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1 α ,25-Dihydroxyvitamin D Inhibits *de novo* Fatty Acid Synthesis and Lipid Accumulation in Metastatic Breast Cancer Cells through Downregulation of Pyruvate Carboxylase

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Abstract

Both increased *de novo* fatty acid synthesis and higher neutral lipid accumulation are a common phenotype observed in aggressive breast cancer cells, making lipid metabolism a promising target for breast cancer prevention. In the present studies we demonstrate a novel effect of the active metabolite of vitamin D, 1 α ,25-dihydroxyvitamin D (1,25(OH)₂D) on lipid metabolism in malignant breast epithelial cells. Treatment of MCF10CA1a breast epithelial cells with 1,25(OH)₂D (10 nM) for five and seven days decreased the level of triacylglycerol, the most abundant form of neutral lipids, by 20% ($\pm$ 3.9) and 50% ($\pm$ 5.9), respectively. In addition, 1,25(OH)₂D treatment for five days decreased palmitate synthesis from glucose, the major fatty acid synthesized *de novo* (48% $\pm$ 5.5 relative to vehicle). We have further identified the anaplerotic enzyme pyruvate carboxylase (PC) as a target of 1,25(OH)₂D mediated regulation and hypothesized that 1,25(OH)₂D regulates breast cancer cell lipid metabolism through inhibition of PC. PC mRNA expression was downregulated with 1,25(OH)₂D treatment at two (73% $\pm$ 6 relative to vehicle) and five (56% $\pm$ 8 relative to vehicle) days. Decrease in mRNA abundance corresponded with a decrease in PC protein expression at five days of treatment (54% $\pm$ 12 relative to vehicle). Constitutive overexpression of PC in MCF10CA1a cells using a pCMV6-PC plasmid inhibited the effect of 1,25(OH)₂D on both TAG accumulation and *de novo* palmitate synthesis from glucose. Together, these studies demonstrate a novel mechanism through which 1,25(OH)₂D regulates lipid metabolism in malignant breast epithelial cells.

Keywords

1 α ,25-Dihydroxyvitamin D; Breast Cancer; Prevention; Lipid Metabolism

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Conflicts of Interest: None

I. Introduction

Breast cancer continues to be the most commonly diagnosed cancer in women worldwide, with over 1.6 million cases diagnosed annually [1]. When diagnosed early, treatment of breast cancer is highly effective with five year survival rates reaching 99%. However, when cancer progresses to an aggressive phenotype and invades secondary organs, survival rates drastically decrease to 26% [2]. Therefore, preventing the progression of breast cancer is of primary importance in prolonging the life of breast cancer patients and survivors.

Growing evidence suggests that vitamin D may be a promising preventive agent against breast cancer [3]. Vitamin D is unique because it can be obtained from the diet or synthesized endogenously in the skin upon adequate sunlight exposure [4]. Regardless of its source, vitamin D is first hydroxylated in the liver to 25-dihydroxyvitamin D (25(OH)D), a stable metabolite found in circulation and used as a marker for vitamin D status. Mohr *et al.* [5] recently demonstrated that circulatory levels of 25(OH)D inversely correlate with mortality among breast cancer patients, suggesting that vitamin D may be preventive at later stages of breast cancer progression. Studies into the mechanisms of vitamin D-mediated cancer prevention have primarily focused on the effect of the bioactive vitamin D metabolite, 1 α ,25-dihydroxyvitamin D (1,25(OH)₂D). The mechanism of action of 1,25(OH)₂D is through binding the vitamin D receptor (VDR) and regulating transcription or repression of target genes [6].

An emerging hallmark of cancer is its extensive reprogramming of energy metabolism [7]. In particular, upregulated *de novo* fatty acid (FA) synthesis and increased lipid accumulation [8] are a common phenotype observed in aggressive breast cancer cells. Furthermore, alterations in lipid metabolism correlate with cancer progression, with more aggressive tumors harboring mutations in genes involved in FA synthesis, uptake and oxidation [9]. This suggests that targeting lipid metabolism may be an effective strategy for inhibiting breast cancer. Specifically, inhibition of *de novo* FA synthesis was previously shown to delay progression and increase survival in animal models of multiple cancers [10–12], demonstrating the potential for targeting lipid metabolism in breast cancer prevention.

Sustaining high rates of *de novo* FA synthesis in cancer cells is primarily orchestrated through upregulation of lipogenic enzymes. The regulation of FA synthesis is under the control of sterol response element binding protein 1 (SREBP-1), which when activated, stimulates transcription of lipogenic enzymes and coordinates lipid synthesis [13]. The major enzymes involved in *de novo* FA synthesis are ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). All three of these enzymes have been shown to be overexpressed in several cancers including breast, with most studies focusing on FASN [14].

In addition to overexpression of lipogenic enzymes, sustaining high levels of FA synthesis by cancer cells requires an adequate supply of metabolic substrates. One of the primary substrates for FA synthesis is glucose converted to citrate in the tricarboxylic acid (TCA) cycle. The anaplerotic enzyme pyruvate carboxylase (PC) catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate and therefore replenishes TCA cycle intermediates

[15]. PC was shown to be overexpressed in breast cancer cells and its expression correlates with breast cancer aggressiveness in clinical patients [16]. Interestingly, PC also plays a role in regulation of lipid accumulation and FA synthesis. Inhibition of PC in 3T3-L1 adipocytes decreased triacylglycerol (TAG) accumulation [17], the most abundant form of neutral lipids, while PC overexpression in the same cell line increased *de novo* FA synthesis from glucose [18]. The role of PC in FA synthesis was further confirmed in non-small-cell lung cancer (NSCLC), where higher PC activity was observed in tumors of lung cancer patients. In addition, *in vitro* studies in A549 NSCLC cells confirmed that PC is necessary for maintaining high rates of FA synthesis in this cell line [19]. Taken together, this evidence suggests that PC inhibition may be an effective strategy for prevention of breast cancer.

Previously, we have shown that 1,25(OH)₂D regulates glucose metabolism in breast epithelial cells at early stages of cancer progression [20]. In the present studies, we investigated the effect of 1,25(OH)₂D on FA synthesis, which is closely linked to glucose metabolism, at later stages of breast cancer. We have identified PC as a target of 1,25(OH)₂D mediated regulation and hypothesized that 1,25(OH)₂D inhibits *de novo* FA synthesis and lipid accumulation through downregulation of PC in malignant breast epithelial cells.

2. Materials and Methods

2.1. Chemical and reagents

The 1,25(OH)₂D was purchased from Biomol (Plymouth Meeting, PA). Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F12) media, horse serum, trypsin and penicillin/streptomycin were obtained from Life Technologies, Gibco-BRL (Rockville, MD).

2.2 Cell Culture

In these studies we utilized the MCF10 model of breast cancer progression which consists of untransformed immortalized MCF10A cells, Harvey-*ras* oncogene transfected MCF10A cells representing the stage of initiation, as well as the MCF10CA1h and MCF10CA1a cells which were obtained through serial passage of MCF10A-*ras* cells *in vivo* and represent progressively later stages of breast cancer. While MCF10A cells do not form tumors *in vivo*, MCF10A-*ras* cells are able to form tumors in xenografts 25% of the time. MCF10CA1h cells consistently form well differentiated tumors *in vivo* while MCF10CA1a cells, representing the latest stage of cancer progression in the model, form poorly differentiated tumors and have the capability to invade distant secondary sites [21]. Therefore, together, the MCF10 model allows for the study of multistage breast cancer from initiation up to and including invasion of secondary organs. MCF10A and MCF10A-*ras* breast epithelial cells were a gift from Dr. Michael Kinch, Purdue University. Both cell lines were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, 1:1) containing 5% horse serum and supplemented with 10 mg/L insulin, 20 µg/L epidermal growth factor, 50 µg/L cholera toxin, 50 mg/L hydrocortisone, 100 units/mL penicillin, and 100µg/mL streptomycin in a humidified environment at 37°C with 5% CO₂. MCF10CA1h and MCF10CA1a cells were a gift from Dr. Julia Kirshner (Purdue University). Murine 4T1 cells were a gift from Dr. Michael Wendt (Purdue University). MCF10Ca1h and

MCF10CA1a cells were cultured in DMEM/F12, 1:1 supplemented with 5% horse serum, 100 units/ml penicillin, and 100µg /mL streptomycin in a humidified environment at 37°C with 5% CO₂. Murine 4T1 as well as MDA-MB-231 breast epithelial cells were cultured in the same temperature and CO₂ level as the MCF10CA1a cells but in DMEM with 10% fetal bovine serum Gibco-BRL (Rockville, MD), 100 units/ml penicillin, and 100µg /mL streptomycin.

2.3 TAG Assay

For the TAG assay, cells were plated in six-well plates at a concentration of 21,000 cells/cm² and allowed to attach overnight. Cells were then treated with media containing either vehicle (100% ethanol>0.1% final volume) or 1,25(OH)₂D (10 nM) for the indicated number of days, with media changed every 24 hours. At the end of the indicated time point, cells were washed with Tris-buffered saline (TBS) solution containing 0.2% bovine serum albumin (BSA). Lipids were then extracted using 3:2 hexane: isopropanol solution and dried under stream of nitrogen. After drying, cell extracts were resuspended in 200µl of 1% Triton-X-100 chloroform and dried again with nitrogen. The final cell lipid extract was resuspended in 40 µl of 1% Triton-X-100 in water. TAG was quantified using a colorimetric assay according to the manufacturer's instruction (Wako Diagnostics, Richmond, VA) with absorbance measured at 600 nm. To normalize TAG to protein content, 0.1 N NaOH solution was added to each well of the 6-well plate after hexane:isopropanol extraction and evaporation of excess solvent. Protein content was quantified using the Pierce BCA method (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Final TAG concentrations are represented as µg TAG per mg protein.

2.4 BODIPY Neutral Lipid Quantification

To visualize the effect of 1,25(OH)₂D on neutral lipid accumulation, MCF10C1a cells were plated onto 8 well Permanox[®] chamber slides (Sigma-Aldrich, ST. Louis, MO) and treated for 5 days with either vehicle or 1,25(OH)₂D. After 5 days of treatment, cells were fixed using 10% neutral buffered formalin for 15 minutes at room temperature. Neutral lipid droplets were next stained with BODIPY[®] 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene), a fluorescent dye with specificity for neutral lipids (D-3922, ThermoFisher Scientific, Waltham, MA) at a concentration of 10µg/ml in PBS containing 1 % BSA, 0.1 % saponin, and 0.02 % Sodium azide for one hour. A DNA stain was further applied as a nuclear counterstain (DAPI (4',6-diamidino-2-phenylindole), D21490, ThermoFisher Scientific, Waltham, MA). Imaging was performed using an A1 RMP multiphoton confocal microscope and then analyzed using FIJI, an ImageJ based image processing software available under the General Public License (fiji.asc). For quantification, cell images were taken 1µm apart across the whole cell and stacked onto one image. BODIPY signal per each stacked image was then quantified using FIJI and normalized to the number of stained nuclei in each image.

2.5 De Novo FA Synthesis

MCF10CA1a cells were plated in 6-well plates at a concentration of 21,000 cells/cm² and treated with either vehicle (100% ethanol>0.1% final volume) or 1,25(OH)₂D (10nM) for 4 days with media changed every 24 hours. At the end of day 4, cells were treated for an

additional 24 hours in media containing either 10mM $^{13}\text{C}[\text{U}]$ -Acetate (279315 Sigma-Aldrich, St. Louis, MO) or, in separate experiments, 50% $^{13}\text{C}[\text{U}]$ -Glucose (389374 Sigma-Aldrich, St. Louis, MO). At the end of 5 days, fatty acids were hydrolyzed in the presence of hydrochloric acid and extracted for analysis using Agilent 6460 Triple Quadrupole Liquid Chromatography–Mass Spectrometry (LC-MS/MS) as described previously [22]. Palmitate synthesis was chosen as the primary endpoint since this 16-carbon saturated fatty acid is the main product of FASN and the primary fatty acid synthesized *de novo* [23]. To calculate the percent incorporation of each ^{13}C -substrate into palmitate, area under the curve (AUC) for each stable isotope of palmitate detected by LC-MS/MS was obtained. Further, weighted analysis was performed to account for the number of ^{13}C -labelled carbons contributing to the AUC for each peak detected by LC-MS/MS. The final values are represented as percent of total palmitate pool derived from ^{13}C -substrate after 24 hours of incubation and was calculated by dividing the sum of weighted AUC by the sum of all AUC times 100.

2.6 RNA Isolation and Analysis

RNA isolation and its subsequent analysis were conducted as described previously [20]. Briefly, after the indicated time point cell RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Reverse transcription of total RNA was conducted using MMLV reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed using the Brilliant III SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA). The mRNA expression was normalized to 18S expression and results were expressed as arbitrary units relative to vehicle. The primers used are shown in table 1.

2.7 Western Blot Analysis

Western blot was performed as described previously [24]. Briefly, after washing with CMF-PBS solution, cells were harvested on ice into lysis buffer containing 1% protease inhibitor and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Cells were lysed with sonication and cell debris removed by centrifugation at 12,000 RPM for 15 min at 4°C. The supernatant was collected and protein concentration determined using the Pierce BCA assay (ThermoFisher Scientific, Waltham, MA). Proteins (25 µg) were resolved through SDS-PAGE on a 10% Tris-HCl gel and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). PC (sc-67021) and actin (sc-1616-R) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antigen-antibody complexes were detected using Lumiglo Reagent (Cell Signaling Technology, Inc., Danvers, MA). Density of bands was assessed using UN-SCANIT 6.1 (Silk Scientific Inc., Orem, UT). Band densities were in linear range of detectability and final expression is represented as PC/actin relative to vehicle.

2.8 PC overexpression

PC overexpressing plasmid was obtained from OriGene Technologies (Origene, Rockville, MD). Since previous studies [16], as well as studies in our laboratory, confirmed that breast cancer cells predominantly express transcript variant 1, the PC transcript variant 1 (NM_000920.2) clone was inserted into the pCMV6-Neo vector downstream of the cytomegalovirus (CMV) immediate-early promoter. For transfection, cells were grown in

either 24-well or 6-well plates to 60% confluence and transiently transfected using lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA) for 20 hours according to the manufacturer's instructions.

2.9 Statistical Analysis

Values are presented as mean $\pm$ SEM. Statistical difference between two means was assessed using Student's t-tests, with P-Value <0.05 considered statistically significant. For the TAG assay and PC mRNA in the MCF10 model, results were analyzed using one-way ANOVA and Tukey's HSD test for comparison of multiple means, with P-Value <0.05 considered statistically significant. To investigate the correlation between PC mRNA expression and TAG, simple linear regression was performed with P-Value <0.05 considered statistically significant.

3. Results

Previous studies have demonstrated that aggressive breast epithelial cells have higher neutral lipid accumulation relative to breast epithelial cells at earlier stages of breast cancer progression [8]. We have confirmed the same pattern of neutral lipid accumulation in the MCF10 model of breast cancer with MCF10CA1h and MCF10CA1a cells demonstrating an approximate 4- and 12-fold higher level of TAG than either the MCF10A or MCF10A-*ras* cells, respectively (Figure 1). In addition we measured TAG in MDA-MB-231 breast epithelial cells, a cell line with high metastatic potential, and demonstrated a comparable level of TAG to the MCF10CA1a cells. Therefore, our studies focused on the effect of 1,25(OH) $_2$ D on lipid metabolism in MCF10CA1a cells representing the latest stage of breast cancer progression in the MCF10 model [21]. To investigate the effect of 1,25(OH) $_2$ D on neutral lipid metabolism, we measured TAG accumulation in MCF10CA1a cells treated for two, five and seven days with either vehicle or 1,25(OH) $_2$ D. Treatment with 1,25(OH) $_2$ D significantly decreased TAG at five and seven days in a time dependent manner (Figure 2A), demonstrating that 1,25(OH) $_2$ D inhibits neutral lipid accumulation in malignant breast epithelial cells.

To further confirm the effect of 1,25(OH) $_2$ D on neutral lipid levels in MCF10CA1a cells, we utilized BODIPY® 493/503, a fluorescent neutral lipid dye, to stain and quantify neutral lipid content per cell. MCF10CA1a cells were treated with vehicle or 1,25(OH) $_2$ D for five days and imaged using confocal microscopy. Consistent with results of the TAG assay, treatment with 1,25(OH) $_2$ D resulted in an approximate 26% reduction in neutral lipid accumulation (Figure 2B). Therefore the effect of 1,25(OH) $_2$ D on neutral lipids was confirmed with two independent methods of measurement.

It is estimated that up to 93% of neutral lipids found in cancer cells are derived from fatty acids synthesized *de novo* [25]. Therefore, we investigated the effect of 1,25(OH) $_2$ D on the *de novo* FA synthesis. To further determine where in the pathway of FA synthesis 1,25(OH) $_2$ D exerts its effect, we utilized [U]- 13 C-acetate and, in separate experiments, [U]- 13 C-glucose. Acetate is converted intracellularly to acetyl-CoA and serves as the immediate substrate for *de novo* FA synthesis. Glucose on the other hand is metabolized through glycolysis and then into citrate in the TCA cycle before being transported out of the

mitochondria and converted to acetyl-CoA [23]. After five days of treatment, palmitate synthesis from [U]-¹³C-acetate was not significantly different between vehicle and 1,25(OH)₂D treated MCF10CA1a cells. Interestingly, when the same experiment was repeated with [U]-¹³C-glucose, 1,25(OH)₂D treatment resulted in a significant 48% reduction in palmitate synthesis (Figure 3A). These results demonstrate that 1,25(OH)₂D inhibits *de novo* FA synthesis from glucose upstream of acetyl-CoA and, therefore, upstream of lipogenic enzymes commonly overexpressed in breast cancer cells [14].

Since 1,25(OH)₂D acts through binding to the VDR and regulating transcription of target genes, we measured mRNA expression of genes involved in *de novo* FA synthesis. Neither mRNA expression of *ACC* nor *FASN* was altered with 1,25(OH)₂D treatment after five days (Figure 3B). This is consistent with our experiments utilizing [U]-¹³C-acetate, since acetyl-CoA serves as substrate for both of these enzymes [23]. We also investigated major regulatory enzymes involved in glucose metabolism which may explain the decrease in *de novo* FA synthesis from glucose observed with 1,25(OH)₂D treatment. The effect of 1,25(OH)₂D on multiple genes regulating glycolysis was determined after 48 hours of treatment. The genes studied included glucose transporter 1 (GLUT1), which facilitates intracellular glucose flux and is the major glucose transporter expressed in human breast epithelial cells [26], as well as hexokinase 2 (HK2) which encodes one of the four major isoforms of the enzyme responsible for conversion of glucose to glucose-6-phosphate in the initial step of glycolysis [27]. Additionally, mRNA expression of pyruvate kinase M2 (PKM2) and pyruvate dehydrogenase kinase 1 (PDK1) was determined. PKM2 encodes the enzyme which catalyzes the conversion of phosphoenolpyruvate to pyruvate while PDK1 phosphorylates pyruvate dehydrogenase resulting in its inhibition and decreased glucose flux into the TCA cycle. There was no effect of 1,25(OH)₂D on the mRNA expression of any of the genes mentioned above, suggesting that 1,25(OH)₂D does not regulate *de novo* FA synthesis from glucose at the step of glycolysis (Figure 4). We next focused on the enzyme facilitating pyruvate entry into the TCA cycle and identified PC as a target of 1,25(OH)₂D. PC mRNA expression was increased with breast cancer progression in the MCF10 model, with MCF10CA1a cells having approximately five-fold higher PC mRNA abundance than the MCF10A and MCF10A-ras cells. Furthermore, we conducted simple linear regression to assess the relationship between TAG and PC in the MCF10 model, demonstrating that PC expression positively correlates with TAG accumulation (P-value<0.05, R-square of 0.9956). PC mRNA expression was significantly reduced with 1,25(OH)₂D treatment at two and five days by 27% and 44%, respectively, in MCF10CA1a cells (Figure 5A). Consistent with a decrease in PC mRNA expression, PC protein expression was also downregulated by 46% at five days with 1,25(OH)₂D treatment (Figure 5B). To further investigate the effect of 1,25(OH)₂D on PC in other breast epithelial cell lines, we utilized the murine 4T1 cell line which represents a late stage of breast cancer progression similar to the MCF10CA1a cell line. Consistent with our results, PC mRNA was downregulated by 1,25(OH)₂D in 4T1 cells at two and five days, demonstrating that the effect of 1,25(OH)₂D on PC is not limited to MCF10CA1a cells (Figure 5C).

Given the role of PC in *de novo* FA synthesis in adipocytes [18] and NSCLC cells [19], and our results demonstrating that 1,25(OH)₂D regulates lipid metabolism, we hypothesized that 1,25(OH)₂D regulates FA synthesis and TAG accumulation through downregulation of PC.

To test our hypothesis we employed a pCMV6 plasmid expressing PC transcript variant 1 (NM_000920.2) inserted downstream of the cytomegalovirus promoter (Origene, Rockville, MD). Previous studies have shown the breast cancer cells predominantly express transcript variant 1 [16], with studies in our laboratory confirming the same pattern of expression in the MCF10 cell model (data not shown). Following transfection with the PC containing pCMV6 plasmid, PC mRNA was significantly increased in MCF10CA1a at two and five days compared to the control empty pCMV6-Neo plasmid, with no significant difference between vehicle and 1,25(OH)₂D treated cells (Figure 6A).

We further investigated the effect of PC overexpression on 1,25(OH)₂D mediated decrease in TAG. Consistent with our previous experiments, Neo control transfected cells treated with 1,25(OH)₂D had a 21% decrease in TAG levels relative to vehicle treated cells. In contrast, MCF10CA1a cells overexpressing PC and treated with 1,25(OH)₂D had no significant difference in TAG levels relative to vehicle treated cells at five days (Figure 6b). These results support that 1,25(OH)₂D regulates TAG accumulation in MCF10CA1a cells through downregulation of PC.

To investigate the role of PC in 1,25(OH)₂D mediated inhibition of *de novo* FA synthesis, we measured the effect of 1,25(OH)₂D on palmitate synthesis from [U]-¹³C-glucose in MCF10CA1a cells transfected with either the pCMV6-Neo control or pCMV6-PC plasmid. Consistent with results measuring TAG levels, Neo control transfected cells treated with 1,25(OH)₂D had a significant 29% decrease in palmitate synthesis from glucose. PC overexpression, however, inhibited the effect of 1,25(OH)₂D with no significant difference between vehicle and 1,25(OH)₂D treated cells at five days (Figure 5D). A similar effect of PC overexpression was confirmed on stearate synthesis. Stearate is an 18-carbon long saturated fatty acid synthesized through elongation of palmitoyl-CoA on the cytoplasmic side of the endoplasmic reticulum membrane [28]. Consistent with the effect of 1,25(OH)₂D on palmitate synthesis, Neo control transfected cells demonstrated a 30% decrease in stearate synthesis from glucose while PC overexpression inhibited the effect of 1,25(OH)₂D on stearate synthesis (Figure 4D). These results confirmed that 1,25(OH)₂D decreases *de novo* FA synthesis in breast epithelial cells through downregulation of PC.

4. Discussion

Upregulated *de novo* FA synthesis and, more broadly, the reprogramming of lipid metabolism by cancer cells, is an emerging research area showing therapeutic potential for multiple cancers [29]. In the present study, we identify PC downregulation as a novel mechanism through which 1,25(OH)₂D inhibits FA synthesis and neutral lipid accumulation in malignant breast epithelial cells at late stages of cancer progression.

PC is a relatively newly identified target of cancer research with limited number of studies investigating the role of this anaplerotic enzyme in breast cancer progression. Of the few studies available, Phannasil *et al.* [16] provides strong evidence for the importance of PC in breast cancer. First, the authors demonstrated that PC is overexpressed in metastatic MDA-MB-435 and MDA-MB-231 cells relative to MCF-7 cells, which form primary tumors *in vivo* but do not metastasize to secondary sites [30]. Furthermore, the researchers

demonstrated that PC is necessary for maintaining the migratory and invasive capabilities of MDA-MB-231 cells, while PC overexpression in MCF7 cells promotes their invasive potential *in vitro*. Whether elevated activity of PC promotes breast cancer progression to a metastatic phenotype by sustaining high levels of *de novo* FA synthesis, however, has not yet been investigated.

Although we report a novel mechanism through which 1,25(OH)₂D inhibits lipid accumulation and FA synthesis, previous studies investigating the effect of 1,25(OH)₂D on lipid metabolism in other cell types demonstrate similar results. For example, Chang *et al.* [31] recently demonstrated that 1,25(OH)₂D significantly reduced neutral lipid accumulation in 3T3-L1 adipocytes. Given the role of PC in regulation of lipid accumulation in the same cell line [17], it is possible that 1,25(OH)₂D exerts its effect on adipocyte lipid accumulation through downregulation of PC, similar to what we have characterized in the present study in malignant breast epithelial cells.

In contrast to the effect of 1,25(OH)₂D on lipid accumulation reported in this paper, Wang *et al.* [32] demonstrated that treatment of LNCaP prostate cancer cells with 100nM 1,25(OH)₂D for six days increased neutral lipid accumulation, as measured by Oil Red O staining. A similar result was reported by Lazzaro *et al.* [33]. The researchers demonstrated that treatment with 100 nM 1,25(OH)₂D induced differentiation and subsequent lipid accumulation in T47D breast epithelial cells. Interestingly, 1,25(OH)₂D treatment did not induce differentiation in MCF10A-*ras* cells, the parental cell line of MCF10CA1a cells. Unlike cell lines derived from the MCF10A-*ras* cells, T47D cells are estrogen receptor and progesterone receptor positive. Therefore, 1,25(OH)₂D may have variable effects on lipid metabolism based on breast cancer subtype.

Human PC gene expression is regulated by two alternative promoters, the proximal (P1) and distal (P2), with the level of regulation attributed to these promoters being further tissue specific. Previous studies focusing on breast cancer demonstrated that breast epithelial cells express primarily transcript variant 1 [16], with our laboratory confirming the same phenotype in the MCF10 cell model of breast cancer progression. PC transcript variant 1 expression is under the control of the distal P2 promoter [34]. While the P1 promoter is responsible for regulating PC expression in the liver and adipose tissue, P2 promoter has been shown to be important for anaplerosis and insulin release in pancreatic β -cells [35, 36]. Why breast cancer cells express transcript variant 1 and how this expression is regulated, however, has not yet been investigated.

PC protein expression is suggested to have a relatively long half-life (28–35h) with regulation occurring primarily at the level of transcription of the PC gene [37]. Therefore, it is likely that 1,25(OH)₂D regulates PC expression and activity through inhibiting its level of transcription. However, whether this regulation is orchestrated through a VDR response element located on the P2 PC promoter requires further investigation.

Taken together, our results demonstrate PC as a novel target of 1,25(OH)₂D mediated regulation which results in downstream inhibition of *de novo* FA synthesis. This study contributes to the growing evidence of how vitamin D may prevent breast cancer, potentially

through altering the metabolic changes observed in breast cancer cells in their progression to an aggressive phenotype.

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Abbreviations

25(OH)D	25-hydroxyvitamin D
1,25(OH)₂D	1 α ,25-dihydroxyvitamin D
PC	pyruvate carboxylase
FA	fatty acid
FASN	fatty acid synthase
ACC	acetyl-CoA carboxylase

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Figure 1. TAG accumulation correlates with breast cancer progression in the MCF10 model
 (A) Intracellular TAG was analyzed in four cell lines of the MCF10 model of breast cancer as well as the MDA-MB-231 breast cancer cell line used as a reference. Cells were grown for 48h before TAG analysis. Values are represented relative to the MCF10A cell line. (B) PC mRNA expression was measured in MCF10A, MCF10A-*ras* and MCF10CA1a cells after 48h. Values are expressed as mean±SEM and are a result of three independent experiments (n=3 per group per experiment). Bars with different letters are significantly different (P <0.05).

Figure 2. 1,25(OH)₂D decreases TAG accumulation in MCF10CA1a breast epithelial cells
 (A) MCF10CA1a cells were treated with either vehicle or 1,25(OH)₂D for the indicated time points. Intracellular TAG of vehicle treated cells was not significantly different between time points, hence values were pooled and are represented as a single bar. Values are expressed as mean±SEM and are a result of three independent experiments (n=3 per group per experiment). Bars with different letters are significantly different (P <0.05). (B) MCF10CA1a cells were treated for five days and imaged using the A1 RMP multiphoton confocal Microscope. Representative images are provided (left). BODIPY signal was

quantified and normalized to cell number (right). Asterisk indicates a significant difference relative to vehicle ($P < 0.05$).

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Figure 3. 1,25(OH)₂D inhibits *de novo* fatty acid synthesis upstream of lipogenic enzymes

(A) Conversion of acetate or glucose to palmitate in either vehicle or 1,25(OH)₂D treated cells is represented as percent of total palmitate from each ¹³C substrate. MCF10CA1a cells were treated with either vehicle or 1,25(OH)₂D for five days. [U]-¹³C-acetate or, in separate experiments, [U]-¹³C-glucose containing media was introduced for the last 24 hours of treatment and ¹³C substrate incorporation into palmitate was analyzed using LC-MS/MS. (B) mRNA expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) after five days of treatment with either vehicle or 1,25(OH)₂D is expressed relative to

vehicle and values are normalized to 18S. All Values are expressed as mean $\pm$ SEM and are a result of three independent experiments (n=3 per group per experiment). Asterisk indicates a significant difference relative to vehicle (P <0.05).

Figure 4. 1,25(OH)₂D does not alter the expression of enzymes involved in regulation of glycolysis

mRNA expression of GLUT1, HK2, PKM2, and PDK1 was measured in MCF10CA1a cells treated for 48h with either vehicle or 1,25(OH)₂D using qRT-PCR and normalized to 18S. Values are represented relative to vehicle of each gene.

Figure 5. 1,25(OH)₂D downregulates PC expression

(A) PC mRNA expression was measured in MCF10CA1a cells treated with vehicle or 1,25(OH)₂D for one, two or five days using qRT-PCR and normalized to 18S. Values are represented relative to vehicle of each time point. (B) PC protein expression in vehicle and 1,25(OH)₂D treated MCF10CA1a cells at five days was analyzed by Western Blot analysis. The figure on the right is a representative Western blot and the graph on the left is a quantification of band intensity. (C) PC mRNA expression in 4T1 breast epithelial cells after two and five days of 1,25(OH)₂D treatment. Values for each time point are represented

relative to vehicle of the same time point. All values are expressed as mean $\pm$ SEM and are a result of at least two independent experiments (n=3 per group per experiment). Asterisk indicates a significant difference ($P < 0.05$).

Figure 6. PC overexpression reverses the effect of 1,25(OH)₂D on lipid metabolism

(A) MCF10CA1a cells were transiently transfected with either the pCMV6-Neo control or pCMV6-PC plasmid for 20 hours. Treatment began after transfection for the indicated duration. Values are normalized to 18S and represented relative to pCMV6-Neo vehicle for each time point. (B) After transfection with pCMV6-Neo control or pCMV6-PC plasmid, cells were treated with vehicle or 1,25(OH)₂D for five days. TAG results were normalized to protein content and are represented relative to vehicle of each group. (C&D) After transfection with the pCMV6-Neo control or pCMV6-PC plasmid, cells were treated with

either vehicle or 1,25(OH)₂D for five days. [U]-¹³C-glucose was introduced for the last 24 hours of treatment and ¹³C incorporation into fatty acids was assessed through LC-MS/MS. All values are expressed as mean ±SEM and are a result of at least two independent experiments (n=3 per group per experiment). Asterisk indicates a statistically significant difference (P <0.05).

Table 1

Primers used in QPCR analysis of gene expression

Genes	Primer Information
FASN	Forward: 5'- GGTCTTGAGAGATGGCTTGC - 3'
	Reverse: 5'- AATTGGCAAAGCCGTAGTTG - 3'
ACC	Forward: 5'- GATCAAACCTCTGGGAGTCTATG - 3'
	Reverse: 5'- TCTCGGCCTTCTGGATATT - 3'
PC (human)	Forward: 5'- ATGTTGCCCACAACCTCAGCAAGC - 3'
	Reverse: 5'- AGTTGAGGGAGTCAAACACACGGA - 3'
PC (murine)	Forward: 5'- TCACCAGTGACTCTGTCAAAC - 3'
	Reverse: 5'- GACCAGGTCCACATCTGTAATC - 3'
GLUT1	Forward: 5'- TATCGTCAACACGGCCTTCACTGT - 3'
	Reverse: 5'- CACAAAGCCAAAGATGGCCACGAT - 3'
HK2	Forward: 5'- CTGCAGCGCATCAAGGAGAACAAA - 3'
	Reverse: 5'- ACGGTCTTATGTAGACGCTTGGCA - 3'
PKM2	Forward: 5'- ATTATTTGAGGAACCTCCGCCGCCT - 3'
	Reverse: 5'- CATTCATGGCAAAGTTCACCCGGA - 3'
PDK1	Forward: 5'- TCATGTCACGCTGGGTAATGAGGA - 3'
	Reverse: 5'- AACACGAGGTCTTGGTGCAGTTGA - 3'
18S	Forward: 5'- TTAGAGTGTTCAAAGCAGGCCCGA - 3'
	Reverse: 5'- TCTTGGCAAATGCTTTCGCTCTGG