REGULATION OF ENERGY METABOLISM IN EXTRACELLULAR MATRIX DETACHED BREAST CANCER CELLS

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Nutrition Science

West Lafayette, Indiana

May 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1α,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
ADP	Adenosine diphosphate
αKG	α-ketoglutarate
AMPK	Adenosine monophosphate kinase
ANOVA	Analysis of variance
ASCT2	Alanine/Serine/Cysteine-preferring Transporter 2
ATP	Adenosine triphosphate
AUC	Area under the curve
BCA	Bicinchoninic acid
BMI	Body mass index
CPT1a	Carnitine palmitoyltransferase A1
CYP	Cytochrome P-450 enzymes
DBP	Vitamin D binding protein
DMEM	Dulbecco's modified eagle medium
DRIP	Vitamin D interacting protein
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
$FADH_2$	Flavin adenine dinucleotide
FAK	Focal adhesion kinase
FAO	Fatty acid oxidation
Foxa2	Forkhead transcription factor boxA2
GCASPC	Gallbladder cancer-associated suppressor of pyruvate carboxylase
GLS	Glutaminase
GLUD1	Glutamate dehydrogenase
GLUL	Glutamine synthetase
GLUT	Glucose transporter
GOT	Glutamate oxaloacetate transaminase

GSH	Glutathione
HK2	Hexokinase 2
HNF3β	Hepatocyte nuclear factor 3β
IDH1	Isocitrate dehydrogenase
ILK	Integrin linked kinase
IU	International units
LDH	Lactate dehydrogenase
lncRNA	Long non-coding RNA
MAPK	mitogen activating protein kinase
MARRS	1,25$(OH)_2$D-membrane associated rapid response steroid binding receptor
MEG3	Maternally expressed gene 3
MEK	Mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide dinucleotide phosphate
Nrf2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung cancer
OCR	Oxygen consumption rate
P1	proximal PC promoter
P2	distal PC promoter
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PC	Pyruvate Carboxylase
PCK	Phosphoenolpyruvate carboxykinase
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PDK4	Pyruvate dehydrogenase kinase 4
PEP	Phosphoenolpyruvate
PI3K	phosphoinositide-3 kinase

Poly-HEMA	Poly(2-hydroxyethyl methacrylate)
PPARγ	Peroxisome proliferator-activated receptor γ
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor-κB ligand
Rb	Retinoblastoma
RDA	Recommended dietary allowance
Redox	Reductive-oxidative
ROS	Reactive oxygen species
RXR	Retinoic X receptor
SDH	Succinate dehydrogenase
SLC1A5	Solute carrier family 1A member 5
SOD2	Superoxide dismutase 2
TCA	Tricarboxylic acid
TGFβ	Transforming growth factor β
TRPV	Transient receptor potential vanilloid
UL	Tolerable upper intake level
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factors
VITAL	Vitamin and omega-3 trial
WHI	Women's health initiative

ABSTRACT

Breast cancer is the predominant cancer diagnosed among women, and the second most deadly cancer. The vast majority of cancer-related deaths is caused by the metastatic spread of cancer from the primary tumor to a distant site in the body. Therefore, new strategies which minimize breast cancer metastasis are imperative to improve patient survival. Cancer cells which acquire anchorage independence, or the ability to survive without extracellular matrix attachment, and metabolic flexibility have increased potential to metastasize. In the present studies, the ability to survive detachment and subsequent metabolic changes were determined in human Harvey-*ras* transformed MCF10A-*ras* breast cancer cells. Detachment resulted in reduced viability in a time-dependent manner with the lowest cell viability observed at forty hours. In addition, decreased cell viability was observed in both glutamine and glucose depleted detached conditions, suggesting a dependence on both nutrients for detached survival. Compared to attached cells, detached cells had reduced total pool sizes of pyruvate, lactate, α-ketoglutarate, fumarate, malate, alanine, serine, and glutamate, suggesting the metabolic stress which occurs under detached conditions. However, intracellular citrate and aspartate pools were unchanged, demonstrating a preference to maintain these pools in detached conditions. Compared to attached cells, detached cells had suppressed glutamine metabolism, as determined by decreased glutamine flux into the TCA cycle and reduced mRNA abundance of glutamine metabolizing enzymes. Further, detached glucose anaplerosis through pyruvate dehydrogenase activity was decreased, while pyruvate carboxylase (PC) expression and activity were increased. A switch in metabolism was observed away from glutamine anaplerosis to a preferential utilization of PC activity to replenish the TCA cycle, determined by reduced PC mRNA abundance in detached cells treated with a cell-permeable analog of α-ketoglutarate, the downstream metabolite of glutamine which enters the TCA cycle. These results suggest that detached cells elevate PC to increase flux of carbons into the TCA cycle when glutamine metabolism is reduced.

Vitamin D is recognized for its role in preventing breast cancer progression, and recent studies suggest that regulation of energy metabolism may contribute to its anticancer effects. Vitamin D primarily acts on target tissue through its most active metabolite, 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D). The present work investigated 1,25$(OH)_2$D's effects on viability of detached cells through regulation of energy metabolism. Treatment of MCF10A-*ras*

cells with 1,25$(OH)_2$D resulted in decreased viability of detached cells. While 1,25$(OH)_2$D treatment did not affect many of the glucose metabolism outcomes measured, including intracellular pyruvate and lactate pool sizes, glucose flux to pyruvate and lactate, and mRNA abundance of enzymes involved in glucose metabolism, 1,25$(OH)_2$D treatment reduced detached PC expression and glucose flux through PC. A reduction in glutamine metabolism was observed with 1,25$(OH)_2$D treatment, although no 1,25$(OH)_2$D target genes were identified. Further, PC depletion by shRNA decreased cell viability in detached conditions with no additional effect with 1,25$(OH)_2$D treatment. Moreover, PC overexpression resulted in increased detached cell viability and inhibited 1,25$(OH)_2$D's negative effects on viability. These results suggest that 1,25$(OH)_2$D reduces detached cell viability through regulation of PC. Collectively this work identifies a key metabolic adaptation where detached cells increase PC expression and activity to compensate for reduced glutamine metabolism and that 1,25$(OH)_2$D may be utilized to reverse this effect and decrease detached cell viability. These results contribute to an increased understanding of metastatic processes and the regulation of these processes by vitamin D, which may be effective in preventing metastasis and improve breast cancer patient survival.

CHAPTER 1. INTRODUCTION

1.1 Vitamin D

1.1.1 Structure and Sources

Vitamin D is a unique nutrient that is obtained from dietary sources and synthesized in the skin upon ultraviolet (UV) exposure from the sun (1). Vitamin D is a fat-soluble vitamin, also referred to as calciferol, and is classified as a seco-sterol (2). The two major forms of vitamin D are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Dietary vitamin D_3 may be obtained from animal sources such as oily fish, liver, and egg yolk. Additionally, synthesis of pre-vitamin D_3 from 7-dehydrocholesterol occurs in the skin upon UV irradiation (3). Following which a thermosensitive, nonenzymatic rearrangement of three double bonds produces vitamin D_3 (4). Similar to vitamin D_3 synthesis, vitamin D_2 is synthesized from ergocalciferol via UV irradiation from the sun in plants, most commonly mushrooms (3). The two forms of vitamin D have a single structural difference located in the side chains where vitamin D_2 has a methyl group located at carbon 24 and a double bond between carbons 22 and 23 (Figure 1.1). Vitamin D_3 and D_2 are collectively called vitamin D, and maintain the same function in the body despite the structural difference (3).

Apart from sun exposure, dietary sources provide an alternative source of vitamin D. In the United States, the primary source of dietary intake of vitamin D is provided by supplementation and fortified food. This is due to the low availability of naturally occurring sources of vitamin D. Milk is the primary food product that is voluntarily fortified in the United States with 400 international units (IU)/quart (2). Other vitamin D fortified foods include cereals, yogurts, and juices.

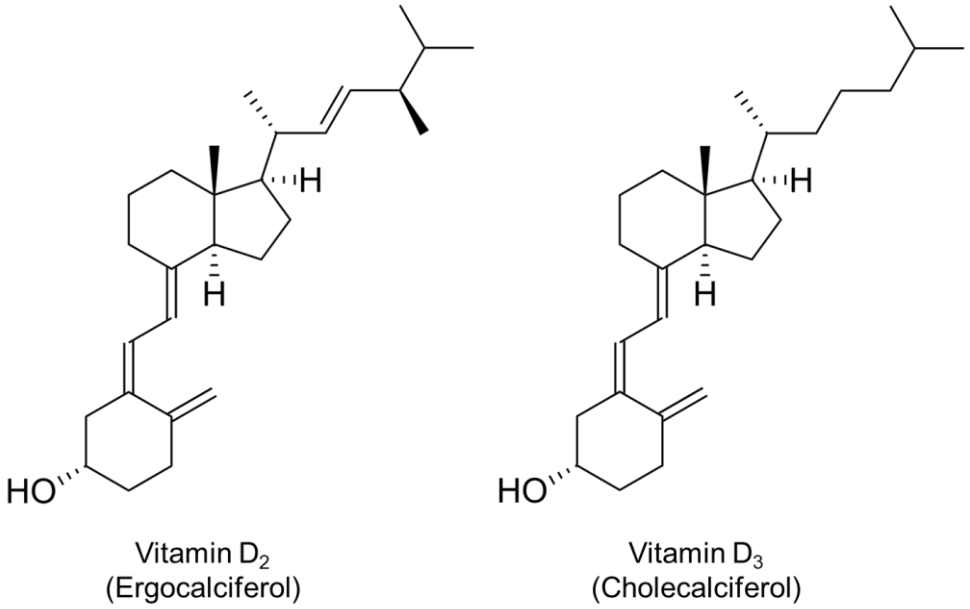

Figure 1.1 Structural Differences Between Vitamin D_2 and Vitamin D_3.

The two major isoforms of vitamin D have one structural difference. At carbon 24 vitamin D_2 has a methyl group whereas vitamin D_3 has a double bond.

1.1.2 Vitamin D Metabolism and Mechanism of Action

Vitamin D synthesis from the sun is dependent on multiple factors, including time of day, latitude, and season which play a role in the ability to synthesize vitamin D in the skin. UVB radiation, which has a wavelength of 291-320 nm, is required for vitamin D synthesis. The amount of UVB photons reaching Earth decreases when the solar zenith angle increases (5). Thus, UVB intensity is greatest during midday when the sun is at its highest point. Additionally, the solar zenith angle increases in latitudes above or below 33° in the winter months leading to a decreased ability to synthesize vitamin D (5). Therefore, dermal synthesis of vitamin D will not occur unless there is sufficient UVB exposure which is suboptimal when the solar zenith angle is increased such as in the morning, evening, and winter months (6). In addition, melanin in the epidermal layer of skin can block the UVB that is required for vitamin D synthesis (2). The use of sunscreen with sun protection factor 8 or more blocks UVB rays from penetrating the skin decreasing the synthesis of vitamin D by 95% and could affect vitamin D status (7). Therefore, factors affecting UVB

exposure including solar zenith angle, skin pigmentation, and use of sunscreen block UVB exposure and decrease the rate of vitamin D synthesis.

Synthesis of the most active form of vitamin D requires further metabolism (Figure 1.2). First, vitamin D is transported by the vitamin D binding protein (DBP) in the blood and is metabolized in two sequential hydroxylation reactions (4). The first reaction takes place in the liver and is performed by vitamin D 25-hydroxylase, which is a member of the cytochrome P-450 enzymes (CYPs) and mediates the hydroxylation of carbon 25, forming 25-hydroxyvitamin D (25(OH)D) (4). The primary CYP involved in synthesizing 25(OH)D is CYP27A1 (8). The predominant form of vitamin D in serum is 25(OH)D. Due to its slow turnover and the low amount of regulation of the 25-hydroxylase, 25(OH)D levels are used as a biomarker to assess vitamin D status (4). In the serum, 25(OH)D is transported by DBP and subsequent hydroxylation of 25(OH)D occurs in the kidneys. DBP interacts with megalin and cubulin, cell surface proteins in renal cells at the proximal tubule, to mediate the uptake of 25(OH)D (9,10). Hydroxylation by 25(OH)D 1α-hydroxylase (CYP27B1) at the first carbon yields 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D) (4). The hormonally active metabolite of vitamin D is 1,25$(OH)_2$D, which mediates the biologically observed effects of vitamin D on calcium homeostasis.

Regulation of gene expression in target tissues by 1,25$(OH)_2$D occurs through the binding of 1,25$(OH)_2$D to the vitamin D receptor (VDR) (11). When VDR is not bound to ligand, it is primarily located in the cytoplasm. The binding of 1,25$(OH)_2$D to VDR induces a conformational change which promotes VDR heterodimerizing with its partner receptor, the retinoic X receptor (RXR). The VDR-RXR heterodimer translocates to the nucleus, where it binds to vitamin D response elements (VDREs) located in the promotors of vitamin D regulated genes (12). VDREs contain imperfect direct repeats of the nucleotide sequence GGGTGA with 3 spacer nucleotides between each repeat (13). VDR-RXR binding to a VDRE requires interaction with coregulators in order to repress or recruit the RNA polymerase and begin or suppress transcription (13). The most characterized coregulator of VDR is the coactivator complex identified in primary keratinocytes as the vitamin D interacting protein (DRIP)/mediator complex (14). The DRIP/mediator complex is a multiprotein complex that directly interacts with VDR to recruit RNA polymerase II to the promoter, thereby initiating transcription.

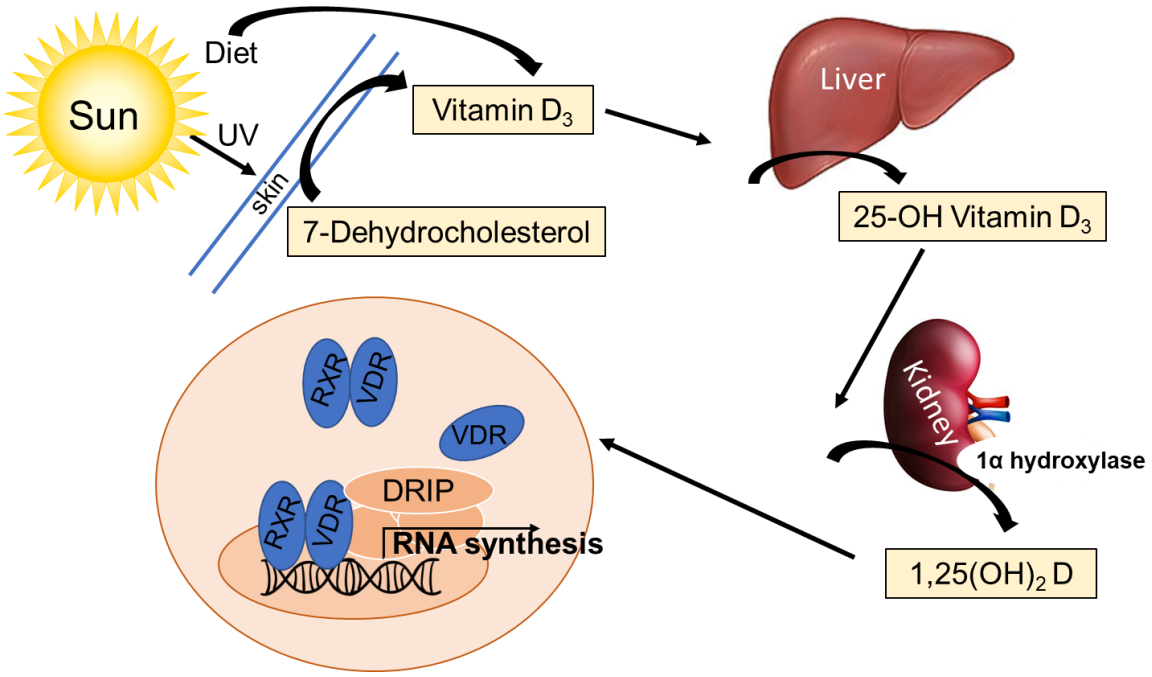

Figure 1.2 Vitamin D Metabolism and Mechanism of Action.

Vitamin D is either obtained from the diet or synthesized in the skin. It is metabolized to its most active form, $1,25(OH)_2D$, in two sequential hydroxylation reactions. Vitamin D's primary mechanism of action in target tissue is through regulation of gene expression through its receptor, VDR.

Inactivation of $1,25(OH)_2D$ occurs in the kidney through further hydroxylation steps (4). Hydroxylation at the 24th carbon of $1,25(OH)_2D$ by the 24-hydroxylase (CYP24A1), produces an inactive form of vitamin D, 1,24,25-trihydroxyvitamin D. The 24-hydroxylase also targets 25(OH)D, synthesizing 24,25-dihydroxyvitamin D, and inactivation by 24- hydroxylation of both $1,25(OH)_2D$ and 25(OH)D targets them for excretion. Further, the action of the 24-hydroxylase to form 24,25-dihydroxyvitamin D reduces 25(OH)D availability for synthesis into the active $1,25(OH)_2D$ form (15). Interestingly, $1,25(OH)_2D$ action via VDR transcriptionally increases expression of the 24-hydroxylase and decreases expression of the 1α-hydroxylase via VDREs, creating a negative feedback loop and regulating its activity (15).

Further evidence suggests a non-transcriptional mechanism of action by $1,25(OH)_2D$ through rapid signaling pathways (16). Studies demonstrate increased calcium and phosphorus uptake in intestinal cells within minutes following vitamin D supplementation in rats and this rapid response cannot be explained by the nuclear actions of $1,25(OH)_2D$ (17). An increase in

$1,25(OH)_2D$ accumulation in the basal lateral membrane fraction of intestinal cells was also observed, suggesting the presence of a membrane receptor for $1,25(OH)_2D$ (18). Further investigation identified the interaction of $1,25(OH)_2D$ with the $1,25(OH)_2D$-membrane-associated rapid response steroid-binding receptor (MARRS), which is a protein that is distinct from the VDR (19). Further evidence of $1,25(OH)_2D$ activity through MARRS signaling comes from a MARRS knockout mouse model (20). In these studies, after 5 minutes of $1,25(OH)_2D$ treatment, intestinal epithelial cells from wild-type mice increased calcium uptake, whereas $1,25(OH)_2D$-calcium uptake in cells isolated from MARRS knock-out mice was reduced. Similarly, following $1,25(OH)_2D$ treatment, the protein kinase A pathway was activated in wild-type cells, but not the MARRS knock-out cells (20). In addition to the regulation of intestinal regulation of calcium uptake $1,25(OH)_2D$-MARRS activity also functions to inhibit the growth of cancer (21,22). However, studies also demonstrate regulation of functions attributed to rapid signals in previous literature occurs in the absence of rapid signals (23). Thus, the role of rapid signaling and the MARRS in $1,25(OH)_2D$ action remains controversial.

1.1.3 Vitamin D's Role in Calcium Homeostasis and Bone Health

Vitamin D is most known for its role in bone health by moderating calcium homeostasis (4). Calcium sensing receptors in the parathyroid gland initiate an increase in parathyroid hormone (PTH) synthesis when serum calcium levels are low (30). PTH acts at the kidney to increase the synthesis of the 1α-hydroxylase, thereby increasing circulating $1,25(OH)_2D$ levels (31). In addition to the direct downregulation of VDR and the 1α-hydroxylase, $1,25(OH)_2D$ acts to decrease PTH synthesis. Reduced PTH synthesis further negatively regulates kidney 1α-hydroxylase expression (30). The negative feedback of $1,25(OH)_2D$ demonstrates the tight control of serum calcium levels and overall calcium homeostasis.

The binding of $1,25(OH)_2D$ to the VDR regulates genes involved in calcium absorption and resorption in the intestine, kidney, and bone target tissues, thereby upregulating serum calcium levels (4). At the intestine, increasing $1,25(OH)_2D$ levels act to increase transcription and expression of transient receptor potential vanilloid (TRPV) 6, a calcium channel protein located in the apical membrane of enterocytes. Additionally, $1,25(OH)_2D$ through VDR transcriptionally upregulates the intestinal calcium-binding protein calbindin-D_{9k} (32). Following the entry of calcium into the cell, calcium binds to calbindin-D_{9k} and is shuttled across the cells and exported

via the adenosine triphosphatase into circulation (4). Thus, $1,25(OH)_2D$ action at the intestine directly increases calcium absorption and calcium transport in the serum.

In the kidney increasing production of $1,25(OH)_2D$ leads to transcriptional upregulation of TRPV5 (4), a calcium channel protein located in the distal tubule which mediates calcium resorption. Upregulation of TRPV5 results in increased calcium resorption in the kidney. Similar to the intestine, $1,25(OH)_2D$ acts at the kidney to increase expression of calbindin-D_{28k} and increase calcium transport in circulation (33). Collectively, $1,25(OH)_2D$ acts at the kidney to maintain calcium homeostasis and increasing serum calcium by increasing calcium resorption and transport into the serum.

Serum calcium is regulated by $1,25(OH)_2D$ at the bone through activation of osteoclast bone resorption (34). Membrane-associated expression of the receptor activator of nuclear factor-κB ligand (RANKL) is induced by both PTH and $1,25(OH)_2D$ in osteoblasts (35). Osteoblasts expressing RANKL bind to RANK expressing osteoclasts by direct RANK/RANKL interactions which subsequently induces bone resorption activity in osteoclasts. VDR knockout mouse models demonstrate $1,25(OH)_2D$'s regulation of osteoclast bone resorption in mice through induction of RANKL in osteoblasts (36). Interestingly, $1,25(OH)_2D$ treatment-induced activity in osteoclasts from VDR knockout mice which were in contact with osteoblasts from wild-type mice, demonstrating $1,25(OH)_2D$ regulation of bone resorption requires VDR expression in osteoblasts, but not osteoclasts.

1.1.4 Extraskeletal Roles of Vitamin D

Vitamin D also regulates extraskeletal processes in the body. Specifically, VDR expression is identified in many cell types, including muscle, immune cells, brain, adipocytes, cancer cells, pancreas, skin, endocrine glands, reproductive tissues, and bronchial epithelial cells (27,37). The expression and activity of VDR present in these cell types suggest vitamin D's regulation of processes in these tissues, beyond its classically defined role in calcium homeostasis.

Vitamin D's regulation of muscle cells is indicated by the presence and activity of VDR in C2C12 myoblasts (38). The activity of VDR and the 1α-hydroxylase was verified as $1,25(OH)_2D$ and 25(OH)D treatment resulted in growth inhibition in C2C12 cells. Inhibition of the 1α-hydroxylase abolished 25(OH)D's effects and $1,25(OH)_2D$ treatment restored the growth inhibition. In addition, vitamin D deficiency and VDR knockout impaired muscle differentiation

in mice (39) and, treatment with 1,25$(OH)_2$D improved myopathy in patients with age-related bone loss (40). After a twelve-week resistance training placebo-controlled clinical trial vitamin D supplementation increased peak muscle power while no improvement was observed in the placebo group (41). Collectively, these results suggest that vitamin D acts at the muscle to regulate muscle differentiation and improve muscle function.

Immune cell regulation by vitamin D is widely characterized. Low serum 25(OH)D levels are associated with numerous immune-related diseases including psoriasis, type 1 diabetes, respiratory infection, rheumatoid arthritis, multiple sclerosis, and more recently COVID-19 (42). Additionally, treatment with nonhypercalcemic levels of 1,25$(OH)_2$D prevents the development of diabetes (43), arthritis (44), and multiple sclerosis (45) in murine models. Immune cells such as activated macrophages and T and B lymphocytes express the 1α-hydroxylase and VDR (42). While the mechanisms by which 1,25$(OH)_2$D through VDR regulates immune cell function is ongoing, a decrease in inflammatory cytokine production was observed in T lymphocytes treated with 1,25$(OH)_2$D and poses one potential mechanism (46). In sum, vitamin D is shown to beneficially regulate the immune system.

Regulation of brain cells by vitamin D is demonstrated as well. Low vitamin D status is correlated to an increased risk of developing neuronal disorders such as autistic spectrum disorder, schizophrenia, Alzheimer's disease, and depression (47). Similar to other tissues regulated by vitamin D, the presence of the 1α-hydroxylase and VDR were identified in various locations of the brain and neuronal cells, suggesting 1,25$(OH)_2$D regulation of brain processes (47). Potential mechanisms of 1,25$(OH)_2$D regulation of the brain includes regulation of reactive oxygen species (ROS) by increasing production of the intracellular antioxidant glutathione (GSH) (48) and decreased calcium uptake through downregulation of voltage-sensitive L-type calcium channels (49). Future work is required to fully understand vitamin D's role in reducing the incidence of neuronal disorders and diseases to recommend its use in these instances.

Studies demonstrate that vitamin D also regulates adipose tissue physiology. A growing body of literature suggests that vitamin D acts to inhibit the differentiation of preadipocytes. For example, treatment of 3T3-L1 adipocytes with 1,25$(OH)_2$D inhibited adipogenesis (50). Further investigation demonstrated that 1,25$(OH)_2$D inhibition of 3T3-L1 preadipocyte differentiation was through downregulation of the transcription factors peroxisome proliferator-activated receptor γ (PPARγ), and CCCAAT enhancer binding-proteins, which are involved in initiating preadipocyte

differentiation. Interestingly, the 1α-hydroxylase was detected in the adipose tissue of Wistar rats fed variable nutrient diets including calcium, vitamin D, fat, and sucrose content, and expression was not dependent on any of these dietary conditions (51). In addition, treatment of 3T3-L1 adipocytes with ^{3}H-25(OH)D resulted in the production of ^{3}H-1,25(OH)$_2$D, suggesting 1α-hydroxylase activity (51). In addition to its regulation of preadipocytes, vitamin D also exerts regulation in mature adipocyte cell biology. Chang et al. demonstrated that 1,25(OH)$_2$D treatment of differentiated 3T3-L1 adipocytes resulted in reduced fat storage, by reducing mRNA abundance of enzymes involved in lipogenesis and increasing mRNA abundance of lipolytic enzymes (52). Additionally, 1,25(OH)$_2$D treatment of 3T3-L1 adipocytes resulted in reduced synthesis of fatty acids from glucose (53). Collectively these studies suggest that adipose tissue is regulated by 1,25(OH)$_2$D and that 1,25(OH)$_2$D inhibits preadipocyte differentiation and mature adipocyte lipid accumulation.

Garland and others created a foundation for vitamin D and cancer research by investigating the incidence rate of colon cancer mortality and the annual mean daily solar radiation across the United States in 1980. They identified that the areas within the United States with the lowest solar radiation exposure had elevated rates of colon cancer (54). These results gave rise to the hypothesis that vitamin D may play a role in preventing colon cancer given that higher solar radiation increases the dermal synthesis of vitamin D. Similar results were discovered for breast cancer incidence rates across the United States (55). These observations lead to further investigation into the role of vitamin D in preventing cancer. An inverse association between vitamin D levels and cancer risk is demonstrated in the colon (56), prostate (57), breast (58), and other cancers (59). Interestingly, evidence also indicates the presence of the 1α-hydroxylase in the colon, pancreas (60), and breast (61), suggesting a mechanistic role of 1,25(OH)$_2$D in regulating cancer through local 1,25(OH)$_2$D production.

1.1.5 Vitamin D and Breast Cancer

Characterization of vitamin D's effects in breast cancer began with observational studies. Given the connection between UVB exposure and reduced breast cancer incidence (55), and that UVB exposure is associated with increased vitamin D status (62), the hypothesis that vitamin D status reduces breast cancer was posed. A systemic review and meta-analysis of observational studies report that higher 25(OH)D levels are associated with improved breast cancer survival and

decreased breast cancer progression (63), giving further support to the hypothesis that vitamin D plays a role in regulating breast cancer. The effects of vitamin D on breast cancer are characterized in a wide variety of studies, including clinical trials, animal models of breast cancer, and breast cancer cell models.

The results of clinical trials investigating vitamin D's effects on cancer incidence and progression are inconsistent. No effect of vitamin D on breast cancer incidence was demonstrated in the Women's Health Initiative (WHI) study, in which 400 IU vitamin D/day was given to participants with a follow-up of seven years (64). However, these results may be explained by the limitations of the study. First, the use of additional personal vitamin D supplementation by participants was not controlled in this study and may confound the effect of additional vitamin D supplementation. In addition, the low dose of vitamin D (400 IU/day) was chosen based on bone health recommendations from the Dietary Guidelines for Americans at the time. These recommendations were not established to prevent cancer and highlight the need to identify safe doses of vitamin D supplementation that are sufficient for cancer prevention. Further, the primary outcome of the study was benign proliferative breast disease which is noncancerous and does not encompass the steps of breast cancer progression which may be regulated by vitamin D. Indeed, analysis of vitamin D's effects on invasive breast cancer, a secondary outcome to the trial, demonstrated a preventative effect (65). These results support that vitamin D may regulate the progression of breast cancer to more advanced stages, based on reduction in invasive breast cancer observed with vitamin D supplementation.

Other clinical trials provided supportive results regarding vitamin D's prevention of breast cancer progression. For example, the protective role of vitamin D against breast cancer was studied in a clinical trial of post-menopausal women (66). This study occurred over a four-year period where participants were given 1100 IU/day vitamin D (1100 IU /day), a higher dose than that in the WHI study, where the primary endpoint was bone health. In a post-hoc analysis, excluding the first twelve months of follow-up, vitamin D reduced breast cancer incidence, a secondary outcome of the study, compared to placebo controls. Further analysis of the WHI randomized controlled trial to stratify results by levels of personal supplementation (67). Among women who did not take a personal supplement, vitamin D reduced the risk of breast cancer, giving strong support to the anticancer effects of vitamin D, but not in those who consumed vitamin D beyond the trial levels. These results demonstrate the importance of considering the status of the participants in assessing

the effects of nutrients on health, including cancer. Similar results to the WHI are also noted in the Vitamin and Omega-3 Trial (VITAL), where 2000 IU vitamin D/day was administered to participants (68). After follow-up for five years, there was no effect of vitamin D on breast cancer incidence compared to placebo. However, when the first two years of follow-up were excluded, the vitamin D group had significantly decreased mortality from breast cancer, suggesting vitamin D inhibition of cancer progression. Collectively, these studies demonstrate vitamin D's preventative role against breast cancer progression.

Vitamin D's preventative role in breast cancer is supported by *in vivo* studies (69). For example, rats fed a low vitamin D diet (0.05 IU/kcal) had more mammary tumor formation compared to rats on a sufficient vitamin D diet (0.5 IU/kcal) after treatment with the carcinogen, 7,12-dimethylbensanthracene (70). In addition, VDR deletion in mammary tumors resulted in increased tumor formation in *in vivo* models of breast cancer, suggesting a role of $1,25(OH)_2D$ action through VDR in reducing breast tumorigenesis (71,72). Further, ablation of the 1α-hydroxylase in mouse mammary tumor virus-polyoma middle tumor-antigen *in vivo* model of breast cancer reduced $1,25(OH)_2D$ levels in primary tumors and increased tumorigenesis (73). Collectively, these studies, among others, support that vitamin D through local tumor production of its metabolite $1,25(OH)_2D$ and VDR inhibits tumorigenesis.

Vitamin D's anticancer role is also demonstrated in mechanistic *in vitro* studies through the use of its most active form, $1,25(OH)_2D$. Treatment of MCF7 breast cancer cells with $1,25(OH)_2D$ inhibited cell growth in culture through inhibition of proliferation and induction of apoptosis (74-76). Cell cycle arrest in the G1 phase was significantly increased in $1,25(OH)_2D$ treated cells relative to control, through increased accumulation of hypophosphorylated retinoblastoma (Rb) protein (74). Rb is a tumor suppressor, and when hypophosphorylated acts to inhibit the cell cycle and proliferation (77). Jensen et al. also observed G cell cycle arrest when MCF7 breast cancer cells were treated with $1,25(OH)_2D$ (75). Again, hypophosphorylated Rb was identified in addition to suppressed expression of E2 factor family of transcription factors which act to increase cyclin A protein expression, a regulator of multiple steps in the cell cycle (75). Increased apoptosis was also observed in MCF7 cells treated with $1,25(OH)_2D$ via downregulation of the antiapoptotic protein, Bcl-2 (74). Interestingly, studies suggest that $1,25(OH)_2D$'s effects to reduce proliferation and enhance apoptosis in breast cancer cells may be through inhibition of the pro-survival Ras/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated

kinase (ERK) signaling pathway (76). Treatment of MCF7 cells with 1,25$(OH)_2$D resulted in decreased expression of RAS, and downstream phosphorylation of MEK and ERK proteins (76). Furthermore, overexpression of ras was sufficient to overcome 1,25$(OH)_2$D's suppression of proliferation and initiation of apoptosis. In addition to vitamin D's regulation of the cell cycle and apoptosis, other cancer-promoting processes have also been recognized. Vitamin D is shown to negatively regulate angiogenesis (78), energy metabolism (79), and oxidative stress protection in cancer cells (80,81), all of which may contribute to vitamin D's inhibition of breast cancer tumorigenesis. In sum, vitamin D mediates its anticancer effects through its bioactive metabolite, 1,25$(OH)_2$D, through a variety of mechanisms in breast cancer cells.

As we discover mechanisms by which breast cancer progresses to metastatic disease, discoveries of vitamin D's negative regulation may likely be applied to develop targeted recommendations for vitamin D or therapeutics. Additionally, vitamin D's effects on late-stage cancer is an ongoing source of research given the protective effects of vitamin D against breast cancer mortality demonstrated in clinical and preclinical studies (65,68).

1.2 Breast Cancer Metastasis

1.2.1 The Metastatic Cascade

In the United States, breast cancer is the most commonly diagnosed cancer in women, with over 280,000 new cases estimated for 2021 (82). Additionally, breast cancer is the second deadliest cancer for women, with the majority of cancer-related deaths caused by metastatic disease (83). Given these statistics, understanding the metastatic process is of utmost importance to prevent and treat metastatic breast cancer. Metastasis of cancer cells from the primary breast tumor to a secondary site follows a multistep process where each step poses new challenges that cancer cells must overcome to proceed to the following metastatic steps (84). The steps include escape from the primary tumor, invasion of the surrounding tissue, intravasation into circulation, systemic dissemination, extravasation into the secondary site, dormancy, and colonization (Figure 1.3). Greater understanding of the cellular requirements of each metastatic step will give direction for antimetastatic treatments.

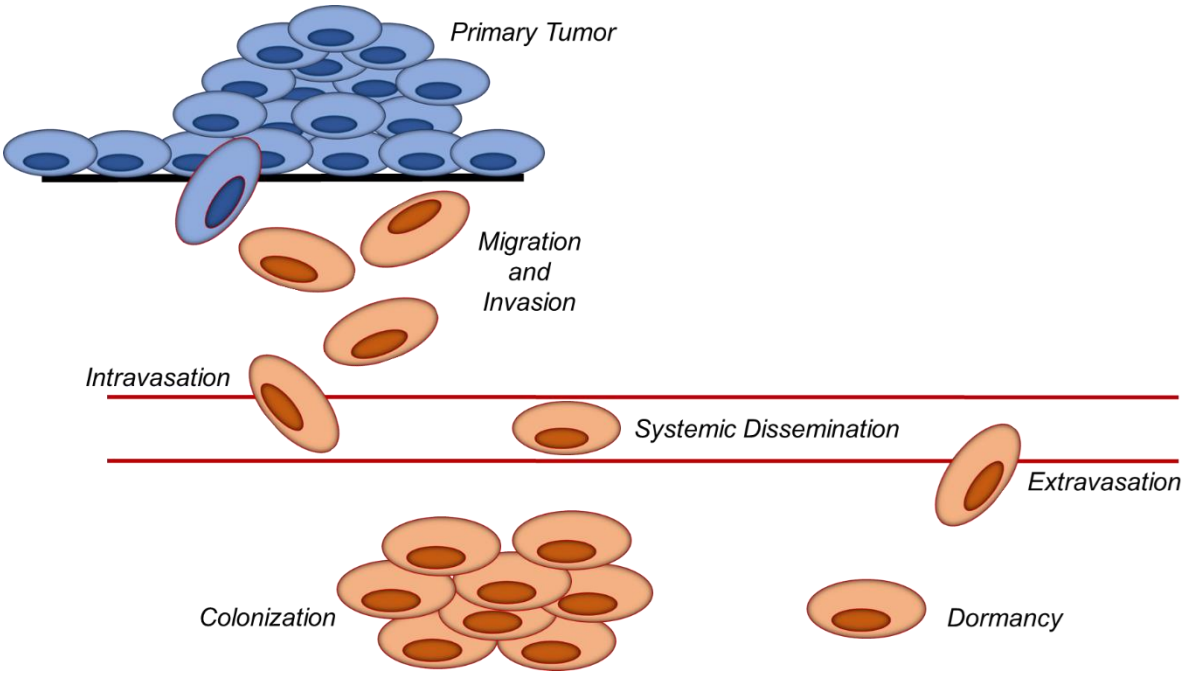

Figure 1.3 Metastatic Progression.

Metastasis from the primary tumor to a secondary site is a multi-step process. The steps include migration and invasion into the surrounding tissue, intravasation, systemic dissemination, extravasation, dormancy, and colonization to form a metastatic tumor.

In order to metastasize, cancer cells escape from the primary tumor by becoming mobile and detaching from the tumor and the extracellular matrix (ECM) at the primary tumor site (85). Normal epithelial cells will undergo anoikis, a programmed cell death, upon detachment from the ECM. However, cancer cells that achieve anchorage independence are resistant to anoikis, as they no longer require attachment to a matrix for continued survival.

Integrins, which mediate cell adhesion to ECM are a complex framework in epithelial cells which regulate cell processes, including survival and energy metabolism based on matrix connections (86). Because of their role in ECM attachment, integrins are involved in initiating the intracellular anoikis signaling cascade upon detachment. Anoikis prevents the survival and growth of epithelial cells in foreign environments in the body. Upon detachment from ECM, cells initiate anoikis through a reduction of integrin signaling through survival pathways and induction of cell death signaling (87). Specifically, integrins reduce signaling through focal adhesion kinase (FAK) and integrin-linked kinase (ILK) when cells become ECM detached. FAK and ILK maintain

signaling through mitogen activating protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) cell survival pathways (87), thus reduction in signaling through these pathways enhances cell death.

To acquire anoikis resistance, cancer cells alter their response to the loss of adhesion by dysregulating integrins and constitutively activating pro-survival signaling pathways (87). Oncogenic mutations obtained by cancer cells allow for permanent activation of growth and survival signaling pathways and overrides integrin-mediated anoikis signaling. Additionally, dysregulation of integrins is demonstrated to confer anoikis resistance. For example, in intestinal carcinoma cells, the downregulation of αvβ3 integrin mediates anoikis resistance in cells grown in suspension, by reducing pro-apoptosis signaling which is initiated when αvβ3 integrin disengages from the ECM (88). Additionally, overexpression of the *ras* oncogene, which is present in 25% of all cancers, in detached intestinal epithelial cells inhibited anoikis by activation of the pro-survival pathway PI3K (89,90). Thus, anchorage independence drives metastasis by resisting anoikis and increasing the survival of cancer cells when they become detached from the ECM.

Following detachment from the primary tumor, metastasizing cancer cells migrate and invade into the tissue surrounding the primary tumor site. Migration involves the alteration of adhesion protein expression involved in cell-matrix and cell-cell connections in tumor cells. For example, loss of the cell adhesion protein, E-cadherin, is associated with increased migratory capability in cancer cells (91). Migration and invasion take place in single cancer cells following transformation to a mesenchymal phenotype, or cancer cells utilize amoeboid cell invasion where mesenchymal and epithelial-like cancer cells collectively migrate (85). In either single or collective invasion of tumor cells, the mesenchymal phenotype drives mobility. This movement is achieved through at least five steps: pseudopod protrusion, contact at focal adhesions, proteolytic breakdown of the matrix by the secretion of matrix metalloproteinases (MMPs), actomyosin contraction, and detachment (85,92). Thus, transformation to a mesenchymal phenotype is required for cancer cells to become invasive.

The epithelial to mesenchymal transition (EMT) drives metastasis by facilitating transformation to the mesenchymal phenotype required for invasion. Mesenchymal characteristics are achieved by repression of epithelial genes and increased expression of mesenchymal genes by transcription factors such as Snail1/2, Twist1, and Zeb1/2 (85). In addition, cancer cells induce EMT as a response to transforming growth factor β (TGFβ), which activates Wnt and Notch cell signaling pathways (93). Wnt and Notch signaling induces a mesenchymal stem-like phenotype

observed in invasive cancer cells. Additionally, a common biomarker for EMT is the decreased expression of epithelial cadherins, which mediate cell-cell adhesion (91). Further, cancer cells that have undergone EMT also resist anoikis, as similar factors which induce EMT also mediate anoikis resistance, such as β-catenin accumulation in the cytosol as a result of decreased expression of E-cadherin (87). For example, β-catenin overexpression mediates anoikis resistance and maintained ECM detached cell growth in MDCK cells (94). As cancer cells undergo EMT they lose adhesion to the matrix, acquire migratory and invasive capabilities, and are resistant to anoikis and each of these characteristics drives metastasis.

Following invasion, tumor cells may undergo systemic dissemination by transiting through the endothelial or lymphatic vessel barrier, termed intravasation, to enter circulation (84). The ability to enter circulation is often accompanied by vascularization of the primary tumor. Tumor cells secrete vascular endothelial growth factors (VEGFs) which stimulate the vasculature to grow within the primary tumor environment to supply the area of growing cells with oxygen and nutrients (95). Interestingly, an invasive phenotype is sufficient for passage through the endothelial cell barrier. For example, *in vivo* breast cancer cells overexpressing TGFβ were able to enter the blood whereas wild-type cells were not (96). Additionally, circulating tumor cells must survive multiple stressors to arrive at a secondary site. For instance, damage from hemodynamic shear stress is acquired by circulating tumor cells (95). In addition, detachment from ECM while in circulation triggers anoikis, so anoikis resistance likely improves survival in circulation. However, it is unlikely that cancer cells remain in circulation longer than a few minutes, due to the large size of cancer cells (20-30 µm) and the relatively narrow diameters of luminal capillaries (~8 µm) in which cancer cells become trapped, potentially before anoikis can occur (95). Extravasation, or passage through the endothelial or lymphatic vessel barrier into a secondary site, occurs after the circulating tumor cells become lodged in the vasculature at the metastatic site. Extravasation is achieved by the utilization of characteristics acquired in previous steps such as anchorage independence, migration, and invasion. *In vivo* analysis of circulating breast cancer cells demonstrated that disruption of endothelial cell-cell junctions by expression of MMP1 and MMP2 is a requirement for metastatic extravasation into the lung (97). Therefore, cancer cells passage through, travel in, and exit from the vascular system to metastasize.

Finally, the cancer cells complete the process of metastasis by invading the tissue of the secondary site and forming a metastatic tumor (85). Prior to the formation of a metastatic tumor,

cancer cell dormancy is often observed (84). Dormancy occurs when cancer cells remain viable but fail to establish a secondary tumor after metastasis and is attributed to the challenges of establishing a tumor in a secondary environment. One challenge is the differences in ECM composition between primary and secondary tumor sites and overcoming these differences permits proliferation and formation of a secondary tumor. For example, Barkan et al. show that metastatic breast carcinoma cells utilize integrin β1 for cell signaling and transition from a quiescent to a proliferative state (98), demonstrating that metastatic cancer cells alter ECM signaling to adapt to the secondary tumor microenvironment. Another challenge in metastatic tumor formation is the tissue specificity of nutrient availability. Metastatic cancer cells must gain metabolic plasticity to utilize nutrients with different availabilities than were experienced in the primary site. For example, breast cancer metastasis to the liver required preferential utilization of pyruvate to lactate, whereas breast to bone and lung metastasis required pyruvate metabolism through the tricarboxylic acid (TCA) cycle (99). These results suggest that metabolic plasticity acquired by differential expression of pyruvate metabolizing genes may support preferential site-specific metastasis. Collectively, cancer cells must adapt to a foreign environment to form a metastatic tumor.

Anchorage Independence Precedes Metastasis

1.2.2 Altered Energy Metabolism in Anchorage Independent Cancer Cells

Energy crisis occurs when normal epithelial cells become detached from ECM as they respond by decreasing glycolysis, pentose phosphate pathway, and oxidative phosphorylation (86). Subsequently, there is decreased adenosine triphosphate (ATP) and nicotinamide dinucleotide phosphate (NADPH) produced as well as decreased fatty acid oxidation (FAO) with increased levels of ROS. This same effect persists in cancer cells, requiring that they reroute metabolism to overcome the ECM detached changes and gain anchorage independence. For example, a study by De Luca et al. confirmed that mitochondrial biosynthesis and function were required for ECM detached survival of MCF7 breast cancer cells (100). Thus, metabolic adaptation to ECM detachment promotes an anchorage independence phenotype and drives metastasis.

Rerouting glucose metabolism is one method that cancer cells overcome the suppression of metabolism in detachment. Upon detachment, nonmalignant human breast epithelial MCF10A cells decreased glucose uptake and glucose flux into glycolysis, the pentose phosphate pathway, and the TCA cycle (101). However, overexpression of the oncogene *ErbB2* rescued flux through

these pathways and consequently improved ATP levels (101,102). Researchers specifically identified that *ErbB2* overexpression-maintained activation of the ERK signaling pathway and ERK signaling suppressed pyruvate dehydrogenase (PDH) kinase 4 (PDK4). PDK4 action reduces the activity of the anaplerotic enzyme PDH. The ERK-dependent suppression of PDK4 allowed PDH activity to proceed and rescued the metabolism of the detached *ErbB2* overexpressing cells. ERK regulation of anchorage-independent metabolism was also identified in other cancer models (102-104), suggesting that detached cancer cells maintain ERK signaling to restore energy metabolism.

Interestingly, like ERK, other signaling pathways which are commonly activated in cancer may be utilized by ECM detached cancer cells to overcome glucose metabolism inhibition. For example, FAK overexpression is common in cancer and has been implicated with maintaining glycolysis in pancreatic ductal adenocarcinoma cell lines (105). FAK overexpression induced glycolysis as measured by increased lactate production and increased expression of enolase, pyruvate kinase M2, and lactate dehydrogenase (LDH) A glycolytic enzymes, as well as increased expression of the membrane transporter for pyruvate and lactate, monocarboxylate transporter. In addition, FAK promoted oxidative phosphorylation suggesting that overexpression of FAK could improve cancer cell survival when detached from ECM by increasing glucose metabolism and energy production. FAK associates with integrins and growth factor receptors to stimulate pro-survival signaling pathways in cells and FAK overexpression promotes anoikis resistance in cancer cell models (106,107). In addition to FAK, SRC is implicated in increasing glycolysis to improve survival in detached ovarian cancer cells (103). These results demonstrate cancer cell's ability through oncogenic transformation to utilize pro-survival signaling pathways to resist anoikis and rescue glucose metabolism in detached conditions.

In addition to glucose metabolism, matrix detached cancer cells also alter the metabolism of pyruvate and glutamine. For example, highly invasive ovarian cancer cells increased pyruvate and glutamine uptake and subsequently increased oxygen consumption and ATP production in detached conditions (108). Additionally, nonmalignant MCF10A cells decreased pyruvate and glutamine uptake, while *ErbB2* overexpressing cells rescued pyruvate and glutamine uptake in detached conditions (101). The rescued glutamine uptake in the detached *ErbB2* overexpressing cells was associated with increased glutamine flux into the TCA cycle relative to detached MCF10A cells. In HepG2 liver carcinoma cells, a preference for glutamine metabolism over

glucose metabolism in detached conditions was observed (109). Detached HepG2 cells increased expression of the glutamine transporter solute carrier family 1A member 5 (SLC1A5) in an adenosine monophosphate-activated protein kinase (AMPK) dependent manner, and this subsequently enhanced ATP levels (109). Silencing of AMPK by shRNA which targeted the AMPK transcript prevented the increase in SLC1A5 expression and ATP levels in detached cancer cells, and supplementation with a cell-permeable analog of the downstream glutamine metabolite, α-ketoglutarate (αKG), rescued ATP levels (109). These studies demonstrate that pyruvate or glutamine may be used by cancer cells as alternate energy sources to glucose in ECM detachment.

Multiple studies have confirmed a drastic increase in ROS production in detached cells (104,110,111). Increased ROS production often results in anoikis if not properly managed by cancer cells. To combat the accumulation of cytotoxic levels of ROS, cancer cells must maintain adequate reductive-oxidative (redox) balance through the synthesis of reducing equivalents such as NADPH. NADPH functions to regenerate the primary cellular antioxidant, GSH, from its oxidized form, GSSG (112). Metabolism of both glucose and glutamine lead to increased NADPH synthesis. Cancer cells can maintain redox balance by increased synthesis of GSH and NADPH or increasing expression of ROS neutralizing enzymes such as superoxide dismutase 2 (SOD2) or catalase.

Multiple cancer models identified increased expression of SOD2 and catalase in anoikis resistant cells to combat the drastic increase in ROS following detachment. For example, detachment of T47D and MDA-MB-231 breast cancer cells induced increased expression of catalase and SOD2 (110). Increasing expression of these enzymes was associated with increased energy production from cellular metabolism, as their overexpression in non-malignant MCF10A cells rescued ATP levels and survival in detachment. Further, silencing of catalase in MDA-MB-231 cells resulted in decreased anchorage-independent survival and reduced ATP production (110). These effects were reversed with the addition of the antioxidant Trolox, a water-soluble vitamin E analog, suggesting that antioxidant defense is required to maintain energy production and survival in detached conditions. Similarly, in the MCF10A non-transformed breast epithelial cells, SOD2 expression was required for detached cells to oxidize glucose and survive in detached conditions (113). Therefore, cancer cells increase SOD2 and catalase ROS neutralizing enzymes to mitigate oxidative stress in detached conditions.

The energy sensor and regulator AMPK coordinates increased energy metabolism with mitigation of subsequent elevated oxidative stress in detached cancer models. In HepG2 liver carcinoma cells, AMPK was activated following detachment and an increase in the antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) was observed (109). The Nrf2 increase was AMPK dependent as depletion of AMPK prevented the detachment-induced increase in Nrf2 and increased ROS levels. Collectively, these results suggest that AMPK signaling increases energy metabolism of detached cancer cells through increased expression of the oxidative stress protection transcription factor Nrf2.

FAO indirectly produces NADPH by providing substrates for NADPH producing metabolic reactions (114). Hence, FAO may be used to increase the synthesis of NADPH in cells by providing substrate for enzymes that produce NADPH, however, FAO also produces oxidative stress as energy production in the mitochondria is a major source of ROS. FAO may contribute to both sides of redox balance in cancer cells which ultimately may determine the cancer cell's ability to survive ECM detachment. In a cell model of colorectal cancer increased expression of carnitine palmitoyltransferase A1 (CPT1a), an enzyme involved in FAO was observed (115). CPT1a driven FAO was observed to confer anoikis resistance, demonstrated when increased anoikis resulted from CPT1a knockdown. Increased ROS levels followed CPT1a knockdown, and antioxidant treatment rescued CPT1a knockdown cells from anoikis. In contrast, antioxidant treatment of *ErbB2* overexpressing breast cancer cells rescued ATP levels by permitting FAO, suggesting that in this cancer model FAO increased ROS (102). These results demonstrate the interconnection between FAO and redox balance in detached cancer cells as they require sufficient redox balance to permit FAO for energy metabolism.

Due to the drastic change in energy metabolism and increased ROS production following ECM detachment, a cancer cell's ability to mitigate these effects are imperative to proceed through the steps of metastasis. Collectively these results demonstrate that cancer cells overcome inhibition of energy metabolism by rescuing metabolism of glucose, glutamine, pyruvate, and lipids and increase oxidative stress protection to achieve anchorage independence. Identification and targeting of these metabolic adaptations may reverse anchorage independence and lead to improved cancer outcomes.

1.2.3 Vitamin D Regulation of Breast Cancer Metastasis

Although a higher vitamin D status, determined from serum 25(OH)D levels, is associated with reduced odds of developing breast cancer (116), vitamin D inhibition of metastasis is also suggested. For example, a meta-analysis investigating serum 25(OH)D levels and breast cancer fatality rates, identified that patients with higher circulating 25(OH)D levels had a 50% lower fatality rate than patients with lower 25(OH)D levels (117). Similarly, higher serum 1,25$(OH)_2$D levels were associated with earlier stages of breast cancer, while patients with breast to bone metastasis had lower 1,25$(OH)_2$D levels (118). In the recent VITAL trial, while vitamin D did not affect cancer incidence, supplementation with vitamin D resulted in a 25% reduction in cancer mortality with the exclusion of the first five years of follow-up (68). Because the majority of cancer deaths result from metastasis (83), these studies suggest that vitamin D reduces death from breast cancer through inhibition of metastasis.

Vitamin D's preventative role in metastasis is also shown in animal and cell models. Williams et al. maintained BALB/c mice on a vitamin D sufficient diet (500 IU vitamin D/kg) to investigate vitamin D and VDR's role in breast cancer metastatic progression (119). In this experiment, researchers seeded 168FARN murine breast cancer cells with and without VDR knockdown into the mammary fat pad of mice to set up primary mammary tumors. Animals with VDR knockdown tumors had larger primary tumors and established secondary tumors in the liver. Similar results were demonstrated in another *in vivo* breast cancer model where VDR knockdown promoted breast to bone metastasis (120), thus displaying that vitamin D prevents breast cancer metastatic progression through VDR. Further, BALB/c mice fed a vitamin D depleted diet had increased breast to bone metastasis when compared to mice on a vitamin D sufficient diet (2000 IU/kg) (121). Wilmanski et al. utilized an *in vitro* cell model representing breast to bone metastasis to analyze 1,25$(OH)_2$D's regulation of metastasis (122). Metastatic MCF10CA1a breast cancer cells treated with 1,25$(OH)_2$D had reduced metastatic cell viability compared to vehicle-treated cells. Taken together these data suggest that vitamin D acts to prevent breast cancer metastasis, however, the mechanism is currently under investigation.

1.3 Vitamin D Regulation of Cancer Cell Metabolism

1.3.1 Introduction

Throughout the process of tumorigenesis and metastasis cancer cells are exposed to environments with varying nutrient statuses. For example, tumor growth often results in outgrowing the vasculature which then limits access to nutrients delivered from circulation to affected areas of the tumor. This exposes cancer cells to low oxygen and nutrient availability thereby changing their metabolism. Many cancer cells won't survive this metabolic stress, however, the cells that survive are observed to have a flexible metabolism or metabolic plasticity. Additionally, as cancer progresses, and cancer cells become more invasive and mobile they are exposed to new environments in the body which maintain different concentrations of nutrients utilized by cancer cells. For example, pyruvate levels are 3-fold higher in lung interstitial fluid compared to blood plasma levels (123). Thus, cancer cells that have phenotypes that facilitate metabolic plasticity are better equipped to survive in environments with a wider range of nutrient statuses.

Given the crucial role of metabolic plasticity in cancer progression, it is proposed that vitamin D may exert its regulation of cancer progression through the regulation of energy metabolism. Vitamin D treatment of cancer cells is observed to regulate the metabolism of glucose and glutamine, the two most consumed nutrients in cancers. Through regulation of these energy metabolizing pathways the cancer cells are then less likely to survive in nutrient-depleted environments and their progression through the stages of carcinogenesis is impaired.

1.3.2 Vitamin D Regulation of Glucose Metabolism

Altered glucose energy metabolism is a common characteristic in cancer cells (124). Otto Warburg was the first to observe that cancer cells have altered glucose metabolism (125), where cancer cells increased glucose uptake, glycolysis, and decreased glucose metabolism via oxidative phosphorylation even in the presence of oxygen (126), termed the "Warburg effect". Although not all cancer cells display the Warburg effect, most cancer cells have altered glucose metabolism to support growth, including energy metabolism and anabolic reactions (127). For example, increased flux of glucose through glycolysis increases the production of carbon-based intermediates which may be used for biosynthetic reactions to support growth, as well as

production of lactate which may be an important fuel source for cancer-associated fibroblasts or cancer cells in select contexts (128,129). Additionally, glucose provides substrates for the pentose phosphate pathway which results in increased synthesis of nucleic acids and reducing equivalents through NADPH production, both of which are required to sustain the rapid cell proliferation observed in cancer (84). Current therapies targeting glucose metabolism, while promising as anti-cancer agents, pose serious concerns for their off-target effects demonstrated in clinical trials (130). Given the prevalence of altered glucose metabolism in cancer and its critical contribution to cell proliferation, identification of agents that target this characteristic with minimal effects on benign tissue, such as vitamin D, may provide a promising strategy for preventing cancer and its progression.

One potential mechanism by which vitamin D may inhibit cancer progression is through inhibition or reversal of altered glucose metabolism, including reducing glucose uptake into cancer cells. Glucose enters cells via different isoforms of the glucose transporter (GLUT1-7), and increased expression of GLUT1 in breast cancer is associated with a worse prognosis (131). In agreement with this, GLUT1 expression is associated with increased invasiveness in various human breast cancer cell lines, while expression of GLUT2-5 is not associated with invasive capabilities (132). Treatment with 1,25$(OH)_2$D decreased GLUT1 mRNA and protein expression in cell models of prostate cancer (133). Additionally, 1,25$(OH)_2$D decreased GLUT1 mRNA and protein expression and glucose uptake in MCF7 and MDA-MB-231 breast cancer cells (134,135). However, the impact of 1,25$(OH)_2$D on GLUT1 expression may be dependent on stage or type of cancer, as 1,25$(OH)_2$D treatment decreased glucose uptake without affecting GLUT1 mRNA levels in MCF10A-*ras* cells, a model of early-stage breast cancer (79). Further, 1-methyl-1-nitrosourea-induced breast cancer in Sprague-Dawley rats, supplementation with vitamin D or the vitamin D analog seocalcitol reduced glucose uptake in tumor tissue, measured using flouro-2-deoxy-glucose (136). Taken together, these results suggest that 1,25$(OH)_2$D suppresses glucose uptake in models of prostate and breast cancer.

In addition to glucose uptake, 1,25$(OH)_2$D also regulates glycolysis in *in vitro* cancer models, although the degree and direction of regulation varies between models. For example, 1,25$(OH)_2$D decreased protein levels of hexokinase 2 (HK2), the enzyme which mediates the first step of glycolysis, in MCF7 breast cancer cells, but increased HK2 protein expression in MDA-MB-231 cells (134). Treatment with 1,25$(OH)_2$D suppressed mRNA levels of *HK2* in both cell

lines (135). In addition, 1,25$(OH)_2$D decreased expression of LDHA, the enzyme that reduces pyruvate to lactate, in breast cancer and prostate cancer cells (133,135). In non-metastatic breast cancer cells, 1,25$(OH)_2$D did not affect mRNA expression of the glycolytic enzymes *HK2* and phosphoglycerate kinase 1, but decreased glycolysis, lactate production, and flux of universally labeled $^{13}C_6$-glucose into 3-phosphoglycerate, pyruvate, and lactate (79). Additionally, 1,25$(OH)_2$D suppressed glycolysis as measured by a biosensor chip system, which uses extracellular acidification as an indicator of glycolysis (133). Decreased acidification was detected over time with 1,25$(OH)_2$D treatment to a greater extent in less progressed cell lines versus highly metastatic cells, suggesting decreased glycolysis (133). In contrast, 1,25$(OH)_2$D treatment did not affect lactate production in MCF7 cells, while a high 1 µM dose of 1,25$(OH)_2$D reduced lactate production in the MDA-MB-231 cells (135). Together, these results demonstrate that 1,25$(OH)_2$D targets glucose metabolism via inhibition of glycolysis; however, the extent of this regulation may depend on the cancer model.

In addition to glycolysis, 1,25$(OH)_2$D is also proposed to regulate the flux of glucose into the TCA cycle (Figure 1.4). Pyruvate, the end-product of glycolysis, enters the TCA cycle as acetyl-CoA through the activity of PDH and oxaloacetate via pyruvate carboxylase (PC) activity. Treatment with 1,25$(OH)_2$D decreased mRNA abundance and protein expression of the negative regulator of PDH, PDH kinase 1 (PDK1), in various stages of prostate cancer (133), suggesting that 1,25$(OH)_2$D may promote flux of pyruvate into the TCA cycle. Similarly, 1,25$(OH)_2$D treatment reduced PDH activity although, in contrast, had no effect on PDK1 mRNA expression in MCF10A-*ras* transfected breast cancer cells (79). Treatment with 1,25$(OH)_2$D reduced PC expression in MCF10A-*ras* transfected breast cancer cells, through direct regulation of a VDRE in the promoter of the *PC* gene (80,137). In agreement with these results, 1,25$(OH)_2$D reduced flux from $^{13}C_6$-glucose to acetyl-CoA and oxaloacetate, and decreased the pool size of succinate, a TCA cycle intermediate, demonstrating that 1,25$(OH)_2$D reduces glucose incorporation into the TCA cycle (79). Following pyruvate entry into the TCA cycle, acetyl-CoA and oxaloacetate condense into citrate. Replenishing the citrate pool provides substrate for energy metabolism through the production of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) in the TCA cycle, as well as fuels fatty acid synthesis, both of which are utilized by cancer cells. MCF7 breast cancer cells had reduced levels of citrate and downstream TCA cycle intermediates when treated with 1,25$(OH)_2$D, while 1,25$(OH)_2$D-treated MDA-MB-

231 breast cancer cells had increased citrate levels (134). Further, 1,25(OH)$_2$D treatment of prostate cancer cells decreased mRNA expression of the TCA cycle enzyme isocitrate dehydrogenase 1 (IDH1), which converts isocitrate to αKG, (133), a metabolite which is pivotal in supporting both oxidative and reductive metabolism in cancer (138). Collectively, these results suggest 1,25(OH)$_2$D treatment decreases glucose utilization in the TCA cycle in cancer cells.

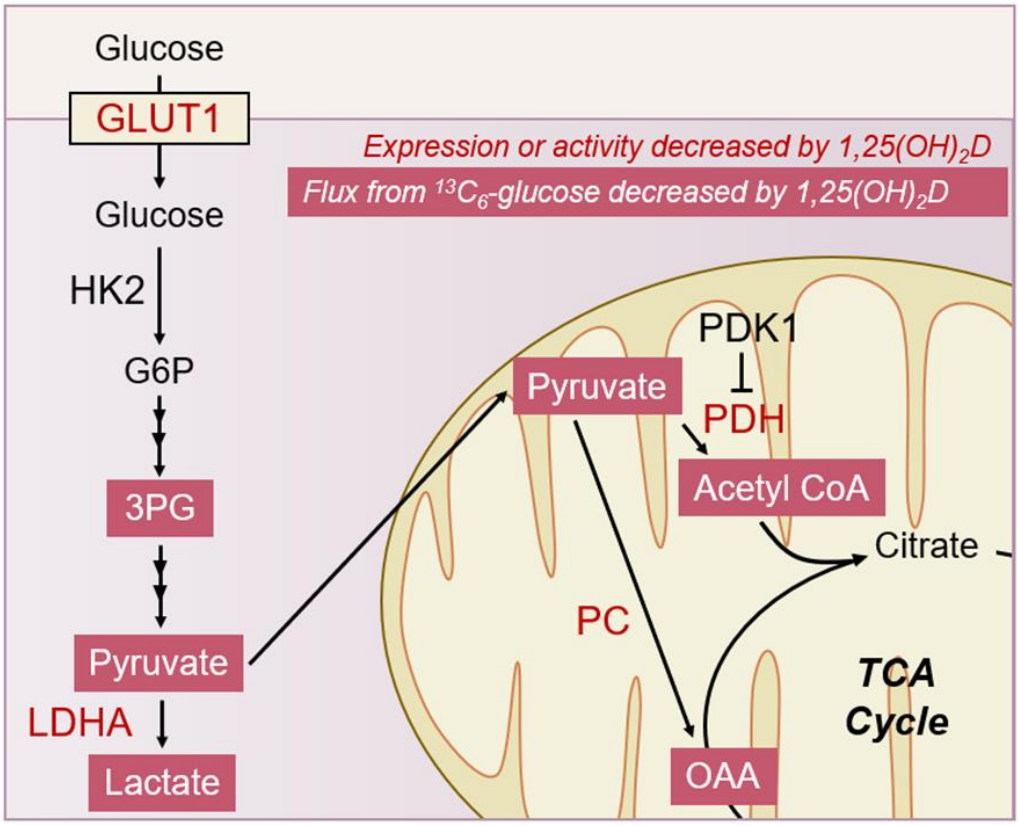

Figure 1.4 Decreased glucose metabolism mediated by 1,25(OH)$_2$D.

An overall reduction in glycolysis and glucose entry into the TCA cycle is observed in cancer cells treated with the active metabolite of vitamin D, 1,25(OH)$_2$D. Treatment of cancer cells *in vitro* with 1,25(OH)$_2$D results in reduced enzyme expression or activity (red font) and U-$^{13}C_6$-glucose flux to metabolites (red shaded boxes).

Oxidative phosphorylation is used by cells to produce ATP from the NADH and FADH$_2$ generated in the TCA cycle. In the context of cancer, oxidative phosphorylation is utilized in oxygenated environments including breast cancer and Hodgkin lymphoma (139-141). Evidence suggests that the effects of vitamin D on oxidative phosphorylation may also have differential

effects depending on stage and type of cancer. For example, 1,25$(OH)_2$D decreased oxygen consumption rate (OCR), a measurement of oxidative phosphorylation in both non-metastatic and metastatic prostate cancer cell lines, although the metastatic cells were more resistant to 1,25$(OH)_2$D-mediated decreases compared to non-metastatic cells (133). However, the opposite effect was observed in breast cancer cells, as 1,25$(OH)_2$D had no effect on OCR in non-metastatic cells, but decreased OCR of metastatic cells (134). Further investigation into understanding the conditions that influence the regulation of oxidative phosphorylation by 1,25$(OH)_2$D is necessary to develop strategies for use of vitamin D as a means of cancer prevention or treatment through targeting oxidative phosphorylation.

With increasing evidence of vitamin D's negative regulation of glucose metabolism, investigations have focused on elucidating the mechanism underlying this effect. Evidence shows that 1,25$(OH)_2$D increases the activation of AMPK in breast cancer cells (134,135). AMPK activity is regulated by the energy status of the cell and is increased in energy-deplete conditions. Phosphorylation targets of AMPK are proteins involved in increasing the bioenergetic status of the cell (142). For example, one AMPK target is the mammalian target of rapamycin (mTOR) pathway where AMPK activity results in inhibition of mTOR activity through direct and indirect mechanisms, thus suppressing anabolic reactions and conserving energy in the cell (143). Indeed, 1,25$(OH)_2$D treatment in breast cancer cells increased levels of the active form of AMPK, and thereby decreased levels of the active form of mTOR (135). In addition, in an *in vitro* colorectal cancer model, 1,25$(OH)_2$D treatment reduced glycolysis and lactate production through induction of the long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3) (144). Further, MEG3 and 25(OH)D serum levels were positively correlated in cancer patients, suggesting that 1,25$(OH)_2$D may increase expression of MEG3, which then may exert its effects to suppress glucose metabolism. Thus, regulation of glycolysis by 1,25$(OH)_2$D may be through regulation of key players in energy metabolism such as AMPK/mTOR signaling or by increasing expression of MEG3 lncRNA.

Collectively, evidence suggests that 1,25$(OH)_2$D regulates glycolysis, the TCA cycle, and oxidative phosphorylation, with differential responses that may be dependent on the cancer type and stage of progression. In some types of cancer, including breast and prostate cancer, regulation of glucose metabolism by 1,25$(OH)_2$D is most prominent in non-metastatic cancer cells with lesser effects in metastatic cells (133,134), suggesting that 1,25$(OH)_2$D may play an important role in

regulating glucose metabolism in early-stage tumors. However, models of breast cancer show that 1,25$(OH)_2$D treatment suppresses oxidative phosphorylation to a greater extent in metastatic cells with less regulation in the TCA cycle (134). This is particularly interesting as the TCA cycle synthesizes the substrates used in oxidative phosphorylation. Further investigation into this differential response may lead to better application of vitamin D as a chemopreventive agent in the treatment of metastatic cancer. In addition, future research addressing the effect of 1,25$(OH)_2$D-mediated changes in glucose metabolism as it relates to cellular processes associated with metastatic progression is needed to provide mechanistic links between 1,25$(OH)_2$D treatment, alterations in glucose metabolism, and metastasis.

1.3.3 Vitamin D Regulation of Glutamine Metabolism

Increased dependence on glutamine metabolism is commonly observed in cancer cells and in certain circumstances is required for cancer cell survival (145,146). Tumor cells use glutamine for several purposes including energy production via entry of carbon into the TCA cycle (Figure 1.5), oxidative stress protection through the production of the antioxidant GSH, as well as the synthesis of non-essential amino acids such as alanine, serine, aspartate, and glycine (146). Given the importance of glutamine metabolism for cell proliferation, identifying strategies to inhibit glutamine metabolism to suppress tumor growth is a promising area of research. Interestingly, 1,25$(OH)_2$D treatment is demonstrated to regulate glutamine metabolism in *in vitro* models of breast cancer through several mechanisms (133,147,148).

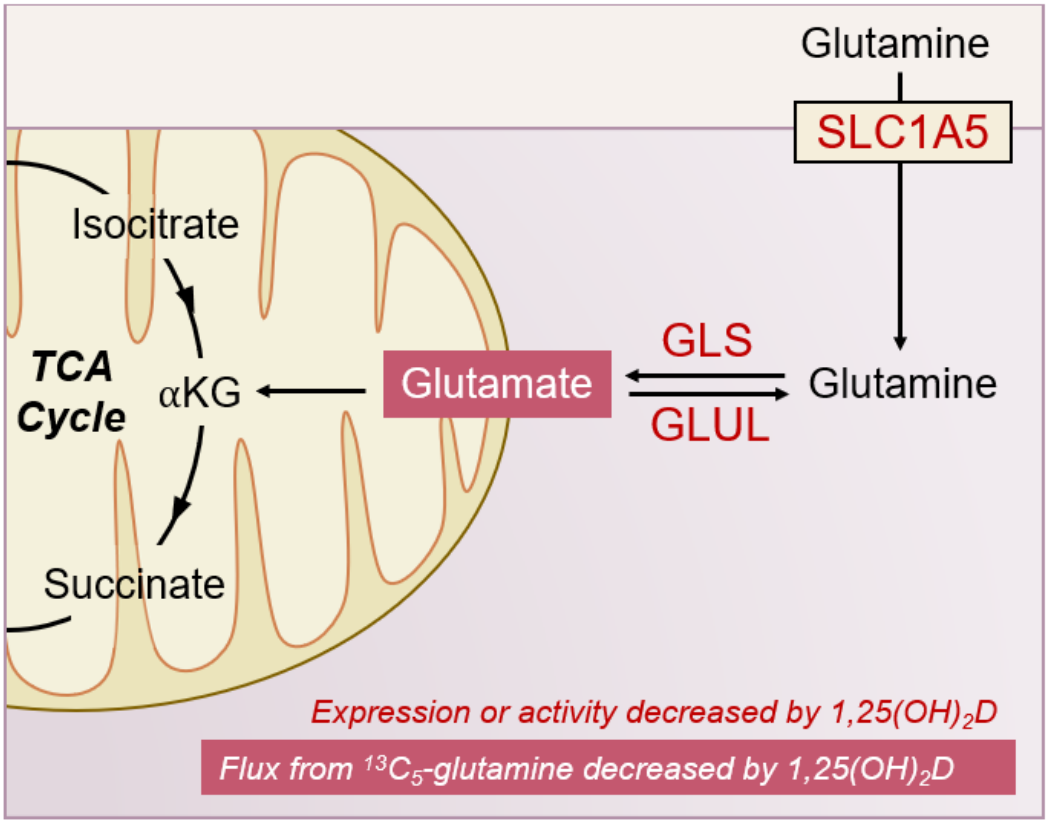

Figure 1.5 Decreased glutamine metabolism mediated by 1,25(OH)$_2$D.

A decrease in glutamine metabolism and replenishment of the TCA cycle by glutamine is observed in 1,25(OH)$_2$D-treated cancer cells. Treatment of cancer cells *in vitro* with 1,25(OH)$_2$D reduces enzyme expression or activity (red font) and [U-13C]-glutamine flux to metabolites (red shaded boxes).

Evidence suggests that treatment with 1,25(OH)$_2$D suppresses glutamine uptake in cancer cells. For example, following 1,25(OH)$_2$D treatment, expression of the major glutamine transporter SLC1A5 is decreased at the mRNA and protein level in MCF10A-*ras* breast cancer cells (148). A VDRE was identified in the promoter of the gene which encodes for SLC1A5 and verified using site-directed mutagenesis, suggesting that 1,25(OH)$_2$D downregulates SLC1A5 directly (148). In agreement with these results, glutamine uptake was decreased with 1,25(OH)$_2$D treatment with no further decrease in uptake observed with the combination of 1,25(OH)$_2$D treatment and the SLC1A5 inhibitor L-γ-glutamyl-p-nitroanilide. In contrast, SLC1A5 mRNA and protein expression were unaffected by 1,25(OH)$_2$D treatment in non-transformed breast epithelial cells (147,148), suggesting that 1,25(OH)$_2$D-mediated regulation of glutamine uptake may occur

specifically in cancer cells. This is particularly interesting, as 1,25$(OH)_2$D's regulation of SLC1A5 in cancer cells may have broader effects on cell metabolism apart from directly inhibiting glutamine uptake. For example, glutamine uptake is linked to the regulation of mTOR signaling, where inhibiting glutamine uptake prevents mTOR activation (149). Given this connection, 1,25$(OH)_2$D-mediated downregulation of SLC1A5 may also lead to reduced mTOR signaling, and therefore a further reduction of cell growth. These data support that 1,25$(OH)_2$D may specifically target cancer cells to reduce glutamine, with minimal effects on this process in non-transformed cells.

Evidence also supports that 1,25$(OH)_2$D regulates intracellular glutamine metabolism. After uptake into the cell, glutamine is metabolized by glutaminase (GLS) to glutamate, following which glutamate can be used for protein synthesis or metabolized into αKG and enter the TCA cycle. In *in vitro* models of breast and prostate cancer, GLS is suppressed by 1,25$(OH)_2$D treatment (133,147,150). In accord with this, several studies show that 1,25$(OH)_2$D inhibits the conversion of glutamine to its downstream metabolites, including glutamate and methionine (148,151). As a result, glutamine oxidation is reduced following 1,25$(OH)_2$D treatment in SV-40 and H-*ras* transfected breast cancer cells (147), suggesting decreased utilization of glutamine for energy production via oxidative phosphorylation in 1,25$(OH)_2$D-treated cells. These results indicate that 1,25$(OH)_2$D treatment reduces glutamine metabolism into glutamate and subsequent utilization in the TCA cycle.

Synthesis of glutamine is also decreased by 1,25$(OH)_2$D in cancer cell models. Synthesis of glutamine from glutamate is mediated by glutamine synthetase (GLUL), and GLUL expression in cancer cells is associated with increased metabolic flexibility, as it supports glutamine independence (146). For example, breast cancer cells that synthesize glutamine through GLUL are less sensitive to glutamine deprivation (147). Interestingly, 1,25$(OH)_2$D treatment decreased GLUL mRNA and protein expression in these cell models, with a subsequent decrease in GLUL activity (147). In agreement with these results, 1,25$(OH)_2$D-mediated inhibition of GLUL causes increased dependence on extracellular glutamine (147). These results suggest that inhibition of glutamine synthesis by 1,25$(OH)_2$D may increase the cellular requirement for extracellular glutamine and result in inhibition of cancer progression.

Regulation of glutamine metabolism in cancer cells by 1,25$(OH)_2$D may be of particular interest as a chemopreventive compound and in therapeutic applications, due to the differential

effect of 1,25$(OH)_2$D across cancer models. For example,1,25$(OH)_2$D treatment had little effect on glutamine metabolism in non-transformed cells, with more prominent effects observed in transformed MCF10A-*ras* transfected breast cancer cells (148), suggesting that 1,25$(OH)_2$D may alter glutamine metabolism with minimal cytotoxic effects on normal tissue. Treatment with 1,25$(OH)_2$D suppressed glutamine uptake as well as glutamine synthesis, suggesting that 1,25$(OH)_2$D's role in targeting glutamine metabolism may be clinically relevant particularly in cancers that display glutamine independence, such as glioblastoma and non-small cell lung cancer (NSCLC) (145). This specificity emphasizes the potential for glutamine metabolism inhibitors such as 1,25$(OH)_2$D to increase cancer cell death in nutrient-restricted environments, thereby inhibiting cancer progression. Further research is needed to determine the impact of 1,25$(OH)_2$D's regulation of glutamine metabolism on specific outcomes in cancer progression, such as migration or invasion, to determine the mechanisms underlying its anti-cancer effects.

1.4 Pyruvate Carboxylase

1.4.1 PC Activity in Normal Tissue

The TCA cycle is central to cell metabolism, and anaplerosis or replenishment of the TCA cycle is required for its continuation. Recent work has highlighted the role of the anaplerotic enzyme, PC, in cancer (152). PC is a biotin-dependent enzyme that synthesizes the TCA cycle intermediate, oxaloacetate, by a two-step pyruvate carboxylation (153) (Figure 1.6). First, PC adds a carboxyl group from bicarbonate to biotin which requires Mg-ATP. In the second step, the carboxyl group is then transferred to pyruvate to form oxaloacetate. In addition to the TCA cycle, PC activity is used in the first step of gluconeogenesis. Given PC's role in central metabolism, PC activity is relied upon for metabolic processes in several tissues including the liver, the kidney, the pancreas, and adipose tissue.

PC's roles in the liver and kidney are similar. In these tissues, PC activity participates in gluconeogenesis in times of fasting or starvation (153). In gluconeogenesis, which occurs predominantly in the liver and kidney, PC plays a primary role in supplying oxaloacetate for phosphoenolpyruvate (PEP) synthesis by PEP carboxykinase (PCK). PC and PCK activity mediate the first steps of gluconeogenesis. In the liver and kidney, the gluconeogenic pathway is critical in times of low glucose, such as fasting or starvation, to support non-gluconeogenic tissues, and

maintain serum glucose homeostasis. For example, PC expression was markedly increased in rat liver and kidney tissue following five-day bouts of starvation (154). Further, liver-specific knockout of PC was sufficient to impair gluconeogenesis in mice (155). Taken together these results demonstrate a critical role of PC in gluconeogenesis in the liver and kidney.

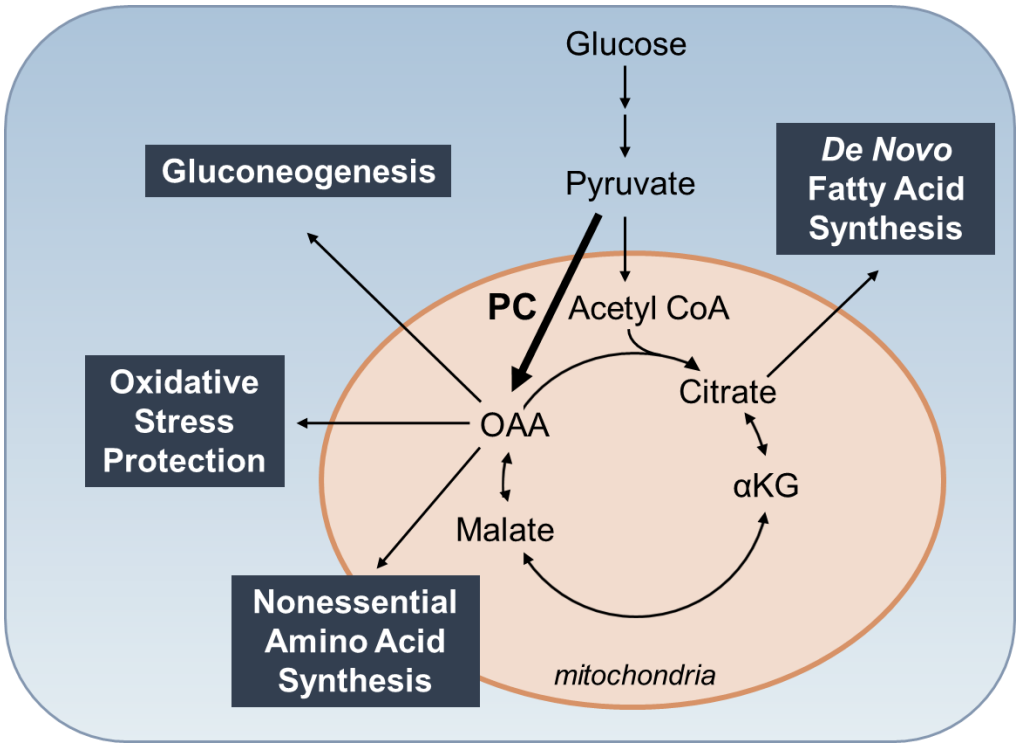

Figure 1.6 PC's Role in Central Metabolism.

PC plays a role in central metabolism, and PC activity contributes to gluconeogenesis, oxidative stress protection, fatty acid synthesis, and biosynthesis of nonessential amino acids.

PC is also involved in maintaining optimal serum glucose levels by its participation in pyruvate cycling in β cells in pancreatic islets. Insulin secretion from pancreatic β cells requires a higher ATP/adenosine diphosphate (ADP) ratio (156). PC activity supports the maintenance of ATP levels by supplying oxaloacetate to the TCA cycle which produces NADH and $FADH_2$ for oxidation and synthesis of ATP. Apart from anaplerosis, PC also participates in pyruvate cycling by supplying oxaloacetate for the synthesis of malate. After malate is shuttled from the mitochondria to the cytosol, malic enzyme mediates the conversion of malate to pyruvate which also synthesizes NADPH. Production of the reducing equivalent NADPH supports ATP

production, and insulin secretion in the pancreatic β cell (156). This concept was demonstrated in insulinoma cells, which were isolated from pancreatic islets (157). Insulinoma cells treated with phenylacetic acid to inhibit PC displayed impaired insulin secretion as a response to glucose. Similar results occurred in animals with pharmacological PC inhibition with phenylacetic acid (158). Therefore, PC's role in pyruvate cycling and maintaining the ATP levels required for insulin secretion in the pancreas is another mechanism by which PC activity supports glucose homeostasis.

In adipose tissue PC activity primarily contributes to lipogenesis, rather than supporting the maintenance of serum glucose levels (156). Oxaloacetate supplied from the PC reaction condenses with acetyl-CoA to form citrate. Citrate is shuttled out of the mitochondria and may be oxidized to cytosolic oxaloacetate and acetyl-CoA. Cytosolic acetyl-CoA is used as a building block for fatty acid synthesis. In support of this, PC inhibition resulted in decreased lipid accumulation in murine 3T3-L1 adipocytes (159). Further, flux analysis using a 1,2-^{13}C-glucose tracer demonstrated that PC-inhibition with phenylacetic acid decreased lipid stores via reduced *de novo* fatty acid synthesis (159). In sum, PC activity supports fatty acid synthesis by providing substrate for production of citrate, a precursor for fatty acid synthesis.

1.4.2 Regulation of PC Activity in Normal Tissue

PC activity is regulated by short and long-term mechanisms in normal tissues. The most well-known short-term regulator of PC is acetyl-CoA's allosteric regulation which enhances PC activity (156). As acetyl-CoA levels increase so does PC activity, which synthesizes oxaloacetate for subsequent condensation with acetyl-CoA to form citrate. Additionally, glutamate, an amino acid that replenishes the TCA cycle through the intermediate αKG, negatively regulates PC activity (160). This regulation maintains TCA cycle flux and prevents the buildup of excess acetyl-CoA or glutamate.

Transcriptional regulation of PC is tissue-specific and allows for its various site-specific roles in energy metabolism. The tissue specificity is made possible by two alternative promoters, the distal (P2) and proximal (P1) (161). Collectively both promotors provide 3 transcript variants of PC, where the P2 promoter regulates transcription of variant 1 and 3, and the P1 promoter regulates variant 2 (161). In liver and adipose tissue PC transcription is regulated by the P1 promotor, and the P2 promotor controls transcription in pancreatic β cells. Thus, the various transcript variants provide differential expression in tissues.

Tissue-specific transcription factor binding sites are also identified to transcriptionally regulate PC expression. For example, in the P2 promotor, hepatocyte nuclear factor 3β (HNF3β)/forkhead transcription factor boxA2 (Foxa2) binds to a Foxa2 site and increases transcription of PC in pancreatic β cells (162). While in adipocytes, PPARγ transcription factor is shown to bind a response element in the P1 promoter and increase PC expression (163). In adipocytes, PPARγ is a master regulator of lipid metabolism where it promotes transcription of enzymes involved in fatty acid synthesis and storage. Thus, PPARγ regulation of PC falls in line with PC's role in adipocytes to promote fatty acid synthesis. Collectively, these studies support the tissue-specific regulation of PC expression.

1.4.3 PC Promotes Metastasis

Metabolic reprogramming is a hallmark of cancer and is a potential target for cancer prevention and therapy (164). In cancer, energy metabolism is utilized to maintain growth through the synthesis of building blocks for proliferating cells, but also to produce energy which is required to support carcinogenesis. While some cancers harbor genetic mutations, which suppress mitochondrial ATP production, maintenance of substantial mitochondrial ATP production is generally selected for in cancer (165). Increasing evidence suggests that anaplerosis of the pools of intermediate TCA cycle metabolites is essential for maintaining the cellular capacity for amino acid, nucleic acid, and lipid synthesis in proliferating cancer cells (166). Given its critical role in TCA cycle anaplerosis, PC may serve as a target to prevent cancer cell proliferation and cancer progression.

PC expression is upregulated in several types of cancer compared with normal tissue, including cancers of the mammary, lung, gallbladder, and papillary thyroid cancer (167-172). For example, PC protein is overexpressed in human mammary cancer compared with normal mammary tissue and increases in abundance with tumor stage, as demonstrated by immunohistochemical analysis (172). In addition, increased PC expression in breast cancer was observed as compared to normal mammary tissue. In contrast, an analysis by Shinde et al. of the METABRIC dataset failed to demonstrate significant differences in *PC* mRNA levels according to tumor stage (173). However, further analysis of outlier groups identified high levels of *PC* expression within tumor stage groups. Increased PC expression in breast cancer is associated with reduced patient survival time (173), suggesting a role of PC in metastatic progression of cancer

cells. While the mechanisms of PC upregulation in cancer remain to be definitively determined, *PC* lies within the 11q13.2 locus, a hotspot for gene amplification. Indeed, analysis of several patient datasets indicates that 16-30% of breast cancer patients harbor copy number gains in the *PC* gene in tumor tissue, and PC gene amplification corresponds with reduced survival (173). Collectively, these results suggest a role of PC in cancer progression and increased mortality.

Cancer metastasis is a multistep process (84), and several of the steps involved likely require metabolic plasticity for successful transit and adaptation to the destination organ. Indeed metastatic cancer cells display an increased requirement for ATP and redox defense, with prominent roles for glucose, fatty acid, and mitochondrial metabolism (174). Thus PC, given its role in regulating glucose-derived anaplerotic carbon supply to the mitochondria, sits at a nexus of numerous metabolic pathways critical to metastasis. For instance, Shinde et al. showed that PC is required for mammary-to-lung metastasis in an *in vivo* model (173). In this model, injection of 4T1 murine mammary cancer cells with genetic PC depletion into the mammary fat pads of BALB/c mice had no effect on primary tumor size, increased nonpulmonary metastasis, but dramatically decreased pulmonary metastases compared to PC-expressing 4T1 cells (173). These results indicate that PC is specifically required for tumor growth within the lungs. Consistent with these results, Christen et al. also observed an increase in PC expression and flux through PC in lung metastatic lesions of mice injected with 4T1 cells (123). Furthermore, PC expression is required for the growth of primary lung tumors (167). These results are supported by mechanistic *in vitro* results showing that PC regulates processes specific to tumor cell growth within the lungs including overcoming oxidative stress (167,171-173). These data collectively suggest an organotropic role of PC in metastatic progression. This notion is consistent with previous literature showing that metabolic reprogramming through modulation of specific enzymes is a requirement for cancer cell growth at specific sites. For example, the metabolic PDK1 and PCK1 are required for hepatic colonization in animal models of breast and colon cancer, respectively (175,176). A better understanding of the mechanisms of PC expression and PC function in particular organ environments will help to refine contexts in which therapeutic targeting of PC might be most beneficial for preventing the emergence of metastatic disease.

1.4.4 PC's Role in Cancer Cell Energy Metabolism

Glucose is a primary source of carbon for energy production and biosynthesis in cancer cells. Increased flux of glucose-derived pyruvate into the TCA cycle through the PC reaction, providing energy and carbon backbones from glucose, appears to be a hallmark of certain cancers. The flux of glucose through pathways, including the TCA cycle, can be assessed using [^{13}C]-labeled glucose and is useful in understanding alterations in cell metabolism with changing PC activity in cancers. For example, infusing NSCLC patients with universally labeled $^{13}C_6$-glucose before tumor resection showed increased M+3 and M+5 labeling of aspartate, citrate, and malate, indicative of increased PC activity in tumor tissue compared to normal tissue (167,170). Likewise, an animal model of Kras^{G12D}-driven NSCLC showed increased $^{13}C_6$-glucose flux through PC in tumor tissue compared to non-tumor tissue (169). Depletion of PC in this model led to impaired primary tumor formation pointing to a requirement for PC in Kras^{G12D}-driven NSCLC (169). Furthermore, in A549 lung adenocarcinoma cells and MDA-MB-231 mammary cancer cells, PC depletion did not affect glycolysis but decreased $^{13}C_6$-glucose flux to aspartate, citrate, malate, and succinate (167,177). Finally, in 4T1 mammary cancer cells, PC depletion resulted in decreased glycolytic activity and OCR (173). Taken together, these results demonstrate a critical anaplerotic role of PC in the utilization of glucose metabolites in a spectrum of cancer models.

The role of PC in replenishing the TCA cycle intermediates in cancer models that are deficient in glucose or display impaired TCA cycle enzyme activity has been investigated extensively. For example, reduced expression of IDH1 in glioma models is associated with increased PC and decreased PDH activity (178). In addition, PC plays a critical role in overcoming deficiencies in succinate dehydrogenase (SDH), an electron transport chain/TCA complex that is commonly mutated in cancer (179-181). SDH converts succinate to fumarate in the TCA cycle, and inhibition of its activity can lead to depletion of downstream TCA intermediates. SDH ablation increases diversion of glucose to aspartate biosynthesis through PC activity in models of renal cell carcinoma (181). Importantly, PC depletion reduces the viability of renal cell carcinoma cells with SDH ablation, and the addition of aspartate *in vitro* rescues cell growth in this model, suggesting that PC's role in aspartate biosynthesis is crucial for overcoming SDH deficiency. Similarly, inhibition of SDHA or SDHB is sufficient to increase PC activity in prostate and neuroendocrine cancer cell models, leading to the replenishment of cellular aspartate pools (179,180). Further, the use of the BRAF inhibitor, vemurafenib, in melanoma cells suppresses glycolysis but does not

affect the flux of glucose-derived pyruvate through PC and results in an increase in the PC/PDH activity ratio. The addition of the PC inhibitor phenylacetic acid to vemurafenib-treated cells decreases cell growth, suggesting that blocking PC may sensitize melanoma cells to vemurafenib treatment, however off-target effects of phenylacetic acid may play a role in the observed decrease in cell growth (182,183). Given these data, PC's role in replenishing the oxaloacetate pool for utilization in the TCA cycle or aspartate biosynthesis is crucial to consider when treating cells with therapies that target glucose metabolism. Cumulatively, these results support the importance of PC in replenishing the TCA cycle, which is critical in cancer cell progression.

There is also significant evidence that cancer cells "switch" to PC activity in glutamine deplete conditions for anaplerosis. Glutamine is the most abundant amino acid in circulation and is highly utilized by cancer cells throughout progression (145). Glutamine is employed by cancer cells to replenish the TCA cycle through the intermediate, αKG. Similar to its rescue of TCA cycle impairment, PC activity rescued glioblastoma cells grown without glutamine (184). Additionally, glioblastoma 1,6-^{13}C-glucose flux results show an increase in PC activity for oxaloacetate flux into the TCA cycle when glutamine metabolism was impaired with GLS depletion. In H460 large cell lung cancer cells' PC activity increased as a result of depletion of the citrate transport protein, which is involved in the entry of glutamine sourced αKG into the TCA cycle (185). Taken together, cancer cell regulation of PC activity is utilized to confer metabolic flexibility to cancer cells exposed to changing nutrient statuses, particularly in low glutamine conditions.

1.4.5 Regulation of PC in Cancer Cells

Given the use of PC for adaption to changing nutrient environments throughout progression, understanding the mechanisms that regulate the expression and activity of PC in cancer cells is important to identify targets to prevent metastasis. Several mechanisms of PC regulation are identified in cancer models, including regulation by non-coding RNAs, signaling pathways, and small molecules, further demonstrating the centrality of PC in metastasis.

PC is regulated by non-coding RNAs, including lncRNA and micro-RNAs. Cell and patient-derived tumor tissue models of gallbladder cancer demonstrated the importance of the lncRNA gallbladder cancer-associated suppressor of pyruvate carboxylase (GCASPC) in the regulation of PC expression (168). Immunoprecipitation studies suggest that GCASPC lncRNA

physically interacts with PC protein in the mitochondria leading to its degradation, suggesting that the loss of GCASPC lncRNA that occurs in gallbladder cancer may lead to increased PC protein retention. Overexpression of GCASPC lncRNA decreased PC protein expression and activity, cell proliferation, and tumor growth *in vivo*, and proliferation was rescued when PC was overexpressed. These data highlight the role of PC in primary tumor formation and regulation by GCASPC lncRNA. In MDA-MB-231 mammary cancer cells, overexpression of the microRNA miR-143-3p resulted in decreased PC expression, cell proliferation, and migration (186). A luciferase reporter assay confirmed the direct binding of miR-143-3p to the 3' untranslated region of PC mRNA, resulting in decreased PC mRNA and protein expression. However, re-expression of PC in miR-143-3p-overexpressing MDA-MB-231 cells increased PC protein expression but did not overcome the inhibitory effects of miR-143-3p on proliferation and migration, suggesting inhibition of PC is only partially responsible for the antiproliferative effects of miR-143-3p on mammary cancer cells. Taken together, these studies demonstrate PC regulation in cancer by noncoding RNAs, which may be novel targets for inhibiting PC in conjunction with other cancer treatments.

Cancer-promoting signaling pathways also play a role in modifying PC expression. In OVCAR-3 ovarian cancer cells, the activity of tankyrase enzyme, a member of the poly(ADP-ribose) polymerase (PARP) family, maintains PC expression through Wnt/β-catenin/Snail signaling (187). Similar increases in PC protein expression through Wnt/β-catenin/Snail signaling were observed in MCF7 mammary cancer cells (188). Both studies demonstrated that Snail was required for PC expression, although the mechanism by which Snail stabilizes PC has not been elucidated (187,188). Stage and cell type might play a role in Snail's ability to regulate PC, as overexpression of Snail in MCF10A-*ras* cells, which represent an early model of mammary cancer, did not increase PC expression (173). c-Myc may be an additional signaling molecule that promotes PC expression, as a positive association between c-Myc and PC was found in transcriptome data from The Cancer Genome Atlas breast cancer dataset (189). Further investigation into the regulatory role of c-Myc in PC expression demonstrated that inhibition of c-Myc decreased PC expression in MDA-MB-231 cells through a direct mechanism, but had no effect on PC expression in MCF7 human mammary cancer cells (189). Further, inhibition of c-Myc in MDA-MB-231 cells decreased cell viability, migration, and invasion. PC overexpression partly rescued viability of c-Myc inhibited cells, but PC overexpression did not rescue cell migration or invasion, suggesting that PC plays an important role in cell viability but not migration

or invasion, at least in the MDA-MB-231 breast cancer cell model (189). Given that PC expression is associated with cancer progression, it is not surprising that these data indicate regulation of PC expression by signaling pathways that are commonly upregulated in cancer.

Finally, hormones, including the hormonal form of vitamin D and leptin, are regulators of PC. The bioactive metabolite of vitamin D, 1,25$(OH)_2$D, decreases PC expression in non-metastatic human MCF10A-*ras* mammary cancer cells (80) and in metastatic human MCF10CA1a cells (137). In MCF10A-*ras* cells, 1,25$(OH)_2$D directly decreases PC expression through a VDRE in the promoter region of the PC gene (80). Further evidence of a VDRE in the distal promoter of the human PC gene was identified in MDA-MB-231 metastatic mammary cancer cells (187). Interestingly, treatment of MCF-7 cells with leptin, a hormone secreted by adipocytes, increases PC activity as well as the ratio of PC/PDH activity (190). Increased circulating leptin is associated with obesity and increased cancer risk (191), thus leptin regulation of PC activity is particularly interesting, given the increased mortality from cancer observed in obese individuals (192). Given the prevalence of vitamin D deficiency (193) and the association of low vitamin D status with obesity (194), these studies illuminate a potential strategy to suppress metastasis through reducing PC activity with vitamin D in PC-dependent cancers.

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CHAPTER 2. PYRUVATE CARBOXYLASE SUPPORTS MCF10A-RAS CELL SURVIVAL IN EXTRACELLULAR MATRIX DETACHED CONDITIONS

2.1 Abstract

Throughout metastatic progression, cancer cells acquire anchorage independence, or the ability to survive detached from the extracellular matrix (ECM). While non-transformed epithelial cells reduce energy metabolism when detached, cancer cells display metabolic flexibility to continue important metabolic processes. Glucose and glutamine are predominant nutrients utilized for energy as well as other purposes, and their metabolism is regulated by cancer cells. Therefore, the effects of detachment on glucose and glutamine metabolism in human breast epithelial MCF10A cells transfected with the Harvey-*ras* oncogene (MCF10A-*ras*), a model of early-stage cancer, was determined. Detachment was simulated with poly-HEMA coated plates, and intracellular flux was determined using stably labeled $^{13}C_5$-glutamine and $^{13}C_6$-glucose tracers. Results show reduced glutamine flux in detached cells as determined by reduced levels of labeled glutamate (21%), malate (30%), and aspartate (23%) from labelled glutamine. Detachment also reduced flux of $^{13}C_6$-glucose to pyruvate and lactate pools by 51% and 29%, respectively. Similarly, detachment reduced total intracellular pool sizes of pyruvate (51%), lactate (49%), α-ketoglutarate (43%), fumarate (32%), malate (19%), alanine (35%), serine (35%), and glutamate (28%) compared to attached cells, but citrate and aspartate pool sizes were unchanged. Compared to attached cells, detachment increased pyruvate carboxylase (PC) mRNA abundance and protein expression by 131% and 190%, respectively. In detachment, PC activity, determined by $^{13}C_6$-glucose derived M+3 isotopomers, was shown to preferentially replenish malate and aspartate, but not citrate pools. In addition, Doxycycline-inducible shRNA knockdown of PC significantly decreased, while Doxycycline-inducible PC overexpression significantly increased, detached cell viability. Further, a switch from glutamine to PC activity for anaplerosis was demonstrated, as supplementation with the cell permeable analog of the tricarboxylic acid cycle intermediate, α-ketoglutarate, a downstream metabolite of glutamine, decreased PC mRNA abundance in detached cells. Collectively, these results suggest that detached breast cancer cells increase PC activity in response to decreased glutamine-derived anaplerosis to promote survival.

2.2 Introduction

Metastasis accounts for the majority of cancer-related deaths (1). The five-year survival rate of breast cancer patients is 28% when diagnosed with metastatic disease compared to a 99% five-year survival rate in patients with a localized diagnosis (2). Metastasis is a multistep process that includes cellular modifications in the primary tumor, invasion into surrounding tissue, systemic dissemination, and colonization of a secondary tumor at a distal site (3). Thus, research that identifies regulators of progression through the metastatic cascade may provide targets to improve the survival of breast cancer patients.

Survival and growth of normal epithelial cells are dependent on attachment to the extracellular matrix (ECM). In a primary tumor, cellular changes permitting "anchorage independence," which enables cancer cell survival when detached from the ECM, promotes tumor growth and metastasis (4). Integrins mediate attachment to the matrix and, upon detachment, initiate signaling that stimulates anoikis, a form of programmed cell death (5). For example, integrins reduce activation of focal adhesion kinase (FAK), leading to reduced downstream signaling through pro-survival signaling pathways such as mitogen activating protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) (6). Oncogenic transformation, such as those mediated by mutation to *RAS* or amplification of *ERBB2*, assists cancer cells in gaining "anchorage independence" which enables their survival when detached from the ECM by maintaining activation through survival signaling pathways such as PI3K (7,8). Thus, anchorage independence supports the metastatic phenotype as metastatic steps such as invasion and systemic dissemination involve cellular detachment from the ECM.

Reprogrammed energy metabolism is an emerging hallmark of cancer (9), and evidence supports that metabolic reprogramming plays an important role in maintaining survival of ECM-detached cancer cells. For example, nonmalignant epithelial cells decrease energy metabolism pathways upon detachment (4). However, detached MCF10A human breast epithelial cells with overexpression of the ErbB2 oncogene increased glucose and glutamine flux into the tricarboxylic acid (TCA) cycle compared to detached non-transformed MCF10A cells (10). Additionally, overexpression of FAK increased glycolysis in detached pancreatic ductal adenocarcinoma cells (11). Further, detached MDA-MB-231 breast cancer cells increased the expression of antioxidant enzymes, which was required for fatty acid oxidation and the subsequent production of adenosine

triphosphate (ATP) (12). These results suggest that oncogenic transformation enables adaptations in cellular energy metabolism in ECM detached conditions.

The anaplerotic enzyme, pyruvate carboxylase (PC), is responsible for the conversion of pyruvate to oxaloacetate to replenish the tricarboxylic acid (TCA) cycle. Previous results demonstrate that PC is required for breast to lung metastasis *in vivo* (13). In addition, Phannasil et al. demonstrated that PC activity contributes to metastatic steps such as migration and invasion (14). Interestingly, a preference for PC over glutamine anaplerosis was previously identified in breast to lung metastatic tumors, suggesting a "PC switch" in metastatic progression to adjust to nutrient availability (15). PC activity replenishes the TCA cycle and is linked to increased fatty acid synthesis and oxidative stress protection in breast cancer models (16,17). While PC activity is utilized by cancer cells to supply substrate for various metabolic processes, its role in detached conditions has not been established. In the current work it was hypothesized that PC is required to maintain cell viability of transformed breast epithelial cells by replenishing the oxaloacetate pool to rescue cells from decreased glutamine availability in ECM detached conditions.

2.3 Methods

2.3.1 Cell Culture

MCF10A cells transfected with the Harvey-*ras* oncogene (MCF10A-*ras*), a of model early-stage breast cancer, were a gift from Michael Kinch, Purdue University. Cells were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) containing 5 mM glucose, 2.5 mM glutamine, and no sodium pyruvate. Media also contained 5% horse serum (Gibco, Waltham MA), 1% penicillin/streptomycin (Gibco), 10 mg/L insulin (Sigma), 50 µ/L cholera toxin (Sigma), 20 µg/L epidermal growth factor (Sigma, St. Louis, MO), and 50 mg/L hydrocortisone (Sigma).

2.3.2 Poly-HEMA-Coated Plates

Extracellular matrix (ECM) detachment was simulated using Poly(2-hydroxyethyl methacrylate) (Poly-HEMA, Sigma) coated plates. Plates were coated with 70 µL/cm^2 of 20 mg/mL Poly-HEMA in 95% ethanol. The plates were dried overnight under ultraviolet light after the addition of Poly-

HEMA. The Poly-HEMA coated plates and dishes were rinsed twice with sterile 1x calcium/magnesium-free phosphate-buffered saline (PBS) before use.

2.3.3 MTT Assay

Viability assays were performed according to the manufacturer's instructions using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). Briefly, cells were plated at 20k cells/well with indicated treatment into Poly-HEMA coated 96-well plates. MTT solution was added to each well to a final concentration of 1% for two hours, followed by solubilization in dimethylsulfoxide and absorbance measured at 570 nm.

2.3.4 RNA Isolation and qRT-PCR

Cells were plated into 100 mm Poly-HEMA coated dishes. After 40 hours, Tri-Reageant (Molecular Research Center, Cincinnati, OH) was used to harvest and isolate RNA according to the manufacturer's instructions. RNA was reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed on the subsequent cDNA using a LightCycler 480 instrument using LightCycler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN). All target gene mRNA abundance was normalized to 18S abundance with the comparative Ct method ($2^{-\Delta Ct}$).

2.3.5 Glutamine and Glucose Flux

Following 40 hours in attachment or detachment, media was changed to media containing either 100% (2.5 mM) $^{13}C_5$-glutamine or 100% (5 mM) $^{13}C_6$-glucose. Cells were incubated at 37°C for two hours, followed by harvest into 70% ethanol at 70°C and the addition of 1 µg of the internal standard norvaline to each sample. Intracellular metabolites were extracted from the cells into ethanol by a 5-minute incubation at 95°C. A bicinchoninic acid (BCA, ThermoFisher, Waltham, MA) assay was used to measure protein content in the cell pellet, and the supernatant dried. Methoxylamine hydrochloride in pyridine was used to derivatize metabolites as previously described (18). Following derivatization, metabolites were analyzed with gas chromatography-mass spectrometry (GC-MS) using a TG-5MS gas chromatography column and Thermo TSQ 8000

triple quadrupole mass spectrometer. Mass spectra data were acquired using Chromeleon 7 software (ThermoFisher). Intracellular pool sizes were calculated by dividing the area under the curve for the total metabolite by norvaline and protein in the cell pellet to control for recovery and cell quantity, respectively. Isotopomer percent enrichment of the metabolite pool sizes was used to calculate ^{13}C flux, and results are calculated relative to levels determined for attached cells.

2.3.6 Western Blot Analysis

Following two washes with calcium/magnesium-free PBS, cells were harvested on ice into 1x radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling, Danvers MA) supplemented with 1% phosphatase (Sigma) and 1% phenylmethylsulfonyl fluoride protease inhibitors (Cell Signaling). To harvest detached cells, cells were pelleted by 5-minute centrifugation at 1000 RPM and rinsed twice with calcium/magnesium-free PBS. Pelleted detached cells were resuspended in 25 µL of RIPA lysis buffer. Attached and detached cells were sonicated for 15 minutes and centrifuged for 15 minutes at 12,000 revolutions per minute. The supernatant was collected and protein quantified using the BCA assay. A 4-15% gradient polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) was used to resolve 20 µg of protein by electrophoresis. Primary antibodies for PC and actin were purchased from Sigma. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and antigen-antibody complexes were detected by the Li-Cor Odyssey imaging system.

2.3.7 PC Knockdown and Overexpression

Doxycycline (Dox) inducible TRIPZ lentiviral human shRNA which targeted PC and was purchased from GE Dharmacon (Lafayette, CO) and Dox inducible lentiviral plasmid containing the human PC gene (NM_000920.2) was purchased from VectorBuilder (Chicago, IL). Polyethylamine was used to transduce HEK293T cells with lentiviral plasmids and the lentiviral particles harvested. MCF10A-*ras* cells were transfected for 48 hours with 10 µg/mL polybrene, and stably transfected cells were selected using puromycin (5 mg/mL). For PC overexpression, hygromycin (450 ug/mL) was used in addition to puromycin to select for stable transfection. PC knockdown or overexpression was verified following treatment with Dox (0.5 ug/mL) for three days by PCR analysis of PC mRNA abundance.

2.3.8 Statistical Analysis

Results are expressed as mean ± S.E.M. Comparisons between two groups were determined using an independent samples *t*-test, or across groups by analysis of variance (ANOVA). Statistical significance of $P < 0.05$.

Table 1. Primers used for qRT-PCR.

18S	Forward: 5'- TTAGAGTGTTCAAAGCAGGCCCGA-3' Reverse: 5'- TCTTGGCAAATGCTTTCGCTC-3'
ASCT2	Forward: 5'- TGAACATCCTGGGCTTGGTAG-3' Reverse: 5'- AGCAGGCAGCACAGAATGTAC-3'
GLUD1	Forward: 5'- TTAGAGTGTTCAAAGCAGGCCCGA-3' Reverse: 5'- TCTTGGCAAATGCTTTCGCTC-3'
GOT1	Forward: 5'-CAACTGGGATTGACCCAACT -3' Reverse: 5'- GGAACAGAAACCGGTGCTT -3'
GOT2	Forward: 5'-ACCCATGTGGAAATGGGACC -3' Reverse: 5'- GACTTCGCTGTTCTCACCCA-3'
GLUT1	Forward: 5'-TATCGTCAACACGGCCTTCACTGT -3' Reverse: 5'- CACAAAGCCAAAGATGGCCACGAT -3'
HK2	Forward: 5'-CTGCAGCGCATCAAGGAGAACAAA -3' Reverse: 5'- ACGGTCTTATGTAGACGCTTGGCA -3'
LDHA	Forward: 5'- TGGTCCAGCGTAACGTGAACATCT -3' Reverse: 5'-TTGCAACCGCTTCCAATAACACGG -3'
PC	Forward: 5'- ATGTTGCCCACAACTTCAGCAAGC -3' Reverse: 5'- AGTTGAGGGAGTCAAACACACGGA-3'

2.4 Results

To evaluate the response of the MCF10A-*ras* cells to ECM detachment, cell viability was measured over a time course of 0, 24, 40, and 48 hours (Figure 2.1A). Cell viability decreased with time in detached conditions up to 40 hours, with an increase at 48 hours, suggesting growth of the

surviving detached cells. Given that 40 hours was the time point with the lowest level of viability, this time point was selected for further study.

Because previous studies demonstrate the importance of energy metabolism for cell survival in ECM detached conditions (10,19), the dependence of detached MCF10A-*ras* cells on glutamine and glucose was determined. Depriving cells of either glutamine or glucose decreased detached cell viability by 57% or 69%, respectively, and deprivation of both glucose and glutamine decreased cell viability by 82% (Figure 2.1B). These results demonstrate a dependence on glutamine or glucose for the survival of MCF10A-*ras* cells in detachment.

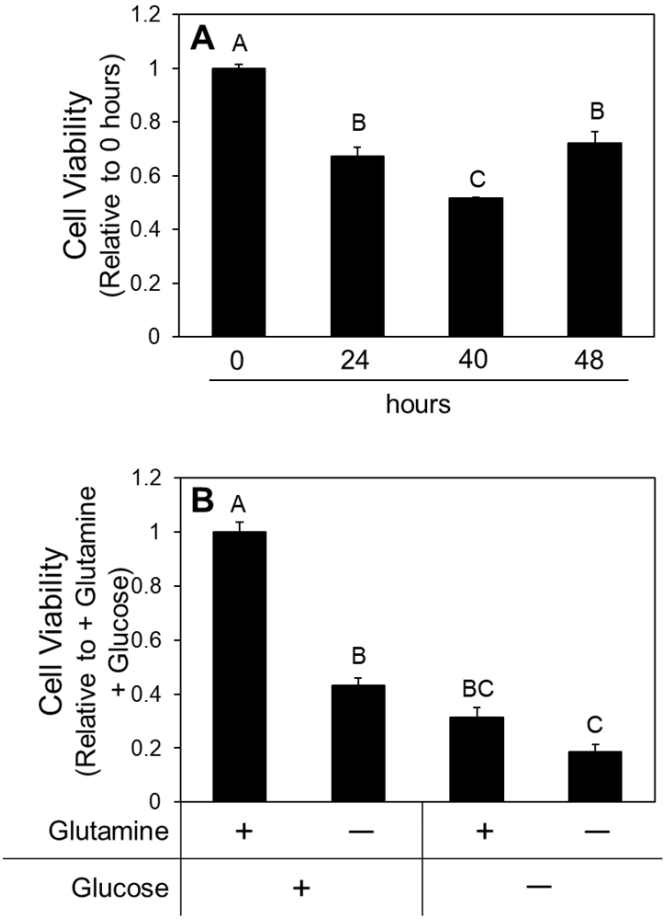

Figure 2.1. Effect of detachment on cell viability.

Viability of MCF10A-*ras* cells was assessed by MTT after 0, 24, 40, and 48 hours in detached conditions (A) and in glutamine and glucose depleted conditions (B). Results are expressed as ± SEM. Different letters indicates significance $P < 0.05$.

Due to the dependence on glutamine for survival in detached conditions, the metabolism of glutamine in detached cells was determined. Compared to attached cells, detached cells had decreased mRNA levels of the membrane glutamine transporter, Alanine/Serine/Cysteine-preferring Transporter 2 (ASCT2) (Figure 2.2C). Flux analysis of $^{13}C_5$-glutamine showed that detachment decreased glutamine flux into glutamate, malate, and aspartate, demonstrating reduced TCA cycle anaplerosis from glutamine (Figure 2.2A and 2.2B). In addition, In detached cells, lower mRNA abundance relative to attached cells of enzymes involved in the metabolism of glutamate, which is directly metabolized from glutamine, were also shown, including glutamate dehydrogenase (GLUD1) and the cytosolic glutamate oxaloacetate transaminase (GOT) 1 (Figure 2C). In contrast, mRNA abundance of the mitochondrial isoform *GOT2* was unchanged in detached cells (Figure 2.2C). These results support that there is a decrease in glutamine uptake and catabolism in detached MCF10A-*ras* cells compared to attached cells.

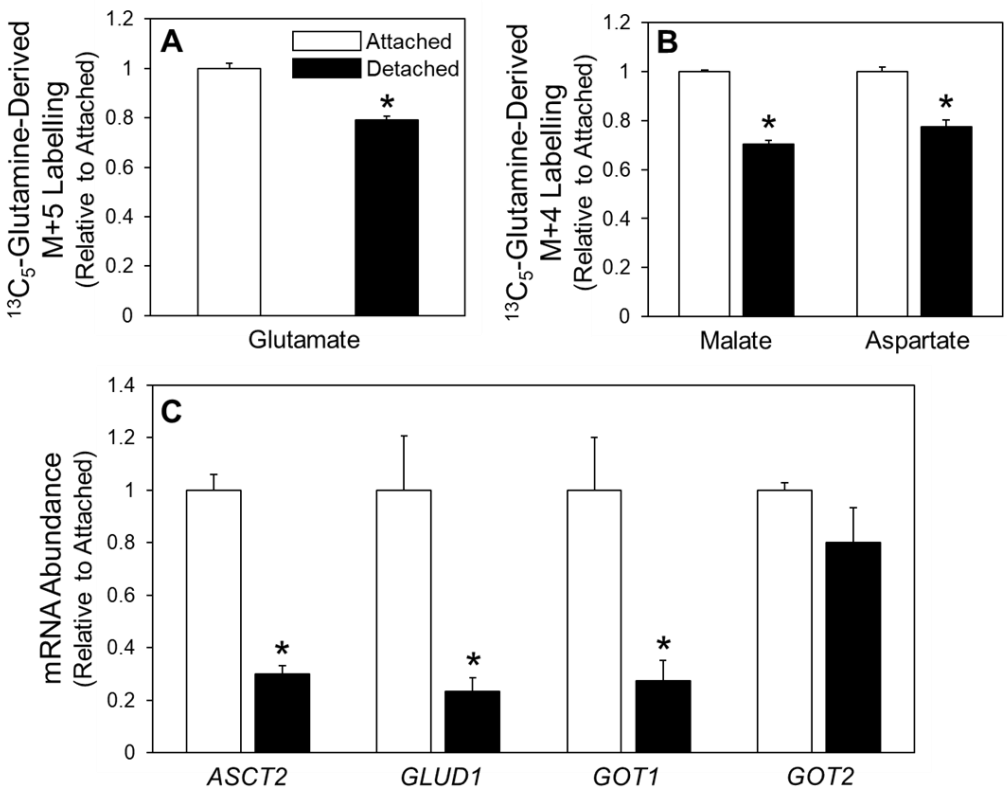

Figure 2.2. Glutamine metabolism in attached and detached conditions.

$^{13}C_5$-Glutamine was used to determine M+5 labeling of glutamate (A), and M+4 malate and aspartate downstream metabolites in attached and detached MCF10A-*ras* cells (B). mRNA abundance of enzymes in glutamine metabolism (C) was assessed in attached and detached MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to attached cells.

To explore glucose metabolism in ECM detached conditions, the effects of detachment on glycolysis were determined. Intracellular pool sizes of the end products of glycolysis, pyruvate and lactate, were both decreased in detachment (Figure 2.3A). Universally labeled $^{13}C_6$-glucose flux, which results in pyruvate and lactate labeled with three ^{13}C's (M+3), were both decreased following detachment (Figure 2.3B). Decreased mRNA abundance of glycolytic enzymes hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA) was observed in detached cells relative to attached cells (Figure 2.3C). However, compared to attached cells, mRNA abundance of glucose transporter 1 (GLUT1) was unchanged following detachment (Figure 2.3C). Similar to glutamine metabolism, these results demonstrate that while glucose is required for detached cell survival,

detached cells decrease glucose metabolism through glycolysis compared to their attached counterparts.

Given the decrease in glycolysis observed in detached cells, it was hypothesized that glucose flux into the TCA cycle was also decreased. Compared to attached cells, detachment decreased pool sizes for all intermediates measured, except citrate which was unchanged (Figure 2.3D). Additionally, free amino acid pool sizes which are metabolically connected to glutamine, glucose, or TCA cycle metabolism were measured. Detachment decreased total pool sizes for alanine, serine, and glutamate, but no change was observed for aspartate (Figure 2.3E). Furthermore, glucose-derived anaplerosis through pyruvate dehydrogenase was measured, using $^{13}C_6$-glucose flux into citrate which results in M+2 labeling, was decreased in detached cells relative to attached (Figure 2.3F). Collectively these results suggest that energy metabolism through glycolysis and the TCA cycle is reduced following detachment. Overall, these results suggest that detachment suppresses TCA cycle anaplerosis from both glutamine and glucose. However, these results also suggest that detached MCF10A-*ras* cells maintain the production of citrate and aspartate in detachment.

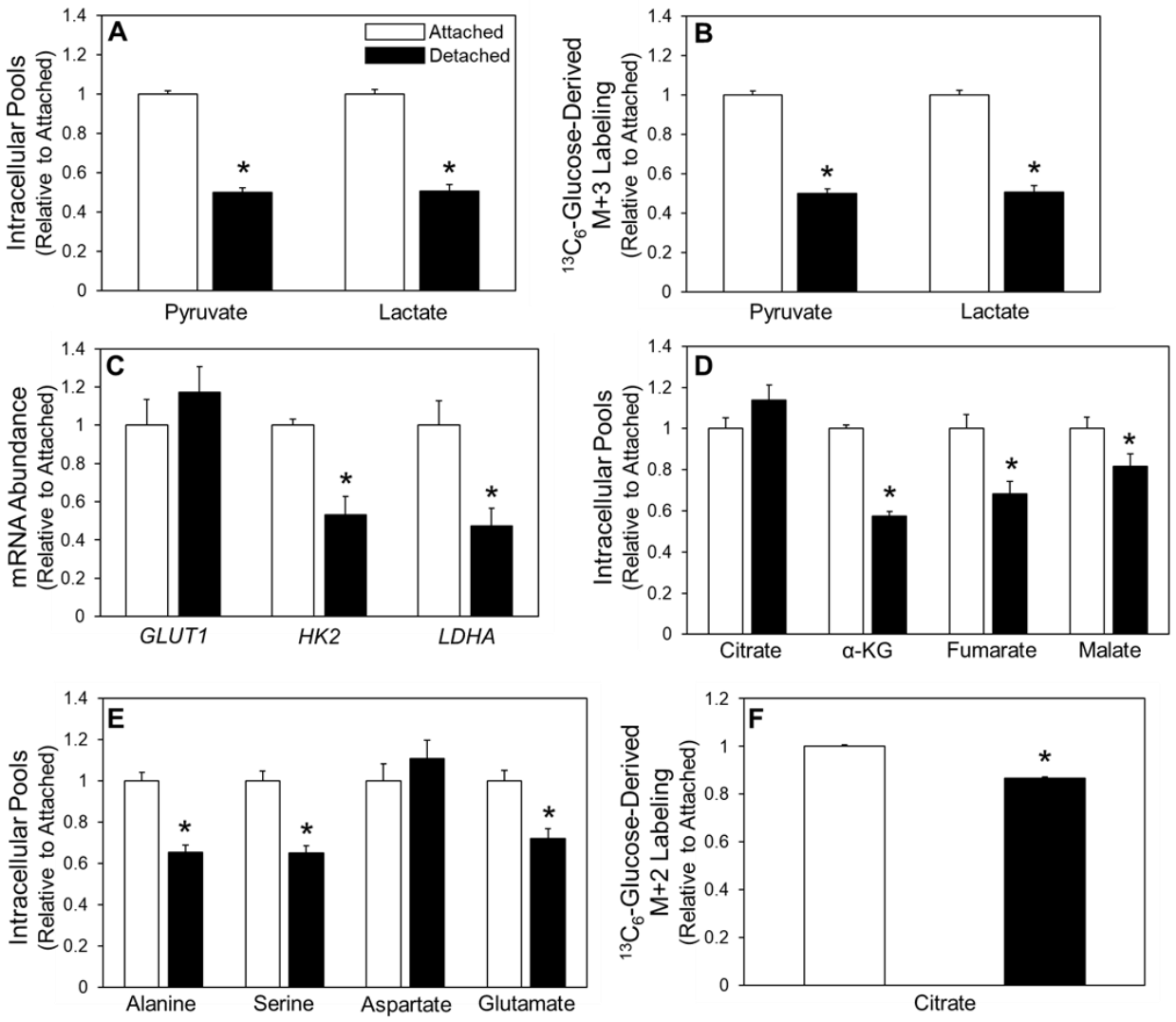

Figure 2.3. Glucose metabolism in attached and detached conditions.

Intracellular pools of pyruvate and lactate were measured (A) and $^{13}C_6$-Glucose was used to determine M+3 labeling of pyruvate and lactate (B) mRNA abundance of enzymes in glucose metabolism was assessed (C) intracellular pools of citrate, αKG, fumarate, and malate were measured (D) intracellular pools of alanine, serine, aspartate, and glutamate were measured (E) $^{13}C_6$-Glucose was used to determine M+2 labeling of citrate (F) in attached and detached MCF10A-ras cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to attached cells.

Replenishment of the oxaloacetate pool to support the TCA cycle can be mediated by PC activity, which synthesizes oxaloacetate from pyruvate and bicarbonate. Because pool sizes of the downstream metabolites of oxaloacetate, citrate and aspartate, were unchanged following detachment, PC activity was assessed. Compared to attached cells, detached cells significantly

increased PC mRNA abundance (Figure 2.4A) and protein expression (Figure 2.4B). Further, the flux of $^{13}C_6$-glucose to M+3 labeled malate and aspartate was significantly higher in detached cells, demonstrating an increase in PC activity. However, there was significantly decreased flux to M+3 citrate in detached cells (Figure 2.4C). These data demonstrate a preferential utilization of PC-derived oxaloacetate into malate and aspartate, rather than citrate, in detached cells. These results suggest that in detached cells, there is reduced forward TCA cycle flux that would replenish oxaloacetate from non-PC sources and are consistent with the decreased glutamine flux into the TCA cycle.

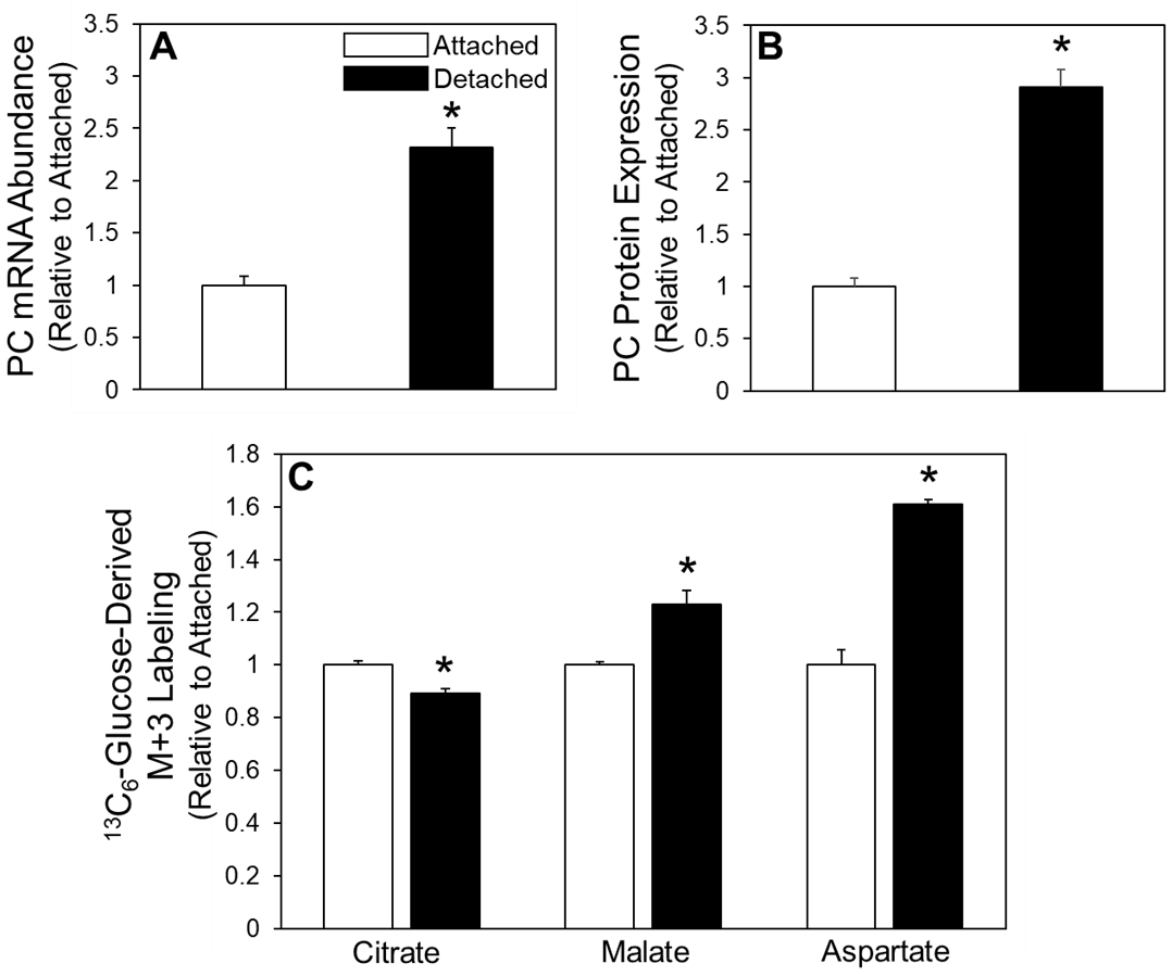

Figure 2.4. Effect of detachment on PC expression and activity.

mRNA abundance of PC (A) and protein abundance of PC was assessed. (B) PC activity determined from $^{13}C_6$-Glucose-derived M+3 labeled citrate, malate, and aspartate (C) in attached and detached MCF10A-ras cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to attached cells.

Due to the increase in PC activity and shift in glucose utilization, the requirement of PC activity for detached cell survival was assessed. Dox treatment of attached Dox-inducible shPC MCF10A-*ras* cells reduced PC mRNA abundance by 73% (Figure 2.5A). PC depletion significantly decreased detached cell viability by 13% (Figure 2.5B). Treatment of PC-depleted cells with exogenous oxaloacetate (2 mM), the product of the PC reaction, rescued cell viability to a level similar to that of PC-expressing cells (Figure 2.5B). In addition, treating PC-depleted cells with 2.5 mM aspartate, a downstream metabolite of oxaloacetate produced via mitochondrial GOT2 activity, also rescued viability to that of PC-expressing cells (Figure 2.5C).

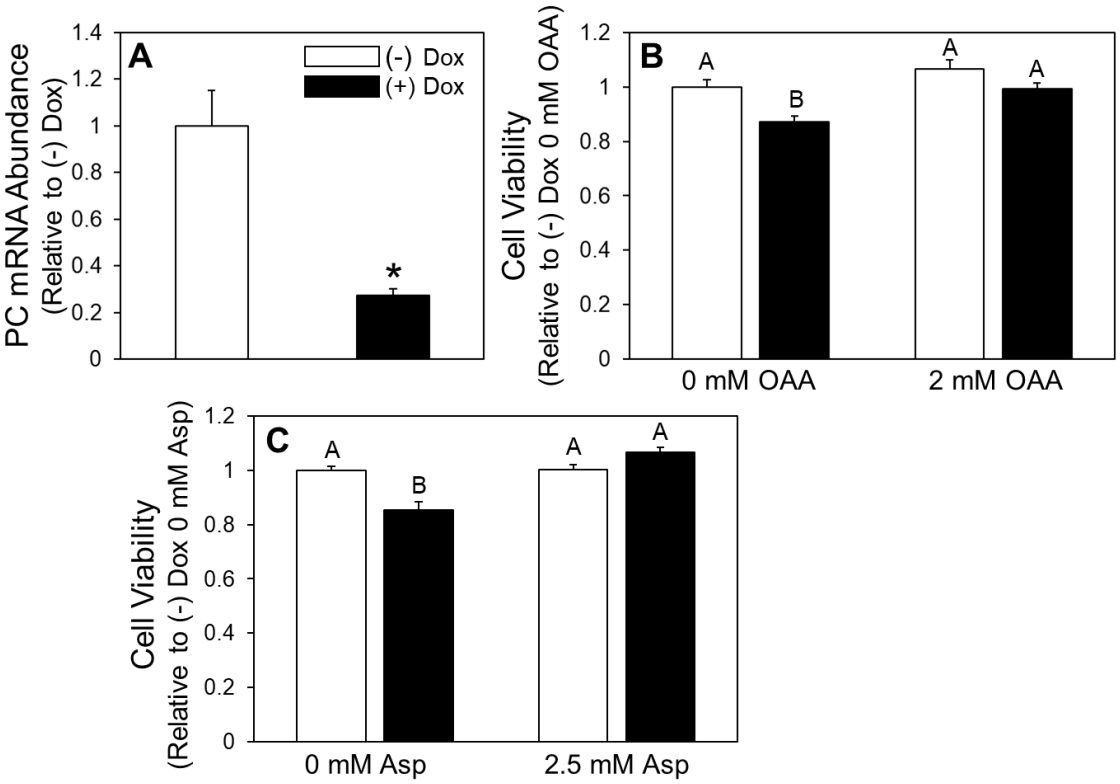

Figure 2.5. Detached cell viability with PC depletion.

mRNA abundance of PC in attached shPC Dox-inducible MCF10A-*ras* cells treated with and without Dox (A) Viability with 2 mM OAA (B) and 2.5 mM Asp supplementation (C) in detached Dox-inducible PC knockdown MCF10A-*ras* cells treated with and without Dox. Results are expressed as ± SEM. Asterisk (*) or different letters indicates significance $P < 0.05$.

Previous work demonstrated that metastatic MCF10CA1a cells have five-fold higher PC expression than MCF10A-*ras* cells (16). Given the increased PC expression observed in cells with higher metastatic capability than the MCF10A-*ras* cells and the requirement for anchorage independence in metastatic cells, the effect of PC overexpression in MCF10A-*ras* cells on cell viability in detached conditions was assessed. Results demonstrate that overexpressing PC in MCF10A-*ras* cells significantly increased their viability in detached conditions compared to detached control cells (Figure 2.6A and 2.6B).

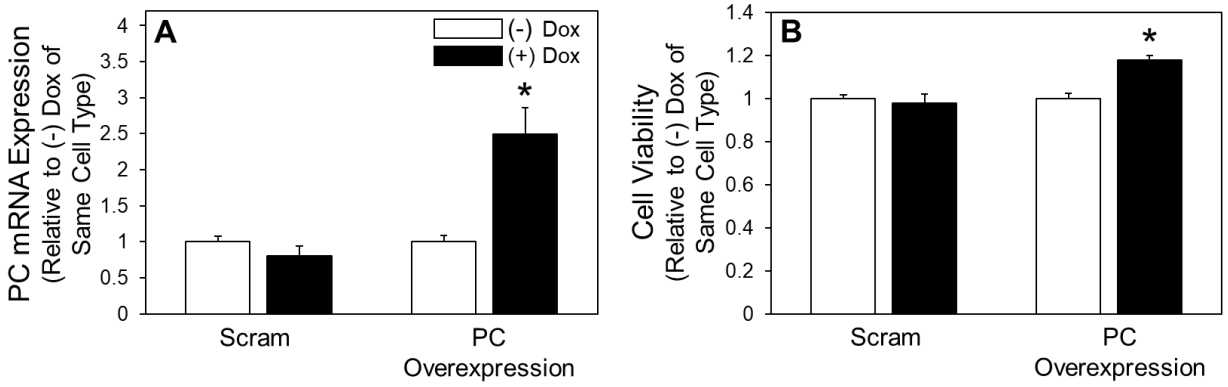

Figure 2.6. Effect of PC overexpression on detached cell viability.

mRNA abundance of PC of attached Dox-inducible PC overexpressing and Dox-inducible scram MCF10A-*ras* cells treated with Dox was assessed (A) Viability with and without Dox treatment (B) of detached Dox-inducible PC overexpressing and Dox-inducible scram MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to (-) Dox of the same cell type.

A recent study identified that glutamine metabolism is required for the survival of detached liver cancer cells (20), and a PC "switch" has been proposed to describe the metabolic flexibility of cancer cells where depletion of glutamine from the microenvironment increases PC activity. To test whether a switch to PC-mediated anaplerosis due to reduced glutamine availability occurs in detachment, detached cells were treated with a membrane-permeable analog of α-ketoglutarate, dimethyl-α-ketoglutarate (DM-αKG), and PC mRNA levels measured. Treatment with DM-αKG, which is the downstream metabolite of glutamine that enters the TCA cycle, decreased PC mRNA abundance in detached cells (Figure 2.7A). These data support that detachment in MCF10A-*ras*

cells reprograms cell metabolism to suppress glutamine flux into the TCA cycle and "switch" to preferential utilization of PC for TCA cycle anaplerosis.

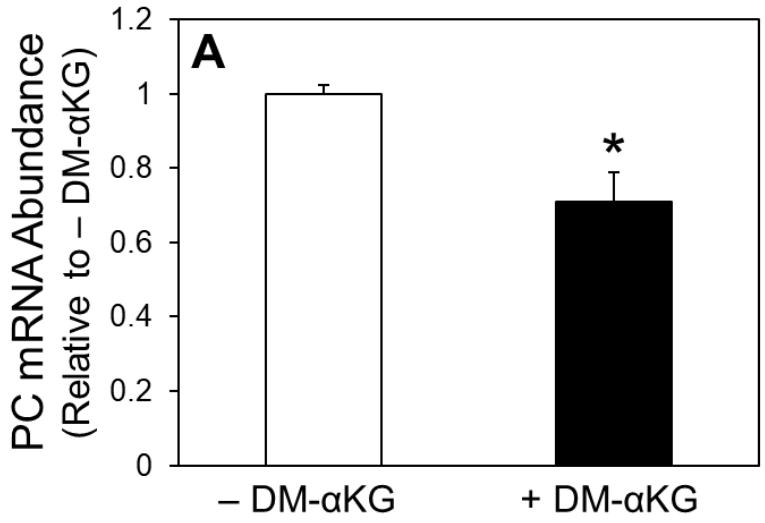

Figure 2.7. Dimethyl α-ketoglutarate's effect on PC expression.

mRNA abundance of PC of detached MCF10A-*ras* cells supplemented with 2 mM dimethyl α-ketoglutarate (DM-αKG) was assessed (A). Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to (–) DM-αKG.

2.5 Discussion

Metastasis of cancer cells is a multistep process, which includes survival in ECM detachment (6). Thus, tumor cells that demonstrate anchorage-independent growth possess a survival advantage. Detachment from ECM in non-transformed epithelial cells induces cellular changes that suppress flux of metabolites, including glucose and glutamine, into energy-producing pathways such as glycolysis and the TCA cycle which contributes to reduced viability in ECM detached conditions (4). Compared to non-transformed cells, oncogenic transformation rescues glucose and glutamine flux into the TCA cycle in detached conditions (10). The flux of metabolites through the TCA cycle generates the coenzymes nicotinamide adenine dinucleotide or flavin adenine dinucleotide, which are then oxidized at the electron transport chain for ATP production, collectively suggesting that enhanced flux of nutrients through the TCA cycle contributes to anchorage independence in transformed cells. Additionally, intermediates of the TCA cycle are

utilized by cells to mediate biosynthetic reactions required for cell proliferation, such as amino acids and lipids. The present study demonstrates that detached MCF10A-*ras* cells display decreased glucose metabolism through glycolysis and decreased anaplerosis from glutamine compared to attached cells. However, detached cells specifically display increased flux of pyruvate, the end product of glycolysis, to oxaloacetate through upregulated expression and activity of PC.

Previous studies identified that PC is required for breast cancer metastasis to the lung. For example, in an *in vivo* model of breast cancer, metastatic lesions at the lung contained high levels of PC expression and activity (15). Additionally PC was required for breast to lung metastasis in an animal model (13). The organotropic role of PC in breast to lung metastasis is consistent with previous literature showing a cellular requirement for PC activity in primary lung tumors (21). Given the requirement for PC in both primary and metastatic cancer cell growth in the lung, these data may highlight PC as an attractive therapeutic target. The present work identified a novel mechanism by which detached breast cancer cells reprogram energy metabolism to increase the expression and activity of PC. Further, depletion of PC in detachment impairs cell survival, suggesting a specific molecular mechanism by which PC mediates breast to lung metastasis. Additionally, overexpression of PC, a characteristic of metastatic breast cancer (14,16), improves detached viability of MCF10A-*ras* breast epithelial cells. Collectively, these results suggest that increased PC expression may promote metastasis by supporting survival in ECM detached conditions throughout the metastatic cascade.

Metabolic flexibility has recently been identified as a contributor to the successful metastasis of cancer cells (22). As cancer cells progress through metastasis, they require adaption to changing nutrient conditions in variable microenvironmental conditions. As a compensatory mechanism, PC may play a crucial role in conferring metabolic flexibility to cancer cells. For example, in healthy BALB/c mice, the ratio of pyruvate/glutamine in the lung interstitial fluid is three times higher than in serum (15). This varying ratio of pyruvate/glutamine may require cancer cells to switch from utilizing glutamine as a means of replenishing the TCA cycle to increasing anaplerosis through PC activity for successful colonization. Indeed, PC activity was increased in lung metastases compared to primary breast tumors (15). In the current study, a PC switch was demonstrated in detached cells as detachment resulted in decreased glutamine incorporation into the TCA cycle and increased flux of glucose into aspartate and malate pools through PC activity. Further support for metabolic reprogramming in favor of PC over glutamine was demonstrated by

the decreased mRNA abundance of PC following the addition of DM-αKG to detached cells. These results demonstrate that replenishing the TCA cycle with a metabolite of glutamine reverses the increase in PC abundance. Therefore, detached cancer cells display metabolic flexibility by increasing PC expression and activity in response to reduced glutamine metabolism.

PC's role in aspartate biosynthesis has been well documented in cancer models. In renal cell carcinoma with succinate dehydrogenase (SDH) ablation, the addition of aspartate rescues cells from PC depletion (23). Consistent with these results, SDHA or SDHB inhibition resulted in an increase in PC activity in both neuroendocrine and prostate cancer cells (24,25). In both of these models, increased PC activity following SDH inhibition resulted in the replenishment of aspartate. Thus, inhibition of the forward TCA cycle by inhibition of SDH activity increases PC-mediated TCA cycle anaplerosis. Consistent with this, the current study identified increased PC activity in detached cells indicated by enrichment of $^{13}C_6$-glucose derived M+3 labeling patterns in the intracellular pools of aspartate and malate. These results suggest that detached cells use PC activity to compensate for an inability to synthesize oxaloacetate from glutamine through the forward TCA cycle.

In summary, these findings demonstrate that detachment of MCF10A-*ras* cells leads to a decreased flux of glucose and glutamine into the TCA cycle compared to their attached counterparts. However, PC expression and activity are increased in detached cells, and this effect is reversed with exogenous addition of the TCA cycle intermediate DM-αKG. These results indicate that PC plays a critical role in promoting the survival of cells in matrix detached conditions and highlights a potential therapeutic target for the prevention of cancer metastasis.

2.6 Acknowledgements

The authors acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute.

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CHAPTER 3. 1,25-DIHYDROXYVITAMIN D REDUCES MCF10A-RAS CELL SURVIVAL IN EXTRACELLULAR MATRIX DETACHED CONDITIONS THROUGH REGULATION OF PYRUVATE CARBOXYLASE

3.1 Abstract

Metabolic reprogramming to adjust to varying cellular environments is an emerging hallmark of cancer. Throughout the process of metastasis, cancer cells gain anchorage independence which confers survival and proliferative characteristics when detached from the extracellular matrix (ECM). Previous work demonstrates that the bioactive metabolite of vitamin D, 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D), suppresses cancer progression, potentially through regulation of energy metabolism. The purpose of the present studies were to determine the impact and mechanism of 1,25$(OH)_2$D effects on cell survival in ECM detached conditions. Treatment of MCF10A-*ras* breast cancer cells with 1,25$(OH)_2$D reduces the viability of cells in detached conditions by 11%. Glucose and glutamine metabolic flux analysis utilized $^{13}C_5$-glutamine and $^{13}C_6$-glucose tracers, respectively. Results show that 1,25$(OH)_2$D treatment reduced glutamine flux into downstream metabolites glutamate (6%), malate (8%), and aspartate (13%), and reduces glucose flux through the anaplerotic enzyme pyruvate carboxylase (PC) determined by decreased $^{13}C_6$-glucose derived M+3 labeling in citrate (9%) and aspartate (23%) pools. However, 1,25$(OH)_2$D treatment did not affect pool sizes or $^{13}C_6$-glucose flux into the end products of glycolysis, pyruvate and lactate. Further, treatment of detached cells with 1,25$(OH)_2$D reduced PC mRNA abundance and protein expression by 64% and 57%, respectively, relative to vehicle-treated detached cells. While depletion of PC with doxycycline-inducible shRNA reduced detached cell viability, PC knockdown in combination with 1,25$(OH)_2$D treatment did not additionally affect the viability of detached cells. Further, PC overexpression improved detached cell viability, and inhibited the effect of 1,25$(OH)_2$D on detached breast cancer cell survival, suggesting that 1,25$(OH)_2$D mediates its effects in detachment through regulation of PC expression. These results suggest that inhibition of PC by 1,25$(OH)_2$D suppresses cancer cell anchorage independence and may provide a target to inhibit breast cancer metastasis.

3.2 Introduction

Breast cancer is estimated to be the most common cancer diagnosed among American women in 2021 (1). In addition, breast cancer is the second most fatal cancer for women, with metastatic disease accounting for the majority of breast cancer deaths (1). Because of the prevalence and mortality of breast cancer, it is critical to identify strategies to not only prevent breast cancer incidence but also improve breast cancer patient survival by inhibiting metastasis.

Metastasis follows a step-wise progression which includes cellular changes at the primary site, invasion into surrounding tissue, systemic dissemination, and tumor formation at the distal site (2). Acquiring anchorage independence, or the ability to survive in the absence of extracellular matrix (ECM) attachment, is required for progression through the metastatic cascade (3). Survival in ECM detachment is facilitated, in part, by reprogrammed energy metabolism in cancer cells (4,5). For example, compared to matrix-detached non-transformed breast epithelial cells, cancer cells increased glucose and glutamine energy metabolism (6). Further, detached MDA-MB-231 cells increased expression of the antioxidant enzyme catalase, which was required for fatty acid oxidation-derived ATP production and anchorage-independent growth (7). Thus, reprogrammed energy metabolism in response to matrix detachment mediates anchorage-independent survival in breast cancer cells and is a potential target for prevention of metastasis.

A growing body of evidence recognizes vitamin D's preventatitve actions against breast cancer development. Vitamin D is either acquired from the diet or synthesized from 7-dehydrocholesterol in the skin upon ultraviolet B exposure from the sun (8). Two sequential hydroxylation steps are required for synthesis of the primary hormonal and active form in the blood, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$). The action of $1,25(OH)_2D$ is mediated through binding to the vitamin D receptor (VDR) expressed in cells regulated by vitamin D. Once bound to $1,25(OH)_2D$, VDR heterodimerizes with the retinoid X receptor, and the heterodimer subsequently binds to vitamin D response elements (VDREs) in the promoter regions of vitamin D-regulated genes. Thus, vitamin D, through the actions of its most active metabolite $1,25(OH)_2D$, may inhibit breast cancer development through transcriptional regulation of genes.

A relationship between vitamin D status and breast cancer incidence was first identified in observational studies where an inverse association was observed between annual ultraviolet light exposure from the sun and breast cancer incidence (9,10). This observation suggested that vitamin D, which can be synthesized in the skin upon ultraviolet B exposure, may be involved in preventing

breast cancer. For example, higher vitamin D status is associated with a lower odds ratio of breast cancer development (11). In addition, the Women's Health Initiative (WHI) study examined the effect of vitamin D on breast cancer where the participants were given 400 international units of vitamin D/day with an average follow-up of seven years (12). Initial results from the WHI did not demonstrate a lower incidence in breast cancer in women in the vitamin D supplementation cohort. However, in a reanalysis of the WHI study where participants were stratified into groups according to personal supplementation, results identified that vitamin D reduced the risk of breast cancer in the group of participants with no additional vitamin D intake from personal supplementation (13). Therefore, evidence supports that sufficient vitamin D status prevents the development of breast cancer, reducing the incidence.

In addition to preventing initial breast cancer development, vitamin D is also implicated in regulating the metastatic process. For example, a recent study identified an inverse correlation between vitamin D status and metastasis in breast cancer patients (14). Similarly, in the Vitamin D and Omega-3 Trial (VITAL), vitamin D supplementation reduced breast cancer mortality when the first two years of follow-up were excluded, suggesting that vitamin D supplementation may reduce the progression of cancer (15). Further, a meta-analysis demonstrated that higher vitamin D status was associated with reduced breast cancer mortality providing additional evidence that vitamin D may be utilized to prevent breast cancer progression to a fatal metastatic disease state (16,17). Several potential mechanisms by which vitamin D prevents tumor formation and subsequent cancer progression have been identified, including increased cancer cell cycle arrest and apoptosis (9,18); however, the role of $1,25(OH)_2D$ in regulating cellular bioenergetics has not been elucidated.

Evidence supports that $1,25(OH)_2D$ regulates energy metabolism in malignant breast epithelial cells (19-21), including through the enzyme pyruvate carboxylase (PC). PC mediates the conversion of pyruvate to the tricarboxylic acid (TCA) cycle intermediate oxaloacetate, which is a precursor for reactions involved in energy production in the TCA cycle, fatty acid synthesis, and oxidative stress protection. Previous research demonstrates that treatment with $1,25(OH)_2D$ reduced lipid accumulation and fatty acid synthesis in MCF10CA1a breast cancer cells through direct regulation of PC (21). In addition, PC is also involved in oxidative stress protection, which is essential for increased fatty acid oxidation, in MCF10A-*ras* cells, thus $1,25(OH)_2D$ treatment acted as a prooxidant by reducing PC expression (22). In the present study, it was hypothesized

that in detached breast cancer cells 1,25$(OH)_2$D reduces expression of PC, thereby reducing glucose-derived anaplerosis and leading to reduced cell survival.

3.3 Methods

3.3.1 Cell Culture

Harvey-*ras* transformed MCF10A-*ras* cells were a gift from Michael Kinch, Purdue University. Cells were grown in an incubator set to 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) with 5 mM glucose, 2.5 mM glutamine, and no sodium pyruvate. Media was supplemented with 5% horse serum (Gibco, Waltham MA), 1% penicillin/streptomycin (Gibco), 10 mg/L insulin (Sigma), 50 µg/L cholera toxin (Sigma), 20 µg/L epidermal growth factor (Sigma), and 50 mg/L hydrocortisone (Sigma). Cells were pretreated for 72 hours with 10 nM 1,25$(OH)_2$D (Biomol, Plymouth Meeting, PA) in 100% ethanol vehicle (final concentration <0.1%), with media changed every 24 hours.

3.3.2 Poly-HEMA Coated Plates

Seventy µL/cm^2 of 20 mg/mL Poly(2-hydroxyethyl methacrylate) (Poly-HEMA, Sigma) in 95% ethanol was used to coat 96-well plates and 100 mm dishes. Following addition of Poly-HEMA, the plates or dishes were dried overnight under ultraviolet light. Before use, Poly-HEMA coated plates and dishes were rinsed twice with sterile 1x calcium/magnesium-free phosphate-buffered saline (PBS).

3.3.3 MTT Assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay according to the manufacturer's instructions. Briefly, following 72-hour pretreatment, cells were plated into Poly-HEMA coated 96-well plates at 20k cells/well with corresponding treatments. After 40 hours, MTT solution was added, allowed to incubate for 2 hours and cells solubilized in dimethylsulfoxide. Absorbance was measured at 570 nm with a spectrophotometer.

3.3.4 Flow Cytometry

Following treatment and placement into detached conditions, a cellular suspension of 1,000,000 cells was harvested into calcium-rich Annexin V binding buffer containing 5 µL Annexin V (Invitrogen, Carlsbad, CA) and 10 mg/mL propidium iodide (Sigma). A BD Fortessa LSR SORP Cell Analyzer with a 488 nm laser was used to analyze apoptosis in cells. Results were analyzed with FCS Express software (Pasadena, CA). Results are shown as the percent of total cells in each apoptotic stage.

3.3.5 RNA Isolation and Analysis

Pretreated cells were plated into 100 mm Poly-HEMA coated dishes at 1500k cells/dish. After 40 hours, cells were harvested and isolated using Tri-Reageant (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Reverse transcription of RNA was performed using MMLV reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed in a LightCycler 480 instrument using LightCycler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN). mRNA abundance of target genes was normalized to 18S abundance using the comparative Ct method ($2^{-\Delta Ct}$), and results represent arbitrary units.

3.3.6 Glucose and Glutamine Flux Analysis

Pretreated cells were grown in detached conditions for 40 hours, and cell media was changed to 100% (5 mM) $^{13}C_6$-glucose, or 100% (2.5 mM) $^{13}C_5$-glutamine containing media. After 2 hours, in detached conditions, cells were harvested into 70°C 70% ethanol and 1 µg of the internal standard, norvaline. Metabolites were extracted from the cells by a 5-minute 95°C incubation and the supernatant dried. The cell pellet was used to quantify protein with a bicinchoninic acid (BCA Assay) (ThermoFisher, Waltham, MA). Derivatization of metabolites from the dried supernatant was performed as described previously using methoxylamine hydrochloride in pyridine (23). Metabolites were analyzed using gas chromatography-mass spectrometry with a TG-5MS gas chromatography column and Thermo TSQ 8000 triple quadrupole mass spectrometer. Pool sizes were calculated by dividing total metabolite area under the curve (AUC) by norvaline AUC and mg protein to account for variations in recovery and cell quantity.

3.3.7 Western Blot Analysis

For protein analysis, detached cells were washed twice with 1x calcium/magnesium-free PBS. Following rinses, cells were harvested into RIPA lysis buffer, sonicated for 15 minutes, and centrifuged at 12,000 revolutions per minute for 15 minutes. The supernatant was collected and a BCA assay used to quantify protein. Protein was resolved by electrophoresis on a 4-15% gradient polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). PC and actin primary antibodies were purchased from Sigma, and a Li-Cor Odyssey imaging system was used for the detection of antigen-antibody complexes.

3.3.8 PC Knockdown and Overexpression

PC knockdown in cells utilized TRIPZ lentiviral human shRNA which targeted PC and was doxycycline (Dox) inducible, purchased from GE Dharmacon (Lafayette, CO). PC overexpression utilized Dox inducible lentiviral plasmids containing the human PC gene (NM_000920.2) were purchased from VectorBuilder (Chicago, IL). HEK293T cells were transduced with lentiviral plasmids using polyethylamine, and lentiviral particles harvested. Transfection of MCF10A-*ras* cells took place for 48 hours with 10 µg/mL polybrene, followed by puromycin (5 mg/mL) selection. PC overexpressing, stably transfected cells were selected with puromycin followed by hygromycin (450 ug/mL). PC knockdown or overexpressimg cells were pretreated with Dox (0.5 ug/mL) for 3 days with an additional cotreatment of vehicle or 1,25$(OH)_2$D where indicated.

3.3.9 Statistical Analysis

Results are displayed using mean ± S.E.M. Independent samples *t*-test was used to calculate comparisons between two groups. Letters indicate differences determined using analysis of variance (ANOVA) with statistical significance of $P < 0.05$.

Table 2. Primers used for qRT-PCR.

18S	Forward: 5'- TTAGAGTGTTCAAAGCAGGCCCGA-3' Reverse: 5'- TCTTGGCAAATGCTTTCGCTC-3'
ASCT2	Forward: 5'- TGAACATCCTGGGCTTGGTAG-3' Reverse: 5'- AGCAGGCAGCACAGAATGTAC-3'
GLUD1	Forward: 5'- TTAGAGTGTTCAAAGCAGGCCCGA-3' Reverse: 5'- TCTTGGCAAATGCTTTCGCTC-3'
GOT1	Forward: 5'-CAACTGGGATTGACCCAACT -3' Reverse: 5'- GGAACAGAAACCGGTGCTT -3'
GOT2	Forward: 5'-ACCCATGTGGAAATGGGACC -3' Reverse: 5'- GACTTCGCTGTTCTCACCCA-3'
GLUT1	Forward: 5'-TATCGTCAACACGGCCTTCACTGT -3' Reverse: 5'- CACAAAGCCAAAGATGGCCACGAT -3'
HK2	Forward: 5'-CTGCAGCGCATCAAGGAGAACAAA -3' Reverse: 5'- ACGGTCTTATGTAGACGCTTGGCA -3'
LDHA	Forward: 5'- TGGTCCAGCGTAACGTGAACATCT -3' Reverse: 5'-TTGCAACCGCTTCCAATAACACGG -3'
PC	Forward: 5'- ATGTTGCCCACAACTTCAGCAAGC -3' Reverse: 5'- AGTTGAGGGAGTCAAACACACGGA-3'

3.4 Results

In order to determine the effect of 1,25(OH)$_2$D in detached conditions, the metabolite's effect on viability was measured. After 40 hours in detachment, MCF10A-*ras* cell viability was decreased following pretreatment with 1,25(OH)$_2$D compared to vehicle-treated cells (Figure 3.1A). In addition, 1,25(OH)$_2$D pretreatment resulted in significantly fewer live cells, and significantly more cells undergoing apoptosis (Figure 3.1B). Together these results demonstrate that 1,25(OH)$_2$D reduces the viability of MCF10A-*ras* cells by inducing apoptosis.

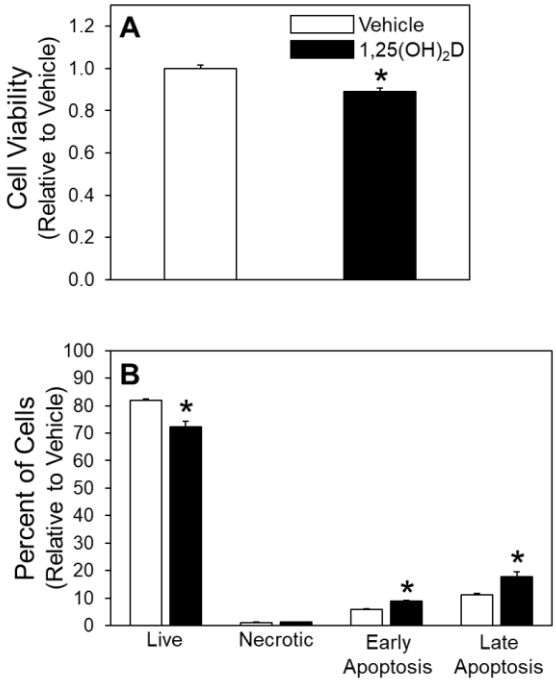

Figure 3.1. Effect of 1,25(OH)$_2$D on detached cell viability.

Following 3-day vehicle or 1,25(OH)$_2$D pretreatment, viability of MCF10A-*ras* cells were assessed after 40 hours in detached conditions for (A) viability by MTT, and (B) apoptosis by flow cytometry. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to vehicle.

Previous studies confirmed an effect of 1,25(OH)$_2$D on regulating energy metabolism in breast cancer cell models (19-21). Given this effect, 1,25(OH)$_2$D regulation of glutamine flux and metabolism in detached cells was examined. Pretreatment with 1,25(OH)$_2$D reduced flux of a universally labeled $^{13}C_5$-glutamine tracer to glutamate, malate, and aspartate, determined by decreased M+5 and M+4 labeling patterns, respectively (Figure 3.2A and 3.2B). In addition, 1,25(OH)$_2$D-treatment did not change *ASCT2* mRNA abundance, the neutral amino acid exchanger

which mediates extracellular glutamine transport of detached cells compared to detached vehicle-treated cells (Figure 3.2C). Additionally, in detached cells 1,25(OH)$_2$D-treatment did not change mRNA abundance of glutamate dehydrogenase (GLUD1) and glutamic-oxaloacetic transferase (GOT) 1 and 2, enzymes involved in the metabolism of glutamate, a downstream glutamine metabolite (Figure 3.2C). While glutamine flux was decreased by 1,25(OH)$_2$D treatment, mRNA abundance of key glutamine metabolizing enzymes was unchanged, suggesting that 1,25(OH)$_2$D regulates glutamine metabolism in detached cancer cells through a mechanism distinct from direct regulation of glutamine metabolizing genes.

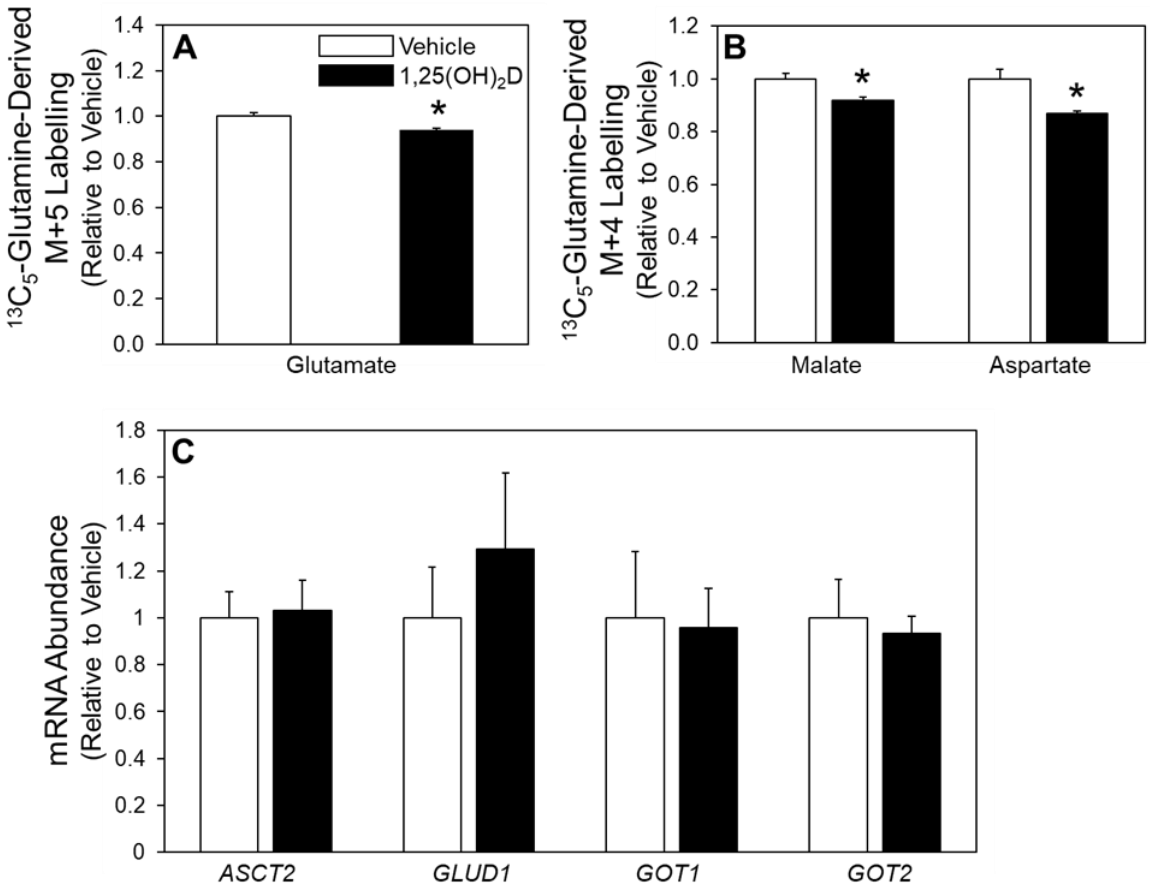

Figure 3.2. Glutamine metabolism in detached cells treated with 1,25(OH)$_2$D.

Following 3 day vehicle or 1,25(OH)$_2$D pretreatment, $^{13}C_5$-Glutamine was used to determine (A) M+5 labeling of glutamate, and (B) M+4 malate and aspartate downstream metabolites (C) mRNA abundance of enzymes in glutamine metabolism was assessed in detached MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to vehicle.

Similar to glutamine metabolism, 1,25$(OH)_2$D was hypothesized to inhibit glucose metabolism in detached MCF10A-*ras* cells, similar to its effects in attached cells (19). Interestingly, 1,25$(OH)_2$D treated cells did not change pyruvate and lactate intracellular pool sizes (Figure 3.3A). Metabolic flux analysis was performed using a $^{13}C_6$-glucose tracer to assess glucose flux. Similarly, there were no differences in $^{13}C_6$-glucose flux into pyruvate and lactate in 1,25$(OH)_2$D treated detached cells relative to vehicle determined by M+3 labelling patterns (Figure 3.3B). In accordance with the $^{13}C_6$-glucose flux results, 1,25$(OH)_2$D did not affect mRNA abundance of glucose metabolizing enzymes. The genes that were measured include the glucose transporter 1 (GLUT1) which transports extracellular glucose into the cells, hexokinase 2 (HK2) which encodes for the enzyme which mediates the conversion of glucose to glucose-6-phosphate in the first step of glycolysis, and lactate dehydrogenase A (LDHA) which catalyzes the conversion of lactate from pyruvate (Figure 3.3C). In accordance with the glucose flux results, 1,25$(OH)_2$D pretreatment had no effect on mRNA abundance of any of the glucose-related genes measured, suggesting that glycolysis is not regulated by 1,25$(OH)_2$D in detached cells.

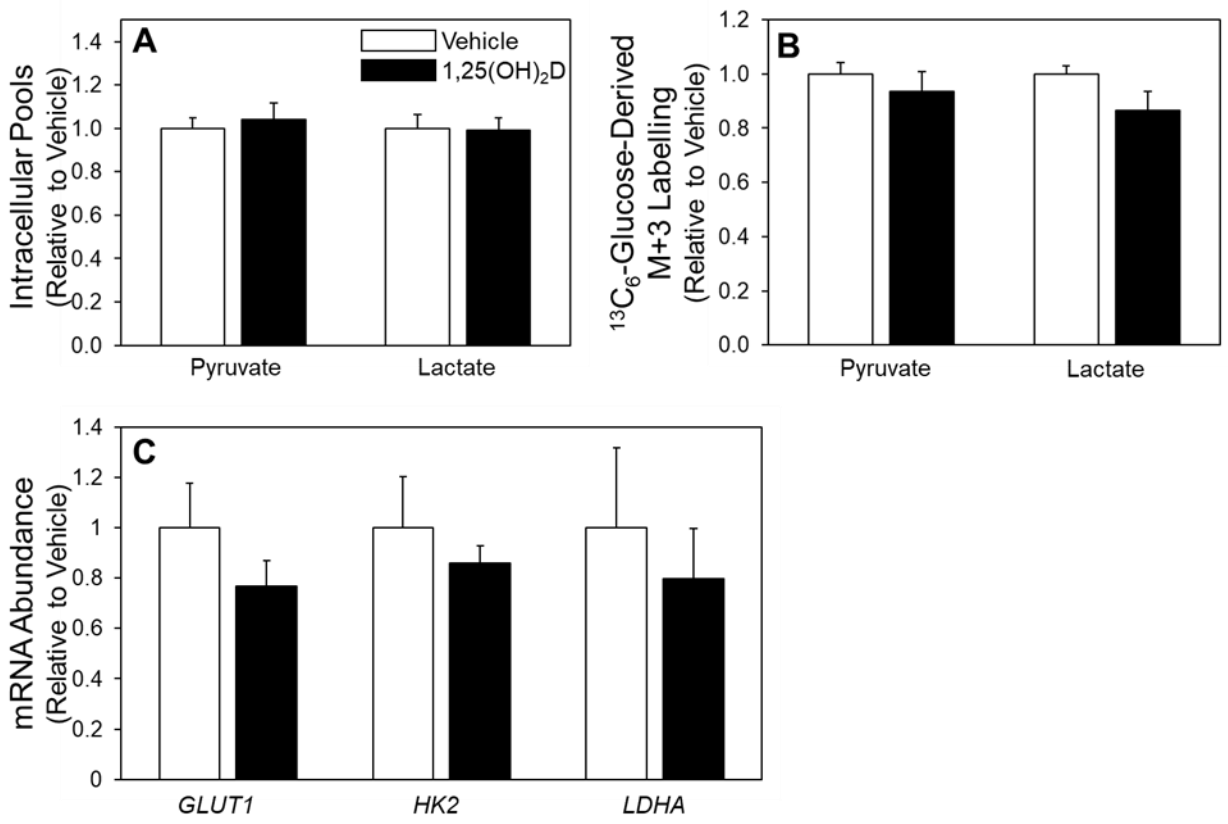

Figure 3.3. Glucose metabolism in detached cells treated with 1,25(OH)$_2$D.

Following 3 day vehicle or 1,25(OH)$_2$D pretreatment (A) intracellular pools of pyruvate and lactate were measured and (B) $^{13}C_6$-Glucose was used to determine M+3 labeling of pyruvate and lactate (C) mRNA abundance of enzymes in glucose metabolism was assessed in detached MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to vehicle.

Glucose replenishes the TCA cycle through pyruvate dehydrogenase (PDH) activity which synthesizes acetyl-CoA from pyruvate, or through PC activity which forms oxaloacetate from the carboxylation of pyruvate. To determine 1,25(OH)$_2$D's regulation of glucose-derived anaplerosis into the TCA cycle a $^{13}C_6$-glucose tracer was used. Treatment with 1,25(OH)$_2$D had no effect on glucose flux into the TCA cycle through PDH activity, determined by M+2 labeled citrate (Figure 3.4A). However, 1,25(OH)$_2$D reduced the entry of glucose-derived pyruvate into the TCA cycle through the PC reaction, determined by decreased M+3 labeled citrate and aspartate (Figure 3.4B). M+3 malate was unchanged in detached 1,25(OH)$_2$D pretreated cells compared to vehicle (Figure 3.4B), suggesting the 1,25(OH)$_2$D-induced reduction in PC activity primarily impacts a reduction

in citrate and aspartate pools. Consistent with the PC activity results, detached cell PC mRNA abundance and protein expression were also reduced with 1,25(OH)$_2$D pretreatment (Figure 3.5A and 3.5B). In sum, 1,25(OH)$_2$D reduced PC expression and activity in detached MCF10A-*ras* cells.

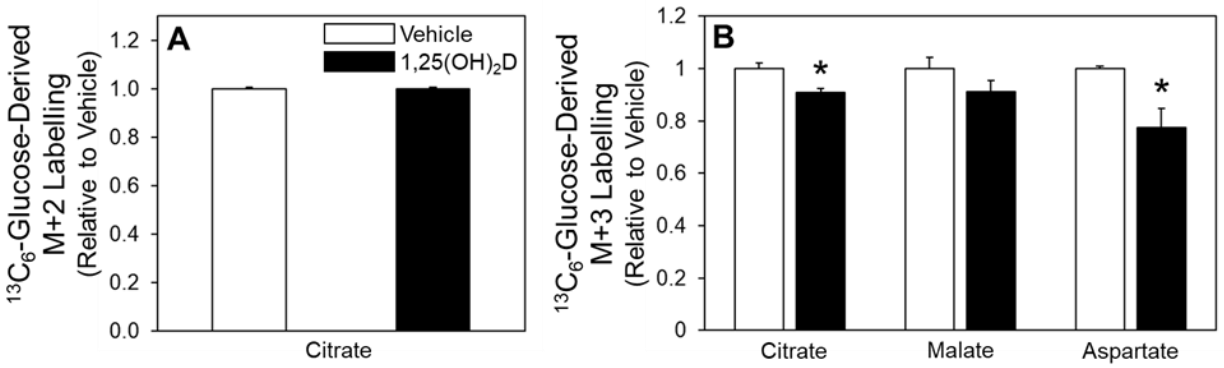

Figure 3.4. Effect of 1,25(OH)$_2$D on TCA cycle intermediates.

Following 3 day vehicle or 1,25(OH)$_2$D pretreatment $^{13}C_6$-Glucose was used to determine (A) M+2 labeling of citrate and (C) M+3 labeling of citrate, malate, and aspartate in detached MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates P < 0.05 relative to vehicle.

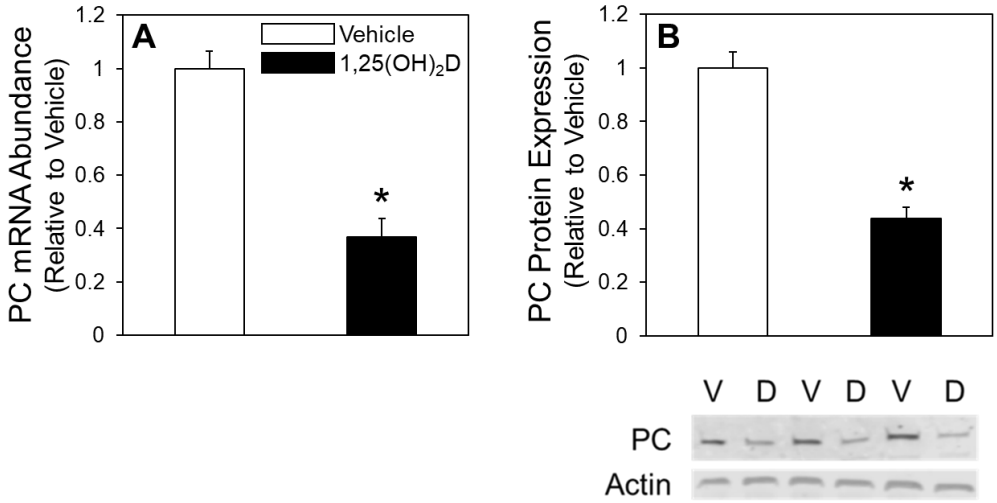

Figure 3.5. Glucose-derived anaplerosis in detached cells treated with 1,25(OH)$_2$D.

Following 3 day vehicle or 1,25(OH)$_2$D pretreatment $^{13}C_6$-Glucose was used to determine (A) PDH activity with M+2 labeling of citrate and (B) PC activity with M+3 labeling of citrate, malate, and aspartate in detached MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates P < 0.05 relative to vehicle.

Given that 1,25(OH)$_2$D downregulates PC in detached cells, the impact of this regulation on survival in detached conditions was evaluated. Dox-inducible shRNA PC knockdown and PC overexpressing MCF10A-*ras* cell lines were utilized to measure cell viability in detached conditions with 1,25(OH)$_2$D treatment (Figure 3.6 A and 3.C). PC depletion reduced cell viability to a similar extent as 1,25(OH)$_2$D treatment with no additional effect on detached cell viability with 1,25(OH)$_2$D treatment and PC knockdown together (Figure 3.6B). In addition, PC overexpression increased detached cell viability, and inhibited the 1,25(OH)$_2$D mediated decrease in detached cell viability (Figure 3.6D). These data demonstrate that 1,25(OH)$_2$D's negative effects on detached MCF10A-*ras* cell viability are dependent on PC downregulation.

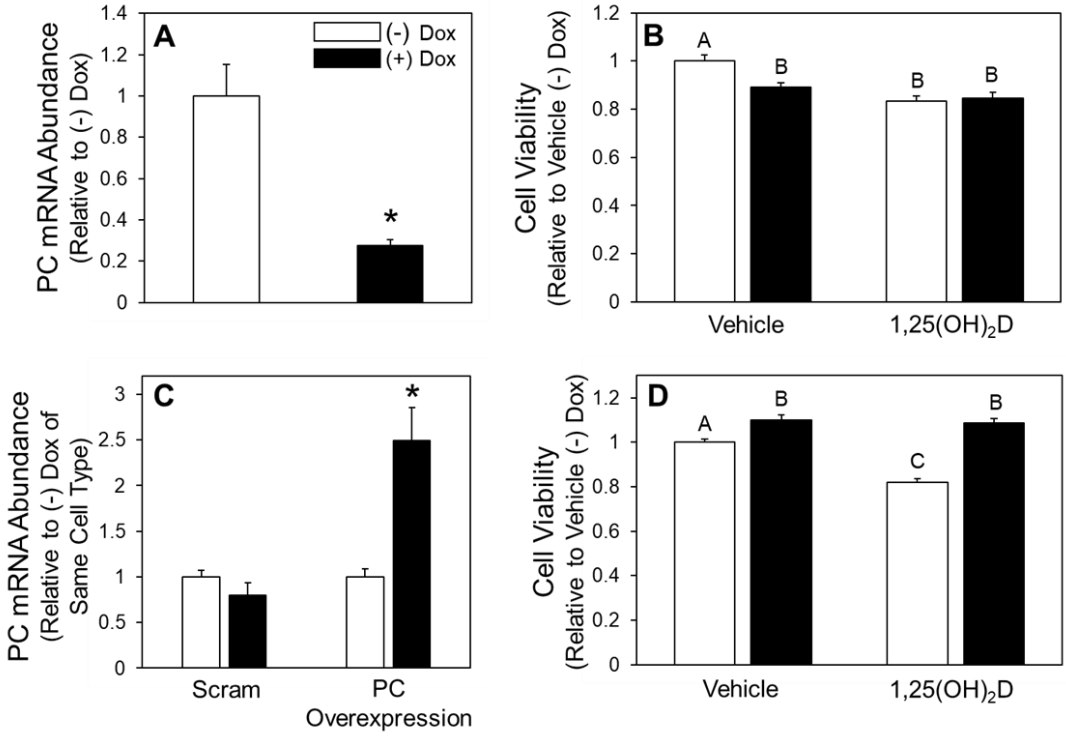

Figure 3.6. Cell viability with 1,25(OH)$_2$D and PC depletion or overexpression.

Following 3 day Dox treatment (A) PC mRNA abundance was measured in attached conditions (B) and MTT was utilized to assess cell viability of detached Dox-inducible PC shRNA MCF10A-*ras* cells with Dox treatment in combination with 1,25(OH)$_2$D. (C) PC mRNA abundance was determined following 3 day Dox treatment and (D) cell viability was assessed with MTT in Dox and 1,25(OH)$_2$D treated detached Dox-inducible PC overexpressing MCF10A-*ras* cells. Results are expressed as ± SEM. Asterisk (*) indicates $P < 0.05$ relative to vehicle (–) Dox of the same cell type.

3.5 Discussion

While breast cancer survival rates remain high when diagnosed early, it is estimated that between 25-50% of breast cancer patients will form metastatic tumors (1,24). Therefore, it is critical to identify safe and effective compounds that prevent metastasis as it results in the vast majority of breast cancer-related deaths (1). Throughout carcinogenesis, metastatic cancer cells acquire anchorage independence and reprogram energy metabolism, both of which support survival and growth at the primary and secondary site (25). The results of the present study demonstrate that the active metabolite of vitamin D, 1,25$(OH)_2$D, downregulation of PC expression reduces cell survival of detached breast cancer cells.

Recent studies have implicated PC in the promotion of breast to lung metastasis. For example, Christen et al. observed an increase in PC expression in metastatic tumors in the lung compared to the primary breast tumor (26). In addition, PC knockdown in the primary tumor significantly decreased the number of breast to lung metastatic outgrowths *in vivo* (27). Further support is provided by analysis of the Molecular Taxonomy of Breast Cancer International Consortium dataset where amplification of PC was detected in 16-30% of breast cancer patients, and PC amplification was associated with decreased patient survival suggesting PC's role in promoting metastasis (27). PC activity replenishes the TCA cycle intermediate, oxaloacetate, which likely supports cancer progression through energy production and synthesis of building blocks such as amino and fatty acids required for proliferation. Studies also suggest that PC expression confers metastatic properties, as PC expression was positively associated with greater invasive and migratory capabilities, and PC inhibition reduced invasion and migration in various breast cancer models (28). Cumulatively, these results demonstrate that PC expression is associated with a metastatic phenotype.

Although others have reported 1,25$(OH)_2$D's long-term negative effects on ECM detached cancer cell survival, a mechanism was not elucidated. For example, Pervin et al observed that SKBR3 breast cancer cells grown in detachment for four and seven days had reduced mammosphere formation with VDR overexpression, and that 1,25$(OH)_2$D treatment alone was sufficient to reduce sphere formation (29). In addition, 1,25$(OH)_2$D treatment reduced mammosphere formation in metastatic SUM159 breast epithelial cells grown in detached conditions for five days (30). Further, treatment with 1,25$(OH)_2$D reduced mammosphere formation after secondary and tertiary passaging, suggesting that 1,25$(OH)_2$D treatment may

prevent tumor resurgence. While the long-term reduction of detached mammosphere formation by $1,25(OH)_2D$ is promising, the mechanism by which $1,25(OH)_2D$ regulates detached cancer growth remained unknown. The present study identifies $1,25(OH)_2D$ regulation of anaplerosis by downregulation of PC as the mechanism by which $1,25(OH)_2D$ decreases viability in detached transformed breast epithelial cells.

While the current results also show a decrease in glutamine metabolism by decreased $^{13}C_5$-glutamine flux to glutamate, malate, and aspartate with $1,25(OH)_2D$, a $1,25(OH)_2D$ target gene was not determined. Gene regulation is the predominant mechanism by which $1,25(OH)_2D$ mediates its effects in target tissues. However, mRNA abundance of key glutamine-metabolizing enzymes was unchanged with $1,25(OH)_2D$ treatment in detached cells, suggesting that $1,25(OH)_2D$ regulates glutamine metabolism indirectly. A potential mechanism may be through reducing signaling through the adenosine monophosphate-activated protein kinase (AMPK) pathway which regulates energy metabolism as previously described (31). The results from the current study are contrary to earlier studies where in attached MCF10A-*ras* cells $1,25(OH)_2D$ treatment reduced glutamine metabolism through direct downregulation of the glutamine transporter ASCT2 (20). Although a $1,25(OH)_2D$ target that regulates glutamine metabolism was not identified in detached cells, the $1,25(OH)_2D$-induced decrease in $^{13}C_5$-glutamine flux likely does not play a significant role in $1,25(OH)_2D$'s reduction of detached cell viability as PC overexpression was sufficient to eliminate $1,25(OH)_2D$'s effects on viability. In sum, these results suggest that regulation of PC, not glutamine metabolism, is the mechanism by which $1,25(OH)_2D$ decreases cell viability in detached conditions.

Regulation of glucose metabolism by $1,25(OH)_2D$ was also previously identified in attached MCF10A-*ras* cells (19,21,22). Similar to attached MCF10A-*ras* cells, in the current study, $1,25(OH)_2D$ treatment did not effect the mRNA abundance of *HK2* or *GLUT1* in detached cells (19). However, previous studies identified in attached cells $1,25(OH)_2D$ treatment reduced intracellular lactate and $^{13}C_6$-glucose flux to lactate, an effect which did not occur with $1,25(OH)_2D$ treatment in detached MCF10A-*ras* cells (19). In addition, $1,25(OH)_2D$ treatment had no effect on detached $^{13}C_6$-glucose-derived anaplerosis through PDH activity, which is contrary to previous work in attached cells where $1,25(OH)_2D$ treatment reduced PDH activity (19). Interestingly, the current work demonstrated a $1,25(OH)_2D$-mediated decrease in PC expression and subsequent activity in detached MFC10A-*ras* cells. These results are in accord with previous studies which

identified 1,25$(OH)_2$D's regulation of PC in attached MCF10A-*ras* cells through an active VDRE, and support that this regulation also occurs in detachment (22). In sum, in contrast to attached cells, detached cells no longer respond to 1,25$(OH)_2$D regulation of glycolysis and PDH anaplerosis, however, similar to attached cells, regulation of PC by 1,25$(OH)_2$D occurred in detachment. Further, results of studies with where PC is depleted or overexpressed demonstrate that regulation of PC was the mechanism by which 1,25$(OH)_2$D reduced detached cell viability. Given PC's role in supporting metastasis, PC downregulation by 1,25$(OH)_2$D may be utilized to inhibit ECM detached cell survival and prevent metastasis.

Taken together, the current results identifies that 1,25$(OH)_2$D's downregulation of PC in detached conditions results in decreased cell viability. These results may contribute to setting vitamin D recommendations to levels which improve breast cancer survival by adding to the growing body of evidence that the bioactive metabolite of vitamin D, 1,25$(OH)_2$D, inhibits cancer progression, potentially through the regulation of energy metabolism.

3.6 Acknowledgements

The authors acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute.

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CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary

The five-year survival rate of metastatic breast cancer is 28% compared to 99% for the diagnosis of localized breast cancer (1). This stark difference is accounted for by the unique difficulty in treating metastatic disease. Metastatic spread may not manifest until many years past the initial cancer diagnosis, at which point metastases have colonized throughout the body (2). Often, resection of metastatic tumors is not possible, thus explaining the low survival rate of metastatic breast cancer. While treatment of metastasis has improved over recent years, only 2-5% of metastatic cancer patients are expected to survive ten years past diagnosis (3). Thus, there remains a clinical need for new treatments which prevent the progression of cancer to metastatic disease.

An emerging hallmark of cancer is reprogrammed energy metabolism (4). One of the first examples of reprogrammed energy metabolism was identified by Otto Warburg in 1927 (5). Warburg observed that cancer cells preferentially metabolized glucose through glycolysis to produce lactate rather than through oxidative phosphorylation in the TCA cycle despite the presence of oxygen. This characteristic was later termed the "Warburg effect." We now understand that cancer cells utilize the carbon backbone of glucose for the biosynthesis of lipids, amino, and nucleic acids to support proliferation (6). In addition, glucose metabolism produces energy and oxidative stress protection by its metabolism through the TCA cycle and the pentose phosphate pathway, respectively. In addition to glucose, cancer cells have an increased dependence on glutamine metabolism (7). After glucose, glutamine is the second preferred nutrient of cancer cells and is the predominant amino acid in circulation (8). Glutamine is used by cancer cells for incorporation into nascent protein synthesis, energy production, and oxidative stress protection by supplying glutamate for the synthesis of the cellular antioxidant glutathione (GSH) (9). Further, metabolic plasticity, or the ability to use one nutrient for various metabolic purposes, and metabolic flexibility, or the ability to utilize different nutrients to fulfill the same metabolic requirement, are highly favored phenotypes in metastasis (10). Metabolic plasticity and flexibility permit cancer cells to reprogram their energy metabolism, such as rerouting glucose and glutamine metabolism, based on changing nutrient status and needs as the cancer cells metastasize to different

environments in the body. Therefore, cancer cells with the ability to reprogram energy metabolism have increased potential for successful metastasis.

Throughout the process of metastasis, cancer cells become detached from the ECM (11). Detachment from the ECM leads to activation of anoikis in normal epithelial cells. Further, attachment to ECM regulates cellular metabolism, and in normal epithelial cells, detachment results in reduced nutrient uptake and energy production and increased oxidative stress levels (12). Cancer cells acquire anchorage independence to survive when detached from the ECM in order to metastasize. While resisting anoikis that accompanies ECM detachment is important for achieving anchorage independence, studies indicate that overcoming ECM detachment-induced metabolic stress is also necessary (13). Interestingly, pro-survival pathways which are constitutively active in cancer cells not only promote anoikis resistance but activate energy metabolism in ECM detached conditions (12). Thus, the discovery of metabolic targets utilized by anchorage-independent cancer cells may be useful for inhibiting metastasis.

The ability to reroute energy metabolism to fulfill cell requirements in ECM detached conditions is a characteristic of anchorage independence (12). Further, metabolic reprogramming may be crucial for cancer cell survival in ECM detached conditions throughout metastasis. In the present work, we identified that anchorage-independent MCF10A-*ras* breast cancer cells displayed metabolic flexibility as they increased expression of the anaplerotic enzyme pyruvate carboxylase (PC) (Chapter 2). Detached cells decreased anaplerosis from glutamine, however, the cancer cells that survived prolonged detachment responded by increasing PC expression and subsequent activity. This metabolic flexibility fulfilled the detached cell requirement for aspartate biosynthesis. Additionally, the PC "switch" from glutamine anaplerosis to PC anaplerosis was demonstrated by decreased PC expression when cells were supplemented with the downstream glutamine metabolite dimethyl-α-ketoglutarate. Thus, anchorage-independent breast cancer cells display metabolic flexibility, and the PC switch may be targeted to reverse anchorage independence and prevent metastasis. However, future research is needed to determine the usefulness of this target in prevention of metastasis.

Vitamin D is associated with reduced breast cancer incidence and progression. For example, breast cancer patients with low vitamin D status have reduced survival, and more progressed disease (14). Given this association, studies have focused on identifying the mechanism by which vitamin D regulates breast cancer to reduce breast cancer mortality. Vitamin D mediates its

regulation in cells through its most active metabolite, 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D). Importantly, treatment with 1,25$(OH)_2$D is not possible due to its hypercalcemic effects, thus it is necessary to determine adequate vitamin D levels which support sufficient local production of 1,25$(OH)_2$D to prevent carcinogenesis (15). While most mechanistic studies focus on 1,25$(OH)_2$D's role in promoting cell cycle arrest and apoptosis in cancer cells (16), recent studies identified an emerging role of 1,25$(OH)_2$D in regulating cancer cell energy metabolism (17-20). While 1,25$(OH)_2$D mediated regulation of energy metabolism is identified, the usefulness of this regulation to prevent progression through the steps of metastasis has yet to be determined. In the present work, we demonstrate that 1,25$(OH)_2$D pretreatment reduces detached MCF10A-*ras* breast cancer cell viability, suggesting that adequate vitamin D may prevent cancer cell survival of metastasis by reducing viability in detached conditions. Further, the reduced viability observed in 1,25$(OH)_2$D treated cells was dependent on PC, as PC depleted detached cells were resistant to 1,25$(OH)_2$D, and PC overexpression overcame the 1,25$(OH)_2$D-induced decrease in detached cell viability. Given the important role, PC plays in central metabolism in normal tissue, utilizing 1,25$(OH)_2$D to inhibit metastasis by increasing vitamin D status to prevent anchorage independence poses a promising low-risk treatment, although future research is required before use in clinical settings.

4.2 Future Directions

4.2.1 Mechanism Behind the PC Switch

Metabolic reprogramming occurs in cancer cells detached from ECM. In Chapter 2, we demonstrate a PC switch utilized by MCF10A-*ras* cells upon detachment to improve survival. Understanding the mechanism that initiates this change in metabolism may be useful for targeting cancer cells with the ability to switch between glutamine and PC anaplerosis.

The cellular ability to switch between glutamine and PC anaplerosis is noted in the literature. For example, in the brain, glutamate, which is directly synthesized from glutamine, negatively regulates PC activity (21). This regulation demonstrates the tight control of the metabolite pools in the TCA cycle. In breast cancer models, PC expression and activity are increased in breast-derived lung metastases compared to the primary tumor (22,23). Increased dependence on PC was also observed in lung cancer models (24,25), suggesting that the lung

environment may stimulate the PC switch. The ratio of available pyruvate/glutamine is hypothesized to drive the increase in PC activity as serum pyruvate/glutamine ratio is decreased compared to the ratio in interstitial lung fluid *in vivo* (22). To test this hypothesis, Christen et al. showed increased PC-dependent anaplerosis with increasing pyruvate supplementation (22). However, our studies were designed to measure glucose-derived anaplerosis, and the PC switch was demonstrated in cancer cells grown without pyruvate supplementation in the media, suggesting that other factors besides environmentally-sourced pyruvate also act to modulate PC. Further, our studies support that the detachment-induced increase in PC activity is due to increased expression of PC as detached cells increased both PC mRNA and protein expression. Thus, environmental factors inducing changes in PC transcription are likely the mechanism underlying the PC switch.

In cancer cells, factors that regulate PC expression have been identified and may be implicated in the PC switch observed in the lung and detached cancer cells. In ovarian and mammary cancer cells, Wnt/β-catenin/Snail signaling was associated with increased PC expression (26,27), although the mechanism by which Snail-induced PC expression was not identified. In contrast, regulation of PC expression by Snail was not observed in MCF10A-*ras* cells (23). In MDA-MB-231 breast cancer cells, PC expression was also increased by c-Myc through direct binding to a c-Myc binding site in the promoter region of the PC gene (28). However, c-Myc was not shown to regulate PC expression in MCF-7 breast cancer cells (28). Also, expression of the micro-RNA, miR-143-3p, in MDA-MB-231 cells resulted in reduced PC expression (29). Decreased PC expression occurred through direct binding of miR-143-3p to the 3'-untranslated region of PC mRNA. While further investigation into these regulators of PC is required to determine their role in the PC switch, the inconsistencies between cancer models indicate the initiator of the PC switch has yet to be discovered. Thus, future research focusing on the molecular regulators of PC expression is needed.

The role of glutamine in the PC switch may provide insight into the mechanism that initiates this switch away from glutamine to PC. For example, in Chapter 2 we observed that detached cells reduced glutamine flux and decreased mRNA abundance of the glutamine transporter, *ASCT2*, which encodes for the enzyme that transports glutamine into the cell. In other models, decreased glutamine uptake is linked to activation of the mTOR signaling pathway (30). mTOR is considered a master regulator of cell growth and metabolism and is commonly

upregulated in cancer (31). Environmental cues such as growth factors, amino acids, and energy status lead to activation of mTOR and subsequent mTOR-induced anabolism, which promotes cell proliferation and survival. Regulation of energy metabolism by mTOR occurs through activation of various transcription factors, such as sterol-regulatory-binding proteins and hypoxia-inducible factor-1-α (32). In addition, the PI3K/Akt/mTOR pathway is activated in detached cancer cells and contributes to anchorage independence (33). Furthermore, mTOR signaling upregulates pyruvate kinase, which mediates the synthesis of pyruvate from phosphoenolpyruvate in glycolysis, in various cancer models (34). These results suggest that in pyruvate depleted detached conditions, cancer cell activation of mTOR as a result of decreased glutamine uptake may increase substrate levels for the PC reaction by increasing pyruvate kinase expression and subsequent pyruvate production. Thus mTOR activation induced by decreased glutamine metabolism may explain the discrepancy between our observed PC switch in pyruvate deplete conditions and the pyruvate-induced PC switch observed in previous studies. PC regulation by mTOR may occur through the activity of transcription factors that upregulate PC expression, as well as increasing substrate production for the PC reaction. Therefore, I hypothesize that the decreased glutamine metabolism observed in detached cancer cells resulting from decreased *ASCT2* expression activates the mTOR signaling pathway and results in increased expression and activity of PC. To test this hypothesis, the active form of mTOR, phosphorylated-mTOR relative to unphosphorylated mTOR can be measured in detached cancer cells. In addition, PC expression measured following inhibition of mTOR will contribute to determining if mTOR is required for the observed increase in PC expression. Assessing activation of mTOR following inhibition of glutamine uptake will connect glutamine metabolism to mTOR activation. Hypothesis supportive results acquired from these studies will demonstrate that mTOR is active in detached cells, inhibition of mTOR will result in reduced PC expression, and glutamine uptake inhibition will also result in increased mTOR activation.

4.2.2 Regulation of Cellular Oxidative Stress by 1,25(OH)$_2$D in Detachment

Detachment from the ECM is associated with a marked increase in reactive oxygen species (ROS) (35). However, cancer cells mitigate increasing ROS levels by reprogramming energy metabolism in favor of reductive-oxidative (redox) balance. Cells maintain redox balance by synthesizing reducing equivalents such as nicotinamide adenine dinucleotide phosphate (NADPH).

NADPH is utilized in the regeneration of the primary cellular antioxidant, GSH, from its oxidized form, GSSG (36). In detachment, reductive glutamine metabolism is used by non-small-cell lung cancer models to cycle citrate into the mitochondria for its use in the NADPH-producing reaction mediated by isocitrate dehydrogenase 2 (37). This cycling contributed to anchorage independence by mitigating mitochondrial ROS. Therefore, increased oxidative stress protection supports anchorage independence in cancer cells and may be targeted to prevent metastasis.

Studies suggest that 1,25$(OH)_2$D acts as a prooxidant in cancer cells by disrupting redox balance. For example, Bao et al. observed increased cell death as a result of 1,25$(OH)_2$D-induced increased oxidative stress levels in prostate cancer cells (38). Similarly, in MCF10A-*ras* cells 1,25$(OH)_2$D treatment decreased the ratios of NADPH/NADP+ and GSH/GSSG thereby increasing oxidative stress by disrupting redox balance and reducing cell viability (39). Interestingly, in non-malignant prostate cancer cells 1,25$(OH)_2$D treatment was protective against hydrogen peroxide-induced oxidative stress, suggesting that 1,25$(OH)_2$D has differential effects which depend on the cell type. Collectively, 1,25$(OH)_2$D protects against oxidative stress in non-transformed cells but acts as a prooxidant in cancer cells which may be a characteristic utilized in treating cancer.

Given the prooxidant effects of 1,25$(OH)_2$D in cancer models and the need to mitigate ROS following detachment, 1,25$(OH)_2$D may be used to reduce cancer cell survival in detached conditions by disrupting redox balance. In the current work, we demonstrate that 1,25$(OH)_2$D treatment reduces viability through downregulation of PC (Chapter 3). Previous work with the MCF10A-*ras* cells identified that 1,25$(OH)_2$D-induced oxidative stress through negative regulation of PC (39). It was proposed, but not tested, that PC activity protects cancer cells from oxidative stress by providing substrate for the NADPH producing malic enzyme 1 reaction. In Chapter 2 of this work, we demonstrate that detached MCF10A-*ras* cells increased PC activity to, in part, replenish intracellular malate pools. Thus, PC activity in detachment may improve redox balance through the malic enzyme 1 reaction. Therefore, I hypothesize that detached cancer cells increase PC expression and activity to provide substrate for NADPH producing reactions and mitigate ROS thereby increasing detached cell survival, and treatment with 1,25$(OH)_2$D reverses these effects. To test this hypothesis, NADPH/NADP+, and GSH/GSSG ratios will be determined in detached breast cancer cells with and without PC depletion. Further, the use of a [1-^{13}C]pyruvate tracer in detached cancer cells will provide insight into which reactions utilize PC-derived

oxaloacetate. This tracer is especially useful for the detection of PC activity as the PC reaction adds all three carbons from pyruvate into the TCA cycle, while pyruvate dehydrogenase activity, the alternative path by which pyruvate may enter the TCA cycle, removes the first carbon. Therefore, the use of a [1-^{13}C]pyruvate tracer results in downstream labeling with PC activity, but unlabeled intermediates arise from pyruvate dehydrogenase activity. Further experiments are also needed to connect PC-derived oxidative stress protection to improved detached cell viability. Therefore, I propose the addition of antioxidants, such as N-acetylcysteine or Trolox, to PC depleted cells and subsequent measurement of detached cell viability. Therefore, if these results support my hypothesis they will demonstrate that PC depletion reduces NADPH/NADP+ and GSH/GSSG ratios, NADPH producing reactions which utilize oxaloacetate synthesized from [1-^{13}C]pyruvate through the PC reaction such as malic enzyme, and addition of antioxidants will rescue cell viability from PC depletion.

4.2.3 Regulation of Lipid Metabolism by 1,25(OH)$_2$D in Detachment

Increased lipid accumulation and dysregulated lipid metabolism are observed in cancer (40). In addition, progressed disease and poor clinical outcomes are associated with lipid accumulation in tumors (41). Indeed, clinical studies are currently testing inhibitors of lipid metabolism as a means of cancer treatment (42). As lipids participate in cellular energy metabolism processes, the observed alteration of lipid metabolism contributes to the overall reprogrammed energy metabolism hallmark of cancer and may also support anchorage independence.

Although few studies have investigated lipid metabolism in detached cancer models, changes in lipid metabolism in detachment are observed. In detached breast cancer cells, fatty acid oxidation (FAO) only occurred when the cells were equipped with sufficient antioxidants to mitigate subsequent ROS produced by mitochondrial oxidation in the electron transport chain (33). Interestingly, in detached colorectal cancer cells, the opposite was observed where cancer cells utilized FAO to provide substrates for NADPH producing reactions (43). While FAO is commonly associated with inducing oxidative stress, it is hypothesized that cancer cells may utilize FAO to transfer reductive potential from the cytosol to the mitochondria when redox balance is needed (44). Thus, detached cells may reprogram their lipid metabolism to maintain cytosolic and mitochondrial redox balance when challenged by detachment-induced increased oxidative stress.

Studies indicate that vitamin D, through 1,25$(OH)_2$D, regulates lipid metabolism in cancer models. For example, 1,25$(OH)_2$D induced an increase in lipid accumulation in BT-474 breast carcinoma cells (45). In contrast, in metastatic MCF10CA1a breast epithelial cells, 1,25$(OH)_2$D treatment reduced lipid accumulation (18). Similar to detached conditions, 1,25$(OH)_2$D may differentially regulate lipid metabolism based on the metabolic demands of the cancer cells. Thus 1,25$(OH)_2$D may disrupt the reprogrammed lipid metabolism observed in anchorage-independent cancer cells, reducing their survival in those conditions. In the current work, 1,25$(OH)_2$D treatment reduced viability through downregulating PC (Chapter 3). Interestingly, in MCF10CA1a cells, 1,25$(OH)_2$D reduced lipid accumulation also by downregulating PC (18). PC activity supported fatty acid synthesis by providing substrate to produce citrate, a precursor of fatty acids. Thus, the downregulation of PC by 1,25$(OH)_2$D reduced lipid accumulation by decreasing fatty acid synthesis. Interestingly, Wilmanski et al. characterized the relative lipid accumulation of the non-transformed MCF10A, MCF10A-*ras*, and MCF10CA1a cells, and determined that MCF10A-*ras* cells did not significantly accumulate lipids relative to MCF10A cells. These results suggest that MCF10A-*ras* cells have not acquired dysregulated lipid accumulation to the extent of the metastatic MCF10CA1a cells. In addition, our current results indicate a decrease in glucose-derived flux through PC to citrate, suggesting that detached cells do not utilize PC activity for fatty acid synthesis (Chapter 2). Thus, future studies investigating 1,25$(OH)_2$D's regulation of lipid metabolism in detached cancer cells may be most productive if focused on the metastatic MCF10CA1a cell line, or other lipid accumulating cells. Therefore, I hypothesize that 1,25$(OH)_2$D treatment disrupts reprogrammed lipid metabolism in detached MCF10CA1a cells resulting in reduced detached cell viability. To test this, 1,25$(OH)_2$D's regulation of lipid metabolism including lipid accumulation, fatty acid synthesis, oxidation, and uptake in detached MCF10CA1a cells will first be identified. Following identification of regulation, the effects of 1,25$(OH)_2$D on genetically manipulated MCF10CA1a cells to constitutively activate or inhibit the targeted lipid metabolism will demonstrate a mechanism. Results supporting my hypothesis from these experiments would demonstrate 1,25$(OH)_2$D's regulation of lipid accumulation such as its previously determined role in reducing fatty acid synthesis (18), and genetic depletion of fatty acid synthase would eliminate 1,25$(OH)_2$D's effects on lipid accumulation.

4.3 Conclusion

Prevention of metastasis continues to be a focus of current research due to the increased mortality associated with metastatic disease (1). Current results from studies targeting specific characteristics of metastatic cancer cells, such as reprogrammed energy metabolism and anchorage independence, identify these characteristics as potential anti-metastatic targets. In Chapter 2 of the current work, the reliance of breast cancer cells on PC activity for survival in detached conditions was demonstrated. Additionally, our data in Chapter 3 support the regulation of PC by 1,25$(OH)_2$D in detached breast cancer cells. Further research into utilizing 1,25$(OH)_2$D to downregulate PC as a means of metastasis prevention is still needed. Given the current vitamin D recommendations are set to maintain adequate bone health, future work is required to establish vitamin D recommendations that optimize its cancer preventative role. The insights from this work contribute to the growing body of evidence that vitamin D prevents breast cancer progression and will hopefully influence setting vitamin D recommendations to levels that reduce breast cancer mortality.

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APPENDIX A. SUPPLEMENTARY DATA

A.1 1,25(OH)$_2$D's Regulation in Metastatic Cells

Understanding regulation of PC by 1,25(OH)$_2$D in metastatic cells is important to understand its differential effects across cells with varying metastatic potentials. In the human metastatic MDA-MB-231 cells, treatment with 1,25(OH)$_2$D for two days resulted in reduced PC mRNA abundance and increased CYP24 mRNA abundance (Figure A.1.A and A.1.B). However, prolonged four-day 1,25(OH)$_2$D treatment resulted in no change in PC mRNA abundance, while CYP24 remained increased relative to vehicle (Figure A.1.C and A.1.D). These results suggest that MDA-MB-231 cells respond to 1,25(OH)$_2$D treatment, although they readily increase expression of CYP24 to inactivate 1,25(OH)$_2$D effects by four days.

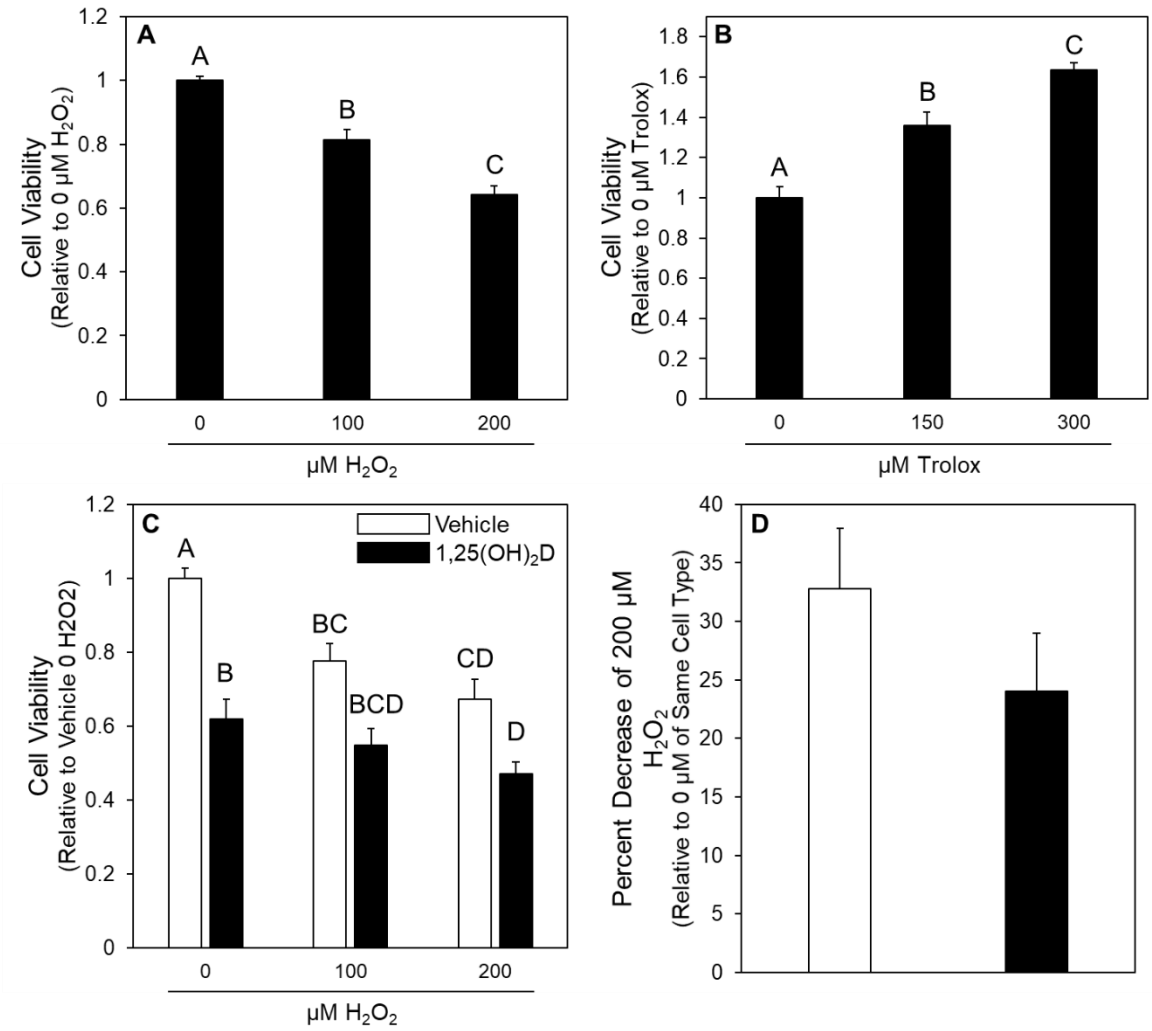

Figure A. 1. Effect of 1,25(OH)$_2$D on MDA-MB-231 mRNA abundance.

Following two-day treatment with 1,25(OH)$_2$D MDA-MB-231 cell mRNA abundance of PC (A) and CYP24 (B) were measured. Following four-day treatment with 1,25(OH)$_2$D MDA-MB-231 cell mRNA abundance of PC (C) and CYP24 (D) were measured.

A.2 Regulation of ROS by 1,25(OH)$_2$D in Detached Cells

A striking increase in ROS is associated with detachment, and previous work indicates 1,25(OH)$_2$D acts to inhibit cancer cell's ability to mitigate ROS. Therefore, the effect of oxidative stress in detached cells, and regulation by 1,25(OH)$_2$D was determined in MCF10A-*ras* cells. Detached cells reduce viability in a dose-dependent manner when stressed with increasing levels of H_2O_2 (Figure A.2.A). Addition of the antioxidant, Trolox, rescues viability

of detached cells in a dose-dependent manner (Figure A.2.B). In addition, 1,25(OH)$_2$D treatment has no additional effect on detached cell viability (Figure A.2.C and A.2.C), suggesting that 1,25(OH)$_2$D's effects on detached cell viability are not through dysregulating redox balance.

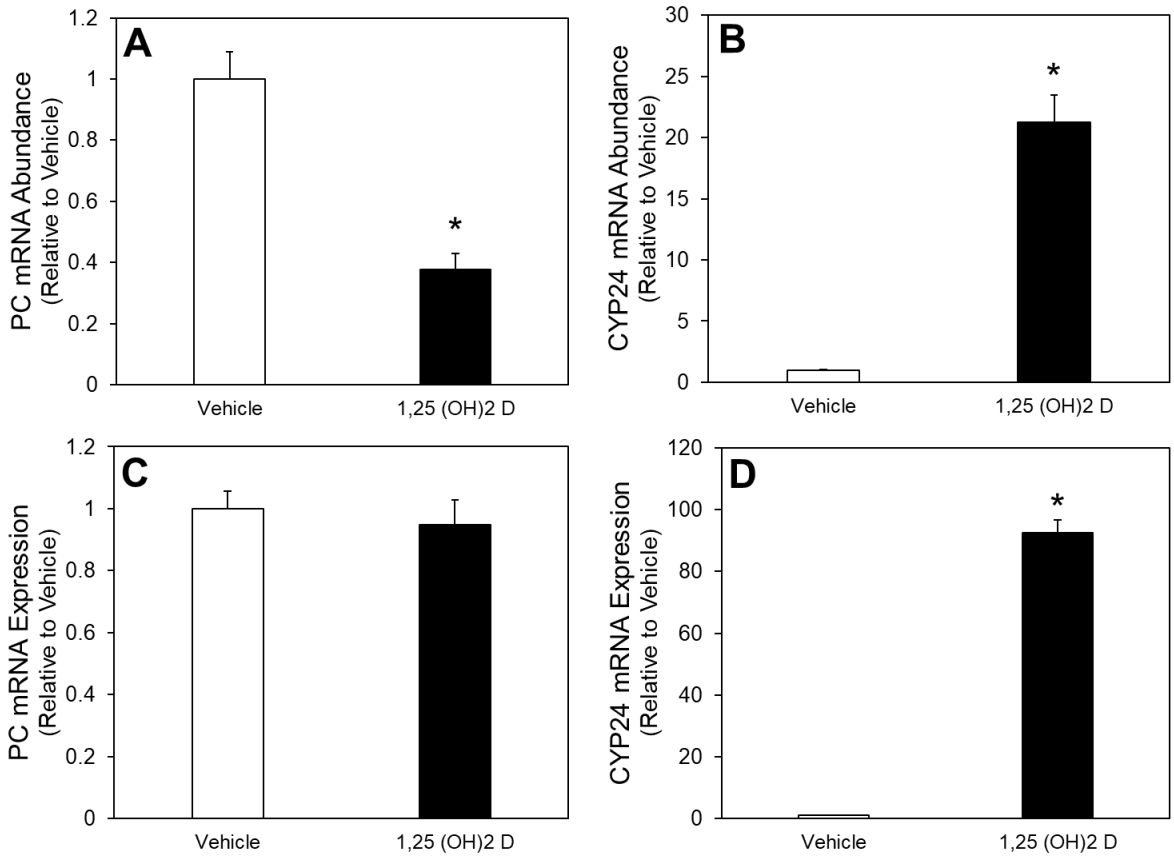

Figure A. 2. Effect of detachment and 1,25(OH)$_2$D on oxidative stress.

Detached MCF10A-*ras* cells viability with 0, 100, and 200 µM H_2O_2 treatment (A). Assessment of viability with treatment of 0, 150, and 300 µM Trolox at the time of detachment (B). Viability of detached cells with co-treatment with 1,25(OH)$_2$D and variable H_2O_2 (C). Percent decrease of vehicle and 1,25(OH)$_2$D pretreated (3 days) cells treated with 200 µM H_2O_2.

A.3 Regulation of Lipid Metabolism by 1,25(OH)$_2$D in Detached Cells

Increased lipid accumulation is associated with increasing metastatic potential. Therefore, lipid metabolism and regulation by 1,25(OH)$_2$D was evaluated in detached MCF10A-*ras* cells. Treatment for three days with 1,25(OH)$_2$D increased lipid accumulation of attached cells (Figure A.3.A). Detached cell viability was unaffected by inhibition of fatty acid oxidation with etomoxir

treatment (Figure A.3.B). The addition of oleic acid did not improve viability of detached cells (Figure A.3.C). This is likely because detached cells reduce fatty acid uptake compared to attached cells (Figure A.3.D). Treatment with 1,25(OH)$_2$D had no effect on fatty acid uptake of attached or detached cells (Figure A.3.D).

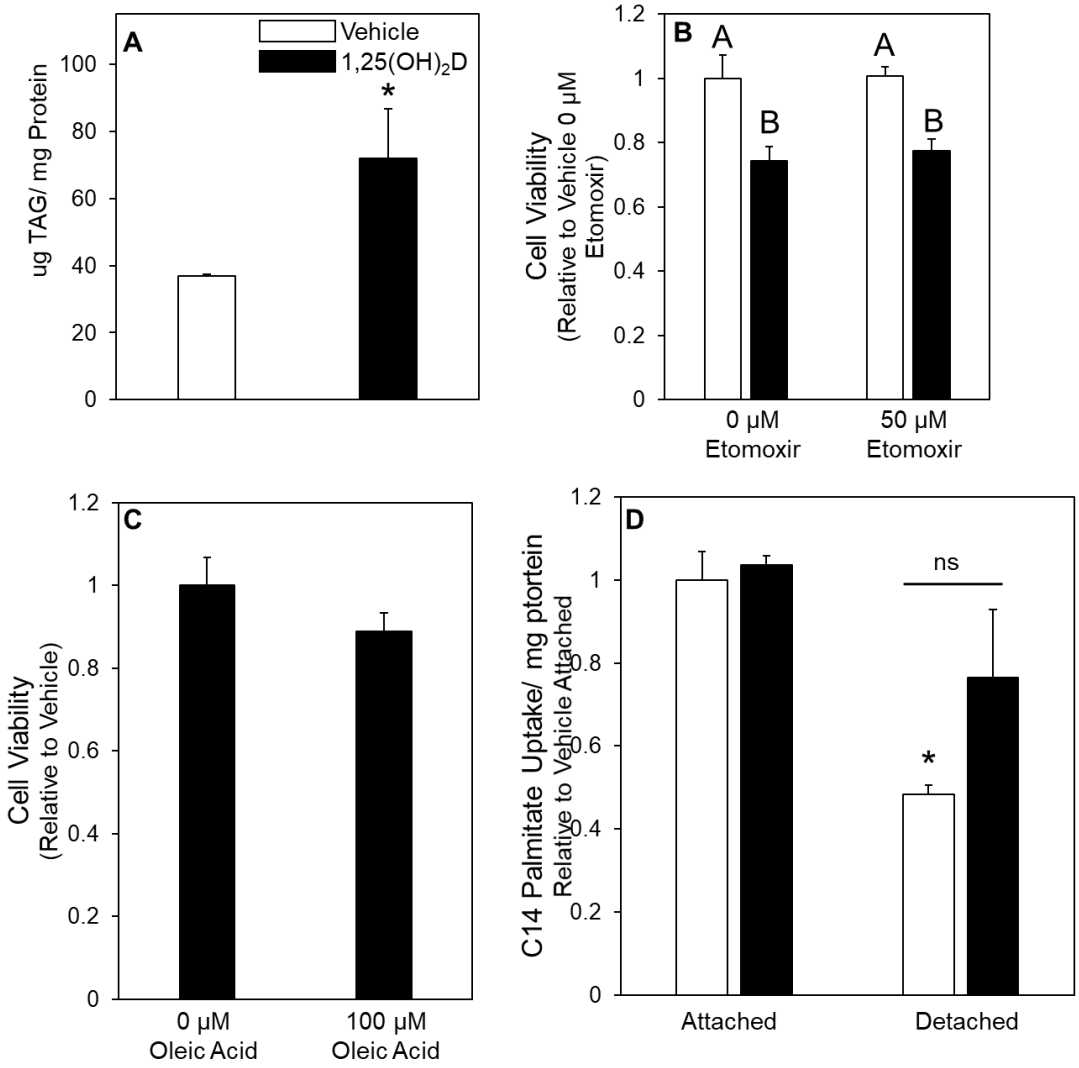

Figure A. 3. Effect of detachment and 1,25(OH)$_2$D on lipid metabolism.

MCF10A-*ras* cells were pretreated for three days with vehicle or 1,25(OH)$_2$D and TAG (A) viability with etomoxir co-treatment (B) were measured. Viability of detached cells supplemented with oleic acid was measured (C). Fatty acid uptake of vehicle and 1,25(OH)$_2$D attached and detached cells.

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