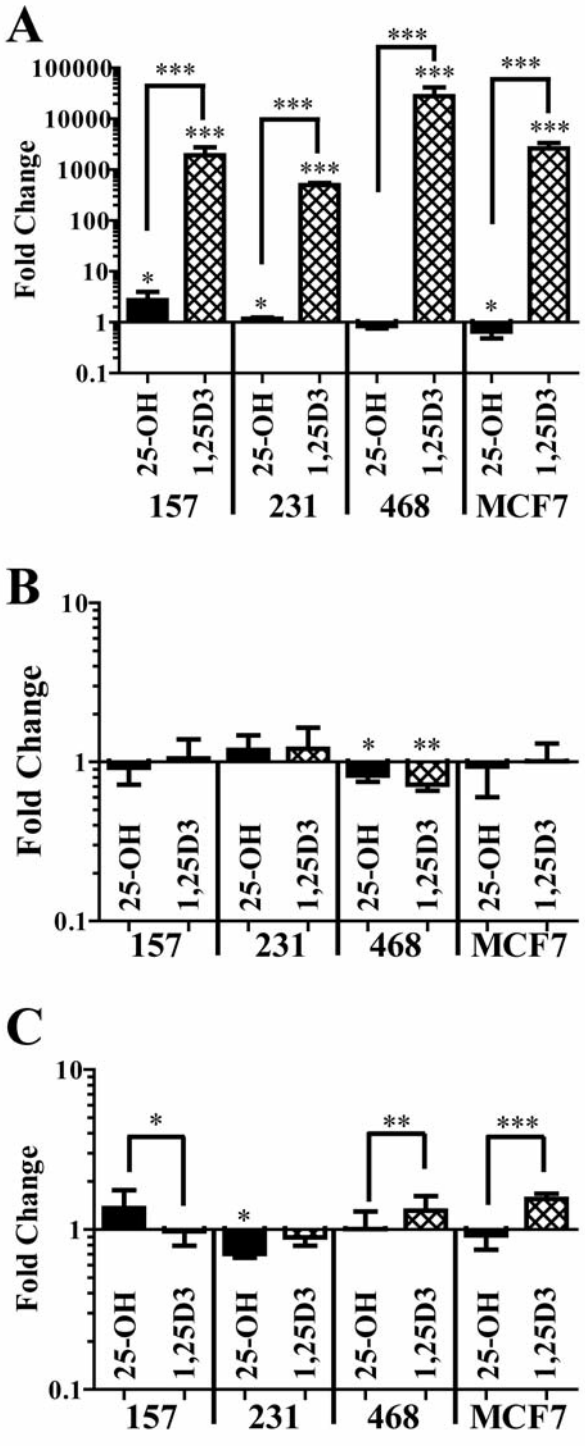

Figure 2. *Effect of Vitamin D on of A) CYP24A1, B) CYP27B1 and C) VDR mRNA levels in MDA-MB-157, MDA-MB-231, MDA-MB-468 and MCR-7 cells. Cells were treated with 200 nM 25-OH or 1,25D3 for 72 h before isolating mRNA. mRNA levels were then quantified by RT-qPCR. Data are presented as fold change to the vehicle control and error bars represent±SEM. p-Values were determined by the Students t-test against the vehicle control using ΔCt values. Brackets indicate comparisons between indicated bars. *p<0.05, **p<0.01, ***p<0.001.*

fuel the public health push to ensure sufficient 25-OH levels in the populace. However, questions still remain about the complete mechanism by which vitamin D provides protection and whether the proposed mechanisms are relevant to all cells under the triple-negative umbrella. Further, it is difficult to ascertain the role of 25-OH, which is the storage form of the vitamin optimized clinically, as most studies focus on 1,25D3, which is the the "active" hormone, or vitamin D analogs.

It has long been established that 1,25D3 results in cell-cycle arrest in breast cancer cells expressing VDR (22, 23). Herein, we showed a drop in cell viability for MCF-7 cells with 1,25D3, which is similar to results of previous studies, although under our culturing and assay conditions the effect is less marked than previously published (22, 24). We also reported a lesser, but still significant, growth inhibition with 25OH using MCF-7 cells. This could be occurring as the MCF-7 cells are converting 25-OH to 1,25D3 through the action of CYP27B1; however, there is at least one study that indicates that 25-OH itself may signal through the VDR as well (25).

In contrast, none of the triple-negative cell lines showed decreases in cell viability at 25-OH and 1,25D3 concentrations of up to 1,000 nM. We found cell number to be increased with 25-OH cell treatment for 72 h at all concentrations tested using MDA-MB-157 cells. This is a rather unexpected result as the effect was not seen with 1,25D3, which is typically the more active form of vitamin D. MDA-MB-468 exhibited a similar increase in cell number with both 25-OH and 1,25D3, with the largest increases seen at the highest concentrations of 1,25D3. MDA-MB-231 exhibited little change in cell number at any of the tested concentrations using either form of vitamin D. It appears these three examples of TNBC cell lines are resistant to the anti-proliferative effects of vitamin D. This has been previously shown with MDA-MB-231 cells that were found by Buras *et al.* to be resistant to growth inhibition at concentrations of up to 1,000 nM (24). This does not appear to be true of all TNBC cell lines, however. It has previously been shown that the SUM159-PT cell line is growth-inhibited by both 1,25D3 and a vitamin D analog (26). A recent report also shows that the WT145 cell line is both triple-negative and also sensitive to growth inhibition by 1,25D3 (27). Based on a recent paper by Stambolsky *et al.* that demonstrated that mutated forms of p53 could turn 1,25D3 from pro-apoptotic to anti-apoptotic (28), we hypothesize that the lack of decreased cell proliferation in the three cell lines presented here is because of a lack of normal *p53*. Studies exist that use MDA-MB-157 as a *p53* null line and MDA-MB-231 and MDA-MB-468 as *p53* non-functional mutants, while using MCF-7 as an example of a wild-type *p53* strain (28, 29).

All four cell lines show dramatically increased amounts of *CYP24A1* mRNA when treated with 1,25D3. This indicates

that these cell lines have functional VDR protein as it has been shown that the VDR is required for *CYP24A1* mRNA up-regulation in response to 1,25D3 (30). As CYP24A1 is the enzyme responsible for 1,25D3 inactivation, this is an expected result. There is a literature precedent for this up-regulation in many types of cells, at both the mRNA and protein levels, though most articles measured mRNA after a shorter incubation than we did (31-34). MDA-MB-157 demonstrated a 2.7-fold increase in *CYP24A1* mRNA with 25-OH treatment, which suggests that either 25-OH is signaling through the VDR or there was enough formation of 1,25D3 during the 72-h incubation to cause this increase in *CYP24A1* mRNA. The changes in *CYP24A1* mRNA levels in the other three cell lines with 25OH are all well below 2-fold. While in two lines the results are statistically significant, this change is small and it is unknown if it would result in a change in the amount in functional CYP24A1 in these cells.

There was very little change in *CYP27B1* mRNA level in these cell lines, except for a 1.2-fold decrease with 25-OH and a 1.4-fold decrease with 1,25D3 in MDA-MB-468. While it may have been expected that 1,25D3 would signal a decrease in the mRNA coding for the protein that forms it, the small changes in the amount of *CYP27B1* mRNA compared to induction of *CYP24A1* mRNA is often demonstrated in cancer cells, including hepatocellular carcinoma and a previously published study focusing on MCF-7 (32, 33). There was also a very little change in *VDR* mRNA levels in most cell lines. In most cell lines, the effects of 25-OH and 1,25D3 were statistically significant from one another but not from the control. There was a 1.4-fold reduction in *VDR* mRNA in MDA-MB-468 but, as with the other changes above that were less than two-fold, it is unknown if this would be physiologically relevant.

In conclusion, our study details the growth inhibition dose response to both 25OH and 1,25D3 in three TNBC cell lines and MCF-7, up to 1,000 nM, which is a supraphysiological dose. The lack of growth inhibition in the TNBC cell lines could be based upon the p53 status of these cells, with all three either lacking p53 (MDA-MB-157) or possessing a mutant form (MDA-MB-231 and MDA-MB-468). In addition, long-term treatment (72 h) with 1,25D3 results in large increases in the amount of *CYP24A1* mRNA. As CYP24A1 inactivates both forms of vitamin D studied here, it is possible that inactivation of 1,25D3 is also a factor in the lack of response to 1,25D3. Further studies are needed to clarify the issue and could include a comparison of growth inhibition with vitamin D between these cell lines and TNBC cell lines with normal p53 status. These studies could be conducted in both the presence and absence of compounds that inhibit CYP24A1. It has been shown that 1,25D3 has increased anti-proliferative activity when combined with CYP24A1 inhibitors (35, 36). It would be interesting to investigate if this effect also extends to treatment with 25-OH.

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