VITAMIN D, OBESITY AND BREAST CANCER METASTASIS

by

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To my beloved family

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Abbreviation	Term
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
18S ribosomal RNA	18S
25(OH)D	25-hydroxyvitamin D
ACAT	Acyl-CoA:cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
ACLY	ATP citrate lyase
ACSS	Acetyl-CoA synthetase
AGPAT	Acylglycerolphosphate acyltransferase
AKG	α-ketoglutarate
AMPK	AMP-activated protein kinase
Angpt14	Angiopoietin-like-4
ANOVA	Analysis of variance
AOA	Aminooxyacetate
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BMI	Body mass index
BODIPY	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene- 3-hexadecanoic acid
BSA	Bovine Serum Albumin
CCL2	C-C motif chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CE	Cholesterol ester
CGI-58	Comparative gene identification-58
CPT	Carnitine palmitoyltransferases
CRP	C-reactive protein

CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1
DAG	Diacylglycerol
DGAT	Acyl-CoA:diacylglycerol acyltransferase
DMEM	Dulbecco's Modification of Eagle's Medium
E-cadherin	Epithelial cadherin
ECM	Extracellular membrane
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT-TFs	EMT-inducing transcription factors
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERK	Extracellular-signal-regulated kinase
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
Fatty acyl-CoA	Fatty acyl-coenzyme A
FBS	Fetal bovine serum
GLUT	Glucose transporter
GPAT	Glycerol-3-phosphate acyltransferase
HER2	Human epidermal growth factor receptor 2
HK	Hexokinase 2
HOMA-IR	Homeostatic model assessment of insulin resistance
HSL	Hormone-sensitive lipase
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor-1 receptor
IL-6	Interleukin 6
JAK	Janus kinase
JNK	Jun N-terminal kinase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase

MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MARRS	Membrane-associated rapid response steroid-binding proteins
MCP-1	Monocyte chemoattractant protein 1
MET	Mesenchymal-epithelial transition
MKP5	MAP kinase phosphatase 5
MMPs	Matrix metalloproteinases
MMTV-PyMT	Mouse mammary tumor virus-polyoma virus middle T antigen model
mTOR	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor-kB
Ob-R	Leptin receptor
PAP	Phosphatidic acid phosphatase
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor γ
PTH	Pituitary thyroid hormone
RANKL	Receptor activator of nuclear factor kappa-B ligand
RXR	Retinoid X receptor
SCD	Stearoyl-CoA desaturase
SREBP	Sterol regulatory element-binding proteins
STAT	Signal transducer and activator of transcription
TCA	Tricarboxylic acid
TGFβ-R1	TGFβ-receptor type 1
TNBC	Triple negative breast cancer

TNFα	Tumor necrosis factor α
TRPV	Transient receptor potential vanilloid
UCP-1	Uncoupling protein 1
UL	Upper limit
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	VDR response elements
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
ZEB	Zinc-finger E-box binding

ABSTRACT

Breast cancer is the second leading cause of cancer death among women in the United States. Many epidemiological studies have demonstrated that obesity is a risk factor for breast cancer. As leptin is an adipokine released from adipose tissue in proportion to the adipose tissue size, obese people generally have higher serum leptin levels than non-obese individuals. Many researchers have shown that leptin exerts migratory and invasive effects through leptin receptors in cancer cells, including breast cancer, and studies on cell models were conducted in conditions with medium containing high glucose concentrations. However, the mechanisms by which leptin induces breast cancer cells migration at physiological glucose levels are not fully elucidated. In the present studies, the effect of leptin on migratory capability was investigated focusing on leptin-mediated altered energy metabolism, as migration is an energy-requiring process. Two murine metastatic 4T1 and metM-wnt^{lung} cell lines are employed in this study. Leptin treatment for 4 days increased migration in 4T1 and metM-wnt^{lung} murine breast cancer cells when cultured in 5 mM glucose medium. We determined, using stably labeled glucose, that leptin did not alter glucose incorporation into palmitate in 4T1 cells, but leptin increased palmitate synthesis from glucose in metM-wnt^{lung} cells. Additionally, in 4T1 cells, leptin treatment increased *Glut1* mRNA level and decreased *Fasn* mRNA level. However, both *Glut1* and *Fasn* mRNA level were increased with leptin treatment in metM-wnt^{lung} cells. These different effects of leptin on glucose and fatty acid metabolism may lead to different energy status regulated through AMP-activated protein kinase (AMPK), a master sensor of energy status. While AMPK was activated with leptin treatment in 4T1 cells, it was inactivated with leptin treatment in metM-wnt^{lung} cells. We also determined that glycolysis is necessary for leptin-mediated increased migratory capability in metM-wnt^{lung} cells, but not in 4T1 cells. However, fatty acid metabolism is not required in leptin-induced migration in either 4T1 or metM-wnt^{lung} cells. Furthermore, glutamine metabolism is also not involved in increased migration with leptin treatment in metM-wnt^{lung}. Thus, leptin-mediated alteration in energy metabolism is differentially regulated in 4T1 and metM-wnt^{lung} cells during leptin-induced migration. Another factor that may regulate breast cancer migration potentially through leptin is vitamin D, as it has been shown to inhibit breast cancer metastasis and vitamin D impacts adipocytes including adipogenesis and inflammation. However, the link between vitamin D's regulation of adipocytes

and the effect on breast cancer metastasis is not understood. Here, we demonstrated that migration of MDA-MB-231 cells was reduced when exposed to conditioned media from differentiated mature 3T3-L1 adipocytes treated with the active form of vitamin D, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$) compared to vehicle-treated controls. In addition, $1,25(OH)_2D$ decreased mRNA level of leptin (*Lep*), adiponectin (*Adipoq*), *IGF*-1, *IL-6*, and *MCP-1*. Consistent with the change in mRNA level, concentrations of leptin, adiponectin, IGF-1, and IL-6, but not MCP-1, in adipocyte conditioned media were decreased with $1,25(OH)_2D$ treatment. Although adipocyte leptin mRNA level and release were decreased with $1,25(OH)_2D$, leptin alone did not induce migration as a chemoattractant, suggesting other factors underly the impact of $1,25(OH)_2D$ on adipocytes to decrease migration of breast cancer cells. In addition to the impact of $1,25(OH)_2D$ on adipocytes, $1,25(OH)_2D$ exerts its effect in breast cancer cells since breast tissue expresses vitamin D receptor (VDR). However, loss of VDR is reported during tumor progression in cancer cells, implicating that vitamin D may not exert its protective effect on more advanced stages of cancer. In this study, MCF-10A series, a series of human cells representing different stages of breast cancer, were employed, including untransformed MCF10A, Harvey-*ras* oncogene transfected early progression of breast cancer cells model (MCF10A-*ras*), and metastatic MCF10CA1a cells. Consistent with previous literature, we demonstrated that the basal VDR mRNA level was reduced in metastatic MCF10CA1a compared to untransformed MCF10A cells, however, treatment with $1,25(OH)_2D$ reduced mRNA level of VDR in MCF10A and MCF10A-*ras* cell but increased VDR mRNA level and induced a trend toward an increase in VDR transcriptional specific activity in MCF10CA1a cells with $1,25(OH)_2D$ treatment compared to MCF10A. Together, these results indicate that vitamin D may induce inhibitory effects on metastatic breast cancer cells through upregulation of VDR level by $1,25(OH)_2D$. Collectively, the present studies provide a novel insight into leptin-mediated changes in energy metabolisms during leptin-induced migration with two murine breast cancer cells. In addition, these studies demonstrate the importance of $1,25(OH)_2D$ regulation of adipocytes in breast cancer cell migration as well as $1,25(OH)_2D$ regulation of VDR level and activity in metastatic breast cancer cells.

1. LITERATURE REVIEW

1.1 Obesity-Associated Metabolic Disorders

Obesity is a growing medical and public health problem worldwide which increases the risk for a multitude of metabolic, cardiovascular, chronic inflammatory, and malignant diseases [1]. The global deaths attributable to excess body weight in 2012 was estimated to be 3.6% of the population, further highlighting the devastating consequences of obesity on health [2]. In addition to the increased risk of multiple diseases, obesity also causes chronic inflammation and increases levels of pro-inflammatory mediators in adipose tissue [3]. Therefore, research into obesity-associated diseases, and developing strategies for counteracting these diseases particularly in the obese, is essential for long term remediation of this growing public health concern.

1.1.1 Changes in Adipose Tissue During Obesity

Excessive accumulation of adipose tissue in response to over-nutrition results in obesity. According to the WHO, overweight and obesity is defined by body mass index (BMI, weight (kg) /height (m^2)) [4], where BMI > 25-29 kg/m^2 is considered overweight and BMI ≥ 30 kg/m^2 obese. Historically, adipose tissue is thought to be a component of energy storage and thermal insulation [5]. However, it is now known that adipose tissue has various roles, including in immune response, inflammation, reproduction, and metabolism [5].

Adipose tissue is traditionally divided into white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores energy as triacylglycerol (TAG) during energy excess and releases energy during energy deprivation. BAT regulates body temperature through heat production from the stored lipids. A high level of uncoupling protein 1 (UCP-1) in the mitochondria of BAT dissipates energy as heat by non-shivering thermogenesis [6]. Evidence suggests that the presence of BAT is inversely associated with BMI and total adipose mass in adult humans [7], highlighting the potential role of BAT in the prevention of obesity. Recently, beige or brite (brown in white) adipocytes have been identified. These multilocular adipocytes express UCP-1 similar to brown adipocytes. Under basal conditions, beige adipocytes act metabolically similar to white adipocytes. However, these beige adipocytes become "browned"

in response to cold stimulation, and beige adipocytes continue to be an area of investigation with the potential to reduce obesity-associated diseases.

Body fat may increase in two ways, hypertrophy and hyperplasia. Hypertrophy refers to an increase in the volume of adipocytes by an excessive accumulation of triglyceride in WAT. On the other hand, the hyperplasia of adipose tissue refers to an increase in the number of adipocytes and only occurs in special situations, including obesity or during periods of differentiation. Adipogenesis is the differentiation of preadipocytes to mature adipocytes that can store lipids. Adipocytes are derived from multipotent mesenchymal precursor stem cells which have the potential to also differentiate into myoblasts, osteoblasts, fibroblasts, and chondroblasts [8]. Mature adipocytes, which is the body's greatest energy reserve, affects not only lipid accumulation, but also glucose metabolism in our body. In addition, adipocytes secrete a variety of factors to regulate feeding behavior, metabolism, and immune function. These various factors are called adipokines and include growth factors, hormones, cytokines, among others. To date, over 400 factors have been reported to be released from mature adipocytes. Therefore, understanding the regulation of mature adipocytes and the release of these factors is important to prevent obesity-associated diseases.

In a state of excess energy, hypertrophy of WAT leads to macrophage recruitment and adipocyte cell death [9]. In this hypertrophic state, pro-inflammatory M1-polarized macrophages form crown-like structures and secrete various inflammatory cytokines and chemokines [10]. These factors lead to development of inflammation in the obese state, which is associated with obesity-related morbidities and mortality by several diseases, including cancers [11]. Therefore, understanding of obesity-associated inflammation is also of interest to investigate the pathogenesis of obesity-associated diseases.

1.1.2 Obesity and Breast Cancer

Evidence from epidemiological studies suggests that obesity is a significant risk factor for several cancers [12-14]. Specifically, excess body weight has been linked to an increased risk of ten different cancer types, including cancer of the oesophagus (adenocarcinoma), colorectum, gallbladder, pancreas, liver, breast, ovary, endometrium, kidney, and prostate (advanced stage) [15]. Risk of oesophageal adenocarcinoma, in particular, increases nearly two-fold in obese

individuals compared to normal weight, demonstrating the dramatic impact adiposity may have on cancer incidence [16].

According to GLOBOCAN 2018, breast cancer is a major cancer frequently diagnosed worldwide among females, followed by colorectal cancer, as well as the leading cause of cancer death globally [17]. Several epidemiological studies show the association between obesity and breast cancer risk. For instance, a prospective analysis within the Nurses' Health Study shows that body weight is positively related with risk of estrogen receptor (ER)+/ progesterone receptor + breast cancer in postmenopausal women [18]. However, in premenopausal women, researchers show inconsistent results in the relationship of obesity with breast cancer risk [19, 20]. For example, a meta-analysis shows a higher risk of developing triple negative breast cancer (TNBC) in obese premenopausal women [19]. In contrast, Suzuki *et al.,* shows that obesity is associated with a decreased risk of developing ER+ breast cancer in pre-menopausal women [20]. As the associations remain conflicting for premenopausal women, further research is needed to provide a convincing association between obesity and breast cancer risk on premenopausal women.

In addition to the link between obesity and breast cancer risk, researchers demonstrate the link between obesity and cancer outcomes. A meta-analysis study shows that high BMI is associated with lower survival in pre- and post-menopausal breast cancer patients [21]. Additionally, Nechuta *et al.,* demonstrate a positive association between obesity and distant breast cancer recurrence [22]. Given that metastasis is the primary cause of mortality in breast cancer, these results suggest that obesity is associated with increased breast cancer metastasis as well as incidence.

1.1.2.1 Obesity and Breast Cancer: Potential Mechanisms

To explain the connection between obesity and breast cancer, various mechanisms have been suggested. Adipocytes are the predominant stromal cell type in mammary tissue responsible for local estrogen production [23], which may contribute to the development of estrogen-dependent breast cancer in postmenopausal women. In one prospective study, urine or blood samples collected from four cohort studies showed that total estrogen levels, including 15 estrogens/estrogen metabolites, are positively related with breast cancer risk in postmenopausal women [24]. This suggests that altering estrogen metabolism is a possible chemopreventive strategy for breast cancer in obese patients.

In addition to estrogen, insulin resistance is associated with increased breast cancer risk. Insulin resistance is found in many obese individuals with high blood insulin level. Insulin promotes the lipogenesis process by increasing glucose and fatty acid uptake in adipocytes [25]. However, in insulin resistance, the overall body respond to insulin is lower and therefore, further increases TAG accumulation in adipocytes. Hernandez *et al.,* show that there is a positive association between higher levels of insulin resistance estimated by homeostatic model assessment of insulin resistance (HOMA-IR) and breast cancer risk in postmenopausal female [26]. In addition, one *in vivo* study shows that in an insulin resistance mouse model, primary breast tumor formation is increased as well as pulmonary metastasis compared to control mice when mammary tumor cells are injected with insulin [27]. These results suggest that insulin resistance promotes breast cancer growth and metastasis.

More recently, adipose tissue has been recognized as an important secretory organ that can produce regulatory molecules with endocrine signaling functions that exert a proximal and distal impact on tumor cells. These regulatory molecules include various hormones, cytokines, and growth factors collectively called adipokines, such as leptin, tumor necrosis factor a (TNFα), interleukin (IL)-6, IL-8, IL-1β, all of which are elevated both locally and/or systemically in obese women [28]. Each of these factors has the potential to create an environment that favors tumor initiation and progression [29]. Thus, multiple mechanisms are proposed to explain the link between obesity and breast cancer risk. However, the mechanism by which excess body fat increases breast cancer risk or how obesity affects the therapeutic approaches to breast cancer remains unclear.

1.1.2.2 Adipokines and Breast Cancer

Adipokines are peptides selectively secreted by adipose tissue that impact targets in the brain, liver, pancreas, immune system, vasculature, muscle, and other tissues. Changes in the secretion of adipokines in an obese state are altered and are a marker for dysfunctional adipose tissue that may lead to obesity-associated mammary carcinogenesis [30]. Secretion of adipokines, including leptin, adiponectin, insulin-like growth factor, cytokines, and chemokines, is altered in dysfunctional adipose tissue and may be key players in obesity-associated diseases [1]. Adipokines have been implicated in cancer progression and metastasis through their effects on insulin resistance and various inflammatory pathways [31]. Thus, adipokines are regarded as

potential targets for novel therapeutic treatment strategies, highlighting that a better understanding of their roles in breast cancer progression is needed.

To investigate the role of adipokines in obesity-associated breast cancer, *in vitro* studies have been conducted to determine the impact of mature adipocytes on breast cancer cells. For example, a study by Lee *et al.* [32] shows that co-culture of mature adipocytes and breast cancer cells induces morphological changes, increases epithelial-mesenchymal transition (EMT) markers and stimulates migration and invasion of breast cancer cells. Another study shows that breast tumor cells previously co-cultivated with mature adipocytes exhibit radioresistance, which suggests that cells or tissues that are less affected than non-treated mammalian cells on exposure to radiation, which complicates therapeutic strategies [33, 34]. Therefore, investigating adipokines is of interest to understand adipocyte-associated breast cancer.

Among adipokines, leptin is mainly produced by adipocytes but also by the enterocyte. Leptin plays a key role in the regulation of energy balance, and evidence suggests that leptin contributes to breast cancer progression and metastasis [35]. The impact of leptin on breast cancer will be discussed in Chapter 1.5.

Another adipokine that has been associated with carcinogenesis is adiponectin, which is mainly released by adipocytes, but also by placenta [36] and cardiomyocytes [37]. Adiponectin is a peptide that is also known as ADIPOQ and is the apM1 gene product [38]. In obese individuals, the plasma concentration of adiponectin is lower than that of non-obese individuals [39], suggesting that lower adiponectin is associated with obesity even though adiponectin is secreted from adipocytes. Unlike leptin, adiponectin has been shown to have anti-tumorigenic activities [40, 41]. For example, one meta-analysis which included 17 studies shows that high adiponectin level is associated with reduced risk of postmenopausal breast cancer [42]. Similarly, Kim *et al.*, show that adiponectin reduces proliferation in colon cancer cells through adiponectin receptor-mediated 5' AMP-activated protein kinase (AMPK) activation [43], highlighting its anti-tumorigenic effect on cancer cells. However, the role of adiponectin is controversial since other research reveals that adiponectin increases expression of the growth factors, vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1), as well as increases cell migration in breast cancer cells [44]. Therefore, the effect of adiponectin on and underlying mechanism by which adiponectin affects cancer cells remains to be further investigated.

Insulin-like growth factor-1 (IGF-1) is mostly produced from hepatocytes but is also secreted from differentiated adipocytes. It is reported that insulin reduces production of IGF-binding protein-1, leading to increase free IGF-1 concentration in serum [45]. Elevated IGF-1 level is regarded as a risk factor in many cancer types including breast cancer [28]. High circulating insulin levels increase the activity of the IGF-1 by the IGF-1 receptor (IGF-1R), activating phosphatidylinositol-3 kinase (PI3K)/Akt pathway as well as the mammalian target of rapamycin (mTOR) complex [46]. For example, Chang *et al.,* has shown that IGF-1R signaling results in activation of the PI3K/Akt/mTOR pathway for survival and maintenance in breast cancer stem cells [47]. These results suggest that targeting of dysregulated PI3K/Akt/mTOR pathway may be an attractive therapy against obesity-associated breast cancer.

The proinflammatory cytokine interleukin 6 (IL-6) is a key molecule produced by both adipocytes and breast cancer cells. It is reported that adipose tissues secrete approximately one-third of IL-6 present in the plasma [48]. While IL-6 is mainly secreted from non-adipose tissue in healthy adipose tissue, IL-6 secretion is increased significantly from adipocytes in conditions of obesity and cancer [49, 50], indicating that IL-6 may exert the impact on obesity-associated breast cancer. IL-6 plays a role in the cell growth and metastasis in breast cancers via janus kinase (JAK) / signal transducer and activator of transcription 3 (STAT3) signaling pathway [51]. It is well known that serum IL-6 level is related to lower survival in breast cancer patients with metastatic lesions [52]. However, recent research finds that IL-6 level is correlated to a better prognosis in an early stage of invasive breast cancer patients [53], highlighting IL-6 may have a different effect on different cell types or different cell status conditions. Therefore, further investigation is required to determine the role of IL-6 in breast cancer progression and metastasis.

Another inflammatory marker that is associated with increased adipose mass is monocyte-chemoattractant protein-1 (MCP-1). MCP-1, also known as C-C motif chemokine ligand 2 (CCL2), is a member of the chemokine superfamily that functions in the recruitment of circulating monocytes. Weisberg *et al.,* show that obese mice have increased expression of MCP-1 compared to lean mice [54]. The depletion of C-C chemokine receptor type 2 (CCR2) reduces the macrophage content of adipose tissue [54], suggesting an important role of MCP-1 via CCR2 in obesity-associated adipocytes. Furthermore, MCP-1 plays a role in breast cancer metastasis. For example, Li *et al.,* reveal that MCP-1 mediates EMT and increased breast cancer migration,

regulated via the extracellular-signal-regulated kinase (ERK)/ glycogen synthase kinase 3β/Snail pathway [55], suggesting regulation of MCP-1 as a potential mediator of breast cancer progression. Taken together, these studies show that adipokines, increased in obesity, may increase progression and metastasis in breast cancer.

1.2 Breast Cancer

According to GLOBOCAN 2018, breast cancer is a leading cancer commonly diagnosed globally, as well as a leading cause of cancer death worldwide [17]. The main cause of death in breast cancer patients is metastases as the five-year survival rate when breast cancer is localized is 99% [56], but the five-year survival rate is 26% when breast cancer is metastasized to a distant organ [56]. Even though the mortality in breast cancer patients have decreased due to early diagnosis and newly developed therapies, the number of cases for breast cancer remains higher than other cancers in women. Therefore, more efforts in breast cancer research to prevent metastasis are still needed.

1.2.1 Breast Cancer Cell Model

1.2.1.1 MCF10A Early Breast Cancer Cell Model

It is critical to choose the right cell model to investigate cancer cell behavior. In order to appropriately investigate cell behavior in early cancer progression, it is important to select early progression premalignant cell models. On the other hand, in order to study cancer metastasis, malignant metastatic cell models are needed. One such model system which spans this spectrum are based in the MCF10A human cell line, which is spontaneous immortalized breast epithelial cells from a patient with fibrocystic disease. When MCF10A cells are implanted subcutaneously in immunodeficient mice, MCF10A cells do not survive *in vivo*. The Harvey-*ras* oncogene was transfected into the MCF10A cells to establish is a premalignant cell line (MCF10A-*ras*) regarded as an early breast cancer model. Upon injection into immunodeficient mice, MCF10A-*ras* cells establish small nodules and grow sporadically to carcinomas at a rate of approximately 25% [57]. The malignant MCF10CA1a was established following several passages of MCF10A-*ras* in immunodeficient mice [58]. When MCF10CA1a cells are injected subcutaneously into immunodeficient mice, MCF10CA1a consistently form primary tumors and spontaneous

metastasis. Taken together, a common genetic background derived from the MCF10A series allows researchers to investigate breast cancer behavior during the progression of human breast cancer from untransformed to metastatic breast cancer.

1.2.1.2 4TO7 and 4T1 Breast Cancer Cell Model

Both 4TO7 and 4T1 are thioguanine-resistant murine breast cancer cells. They are derived from a common parental population of 410.4 cells from BALB/c mice [59]. 4TO7 and 4T1 can form primary tumors when implanted into the mammary gland of BALB/c mice but have different metastatic behaviors. 4TO7 cells can disseminate from primary mammary tumors into the lung but fail to form visible lung nodules [60]. When the primary mammary tumor is removed, disseminated 4TO7 cells in the lung disappear rapidly and 4TO7 cannot colonize in distance sites [60]. On the other hand, metastatic 4T1 cells can hematogeneously metastasize to the lung, liver, brain, and bone [61]. Thus, 4TO7 is regarded as a non-metastatic cell line and 4T1 cells as metastatic mimicking stage IV breast [62], respectively.

1.2.2 A Multistage Model of Cancer Metastasis

Metastasis is a complex process that involves a series of steps including the initial detachment of cells from the primary tumor, local invasion of the basement membrane, intravasation and survival in the circulation, extravasation, survival, and proliferation in a distant site [63]. For local invasion, the primary tumor enters into the surrounding tumor-associated stroma and breaks through the basement membrane which is an extracellular membrane (ECM) that plays an important role for epithelial tissues [64]. Active proteolysis mainly via matrix metalloproteinases (MMPs) facilitates loss of the basement membrane, enabling cell migration [65].

Upon successful invasion the surrounding stroma, locally invasive carcinoma cells enter into blood vessels, termed intravasation. Mammary carcinoma intravasation is enhanced by TGFβ that augments carcinoma cell penetration of the walls of microvesssels [66]. Moreover, tumor-associated macrophages are also involved in intravasation by a positive-feedback loop with the secretion of epidermal growth factor (EGF) and colony stimulating factor-1 [67].

Following intravasation, cancer cells must also survive in the blood stream to successfully metastasize. In order to metastasize, cancer cells must survive various stresses to successfully reach distance sites. For example, they are exposed to conditions without integrin-dependent adhesion to ECM, triggering cell apoptosis. In addition, cancer cells must overcome the damage inflicted by shear forces of blood flow and predation by the innate immune system [64].

After surviving in the circulation, cancer cells become trapped in the microvasculature of distant organs [64]. They may initiate the formation of a microcolony that eventually breaks the wall of surrounding vessels, allowing them to extravasate into distant organ sites. The cancer cells secrete factors that induce vascular hyper-permeability for successful extravasation to the distant site. For example, protein angiopoietin-like-4 (Angpt14) has been shown to increase the extravasation of breast carcinoma cells in the lung but not in the bone, highlighting the critical role of the Angpt14 in a specific tissue for extravasation [68]. After extravasation, cancer cells must survive in the new environment of distant tissues. As the new environment is often different from the primary site, cancer cells may not proliferate, a process called dormancy, characterized by a growth arrest.

For breast cancers, metastatic colonization can be delayed by years or decades [69]. The final step of the metastatic cascade is colonization to form a secondary tumor in the distant organs. Even though metastasis is an inefficient process, metastatic tumors may lead to organ failure and resistance to chemotherapeutic drugs. Thus, it is important to investigate the mechanisms critical at different stages of metastasis in order to design preventive strategies.

1.2.2.1 Epithelial–Mesenchymal Transition (EMT)

In order to proceed with the multiple steps of metastasis, an important phase for the cells is EMT. EMT is a programmed cellular process in which gene expression is altered which permits the phenotypic transition of the cell from epithelial to mesenchymal [70]. During this process, epithelial cells lose their cell-to-cell adhesion to adopt a mesenchymal morphology with a spindle-like shape. EMT has important roles in the malignant progression of various carcinoma cells by remodeling of the cytoskeleton and regulating metabolism.

By inducing EMT, cell surface epithelial cadherin (E-cadherin) expression is repressed, promoting the loss of the epithelial morphology of the cells. E-cadherin is a 120 kD

transmembrane glycoprotein that is involved in cell-cell adhesion in epithelial tissues. By comparing the human breast tissue from breast cancer patients, Singhai *et al.*, show that loss of E-cadherin is a biomarker for the diagnosis of invasive lobular carcinoma and its variants [71]. Consistent with this study, Frixen *et al.*, determined that the loss of E-cadherin expression leads to invasiveness of human carcinoma cells [72], indicating the potential of regulating E-cadherin in the prevention of cancer metastasis. During tumor progression, E-cadherin can be inactivated by the repression of gene expression through the hypermethylation and deacetylation of the gene promoter [73]. While the expression of E-cadherin is decreased during EMT, the expression of markers involved in the mesenchymal state, including N-cadherin, vimentin, and fibronectin, are commonly increased [74]. Together, these studies indicate that changes in protein expression during EMT allow non-metastatic cells to induce metastasis.

There are several EMT-inducing transcription factors (EMT-TFs) identified that are involved in EMT. EMT-TFs repress the genes to maintain the epithelial state and induce the genes to promote a mesenchymal cell state. Many EMT-TFs, such as TWIST1, SNAIL1, SNAIL2 (also named "SLUG"), zinc-finger E-box binding (ZEB)1, and ZEB2, can repress E-cadherin directly or indirectly [75]. For example, SNAIL has been shown to repress E-cadherin expression through binding to E boxes in its promoter region [76]. Similarly, Comijn *et al.*, show that ZEB1 transcriptionally represses E-cadherin expression and induces vimentin and N-cadherin by chromatin-remodeling [77].

EMT-TFs also play important roles in metastatic behaviors in several *in vitro* and *in vivo* models. For instance, ZEB1 is essential for cell plasticity to promote invasion and metastasis in pancreatic mouse models [78]. Similarly, SNAIL is required for the dissemination of mammary tumors in an *in vivo* model [79]. Furthermore, SNAIL upregulates MMPs expression which induces extracellular matrix degradation, promoting cell invasion in hepatocellular carcinoma [80]. Together, these results indicate the important role of EMT-TFs in promoting metastasis.

Several extracellular signals can orchestrate the upregulation of EMT-TFs and EMT. TGFβ is one of the best-characterized inducers of EMT in cancer cells. There are three distinct TGFβ isoforms, and these TGFβs bind to complexes of TGFβ receptor type 1 (TGFβR1) and TGFβR2, allowing phosphorylation of SMAD2 and SMAD3, which form a complex with SMAD4. The trimeric SMAD complex translocates to the nucleus and regulates transcription of target genes, such as vimentin and fibronectin as well as EMT-TFs including SNAIL, ZEB, and

TWIST [81]. The EMT-TFs can upregulate the expression of TGFβ ligands, allowing a positive feedback loop to maintain the expression of EMT programs. The TGFβ pathway also orchestrates with the PI3K-AKT pathway, activating the mTOR pathway as well as nuclear factor-kB (NF-kB) to transition to a mesenchymal cell state. TGFβ also triggers the p38 mitogen-activated protein kinase (MAPK) pathway and Ras-Raf-MEK-ERK signaling axis to activate EMT. Together, several extracellular signals are involved in EMT.

The resulting mesenchymal cells can revert back to the epithelial state, called a mesenchymal-epithelial transition (MET). MET is a reversible transition from spindle-like shaped mesenchymal cells to epithelial cells. To complete the final steps of metastasis, MET is thought to be required to efficiently form metastases at the distance sites [81]. Ocaña et al., demonstrate that loss of Prrx1, an EMT inducer, is essential for metastatic colonization by reverting cells from EMT to MET [82]. Takaishi *et al.*, show that MET reduces cancer malignancy with epithelial properties in squamous cell carcinoma cells [83], highlighting the complexity of MET process. These results indicate the importance of understanding the MET process, which has yet not fully understood.

1.3 Lipid Metabolic Reprogramming in Cancer

Dysregulation of cellular metabolism is regarded as an emerging hallmark of cancer to sustain cellular survival and proliferation in changing and adverse conditions [84]. One metabolic pathway exploited by cancer cells to sustain growth is lipid metabolism. Lipids are hydrophobic biomolecules including di- and tri-acylglycerols, fatty acids, sphingolipids, and sterols. As lipids are used as a source of energy storage, structural components of cell membranes, and cellular signaling, metabolic changes in lipids are associated with cancer development [85]. The following sections discuss lipid metabolic reprogramming in cancer, specifically focusing on fatty acid synthesis, lipid accumulation, and utilization.

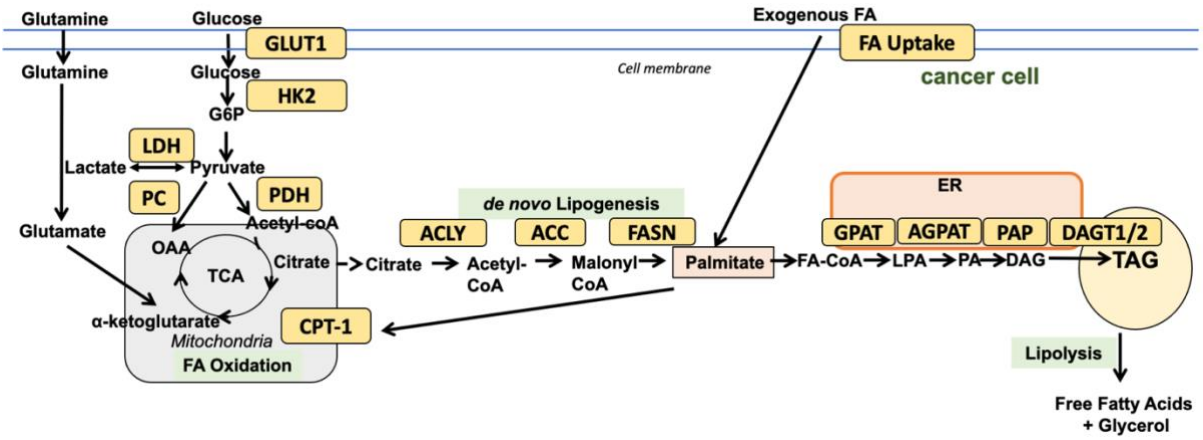

Figure 1.1 Energy Metabolism in Cancer Cells: *de novo* Fatty Acid and TAG Synthesis

Abbreviations: GLUT1: Glucose transporter 1; HK2: Hexokinase 2; LDH: Lactate dehydrogenase; PC: Pyruvate carboxylase; PDH: Pyruvate dehydrogenase; CPT-1: Carnitine palmitoyltransferase 1; ACLY: ATP-citrate lyase; ACC: acetyl-CoA carboxylase; FASN: Fatty Acid Synthase; GPAT: glycerol-3-phosphate acyltransferase; AGPAT: acylglycerolphosphate acyltransferase; PAP: phosphatidic acid phosphatase; DGAT 1 & 2: acyl-CoA:diacylglycerol acyltransferase; TAG: Triacylglycerol; ER: Endoplasmic reticulum

1.3.1 Fatty Acid Synthesis in Cancer

In 1989, Kuhajda *et al.*, first reported a protein in tumor cells that is correlated with poor prognosis for recurrence and death of breast cancer patients [86]. Five years later, researchers identified this molecule by peptide sequencing as fatty acid synthase (FASN) [87]. FASN is a multi-enzyme protein complex that catalyzes *de novo* lipogenesis of fatty acids, predominantly the 16-carbon saturated fatty acid palmitate from acetyl-CoA, malonyl-CoA, and nicotinamide adenine dinucleotide phosphate (NADPH). As overexpression of FASN has been shown in multiple cancers, each step of fatty acid synthesis is described in this chapter (Figure 1.1).

Glucose metabolism provides the majority of substrates needed for *de novo* fatty acid synthesis. Facilitative glucose transporters (GLUTs) are upregulated in cancers, including breast, lung, colorectal, and ovarian cancers [88], suggesting an important role of glucose in cancer progression. After entry into the cell, glucose is metabolized, through glycolysis, to pyruvate. Pyruvate enters into the mitochondria, where it is decarboxylated by pyruvate dehydrogenase complex to produce acetyl-CoA. Pyruvate can also be converted to oxaloacetate (OAA) by pyruvate carboxylase. The products acetyl-CoA and OAA from pyruvate are then condensed to produce citrate by citric synthase.

Glutamine is another substrate for *de novo* fatty acid synthesis through the tricarboxylic acid (TCA) cycle. Geldermalsen *et al.*, show that SLC1A5, a solute-carrying transporter that mediates uptake of glutamine, is highly expressed in most breast cancer subtypes [89], suggesting a potential role of glutamine in breast cancer cells. Glutamine is converted to glutamate via glutaminase, and glutamate is converted to α-ketoglutarate (AKG) by glutamate dehydrogenase. In some cells, NADPH-dependent isocitrate dehydrogenase converts AKG to isocitrate through reductive carboxylation, contributing to citrate production by glutaminolysis [90]. Isocitrate can also be converted to acetyl-CoA which can be a substrate for fatty acid synthesis.

Once citrate is synthesized and transported into the cytosol by SLC25A1 from the mitochondria, citrate is a substrate for the enzyme, ATP citrate lyase (ACLY). ACLY converts cytoplasmic citrate to OAA and acetyl-CoA, a precursor of *de novo* lipogenesis. ACLY is upregulated in various cancers, including breast, colorectal, non-small cell lung cancers [91]. Acetyl-CoA is also produced from acetate by acetyl-CoA synthetases (ACSSs) which have three isoforms, ACSS1, ACSS2, and ACSS3 [92]. One study demonstrates that ACSS2 expression positively correlates with higher tumor stage and lower survival from breast cancer patients [92]. Acetyl-CoA serves as a substrate for both acetyl-CoA carboxylase (ACC) and FASN, two important steps for *de novo* fatty acid synthesis.

ACC enzymes facilitate ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. Malonyl-CoA serves as a substrate for *de novo* lipogenesis and inhibits the activity of carnitine palmitoyltransferases (CPT), a rate-limiting enzyme of the pathway to utilize fatty acids for energy, fatty acid oxidation (FAO). Two isoforms, ACC1 and ACC2, have been identified. While cytoplasmic ACC1 regulates fatty acid synthesis, ACC2 activity negatively regulates FAO by inhibiting CPT1. Svensson *et al.*, shows that inhibition of ACC decreases fatty acid synthesis and tumor cell proliferation in non-small-cell lung cancer [93]. However, one study shows contradictory results that ACC1 depletion worsened tumor recurrence upon primary breast tumor resection in mice model [94], suggesting the role of ACC in cancer needs to be further elucidated.

FASN mediates the final step for the synthesis of the saturated fatty acid palmitate from acetyl-CoA and malonyl-CoA, which are produced by ACLY and ACC. Gonzalez-Guerrico *et al.*, show that FASN expression positively correlates with malignant progression of MCF10A

series breast cancer cells [95]. Additionally, FASN suppression restores a non-malignant to a metastatic breast cancer phenotype [95], highlighting the role of FASN in malignant progression in cancer.

Stearate is synthesized by the process of elongation of palmitate, the principal product of *de novo* lipogenesis, by stearoyl-CoA desaturase (SCD). SCD catalyzes the conversion of saturated fatty acid into monounsaturated fatty acid (MUFA). SCD produces palmitoleoyl-CoA and oleoyl-CoA from palmitoyl-CoA and stearoyl-CoA, respectively. There are five SCD isoforms, SCD 1-5, and two isoforms are found in human tissues, SCD 1 and SCD 5 [96]. SCD 1 is the main isoform expressed in all tissues, including the breast, lungs, and adipose tissue [96]. SCD1 inhibition results in decreased spheroid growth in colon cancer cells partly through downregulating sterol regulatory element-binding proteins (SREBP), basic helix-loop-helix–leucine zipper family, signaling pathways that regulate fatty acid and cholesterol synthesis [97]. Previous evidence shows that the SCD product, unsaturated palmitoleic acid (16:1n-7), in serum is involved in the development of high-grade prostate cancer [98], suggesting MUFA's may be a serum marker for risk of prostate cancer. Therefore, the role of *de novo* fatty acid synthesis involvement in cancer progression continues to be an area of investigation among researchers.

1.3.1.1 Mechanisms Regulating Fatty Acid Synthesis

SREBPs play a role in the regulation of genes involved in lipid metabolism. Three SREBP isoforms, SREBP1(-a and -c) and SREBP2, have been identified in mammalian cells and all isoforms regulate the lipogenic process. Specifically, SREBP1a regulates both fatty acid and cholesterol synthesis, and SREBP1c activates fatty acid synthesis [99]. In addition, SREBP2 activates cholesterol synthesis and uptake in liver and adipose tissue [99]. Furthermore, recent evidence shows that SREBP-1 is overexpressed and essential to maintaining proliferation in various cancers cells [100].

Researchers have proposed various pathways involved in the regulation of SREBPs. Evidence shows that SREBPs are regulated by PI3K/Akt signaling and mTOR in cancer cell lines. For instance, PI3K and K-*ras* oncogene stimulates *de novo* lipogenesis through mTORC1 signaling and SREBP activity in breast epithelial cells [101]. The researchers also show that SREBP promotes *de novo* lipogenesis through regulation of lipogenic enzymes, including FASN and SCD [101]. Consistent with this finding, Yang *et al.*, show that SREBP1c upregulates FASN

expression in MCF-7 breast cancer cells [102]. Ricoult *et al.,* also shows that SCD expression was significantly decreased by SREBP depletion in breast, prostate, colon cancer cell lines [103]. Genetic inhibition of SREBP-1 in glioblastoma cells results in decreased protein expression of FASN and ACC by Akt [104]. These results suggest FASN, SCD, and ACC are canonical SREBP targets genes.

In addition to SREBP-mediated *de novo* lipogenesis, other pathways are involved in the regulation of lipogenic enzymes. For example, human epidermal growth factor receptor 2 (HER2) overexpression breast cancer BT474 cells show increased expression of FASN and ACC by HER2/PI3K/Akt pathway via mTOR activation, independent of activation of SREBP-1 [105]. Collectively, these results support that *de novo* lipogenesis is regulated by multiple pathways in cancer cells.

1.3.2 Lipid Accumulation in Cellular Lipid Droplets

During cancer progression, there is evidence to suggest that fatty acids are synthesized in amounts more than the needs of the cell. To prevent the toxicity of fatty acids, one mechanism that has been proposed is that surpluses of fatty acids and cholesterols are sequestered as cytoplasmic neutral lipids [106], TAG or cholesterol ester (CE), within lipid droplets in cells. TAG consists of three fatty acid esterified to a glycerol backbone. TAG is synthesized from fatty acids by acyl-CoA synthetase conversion of fatty acids to fatty acyl-coenzyme A (fatty acyl-CoA). Glycerol-3-phosphate acyltransferase (GPAT) esterifies fatty acyl-CoA to glycerol-3-phosphate backbone in the sn-1 position to produce lysophosphatidic acid, which is a rate-limiting step of glycerolipid synthesis (Figure 1.1). Lysophosphatidic acid is further converted to phosphatidic acid with an addition of fatty acyl-CoA to the sn-2 position of glycerol backbone by acylglycerolphosphate acyltransferase (AGPAT) located at the endoplasmic reticulum (ER). Phosphatidic acid is then dephosphorylated to 1,2-diacylglycerol (DAG) by phosphatidic acid phosphatase (PAP). Finally acyl-CoA:diacylglycerol acyltransferase 1 and 2 (DGAT1 and DGAT2) esterifies DAG to TAG. Nardi *et al.,* show that DGAT1 inhibition results in the suppression of lipid droplets, decreased proliferation, migration and invasion in prostate cancer cells *in vitro,* and decreased tumor growth *in vivo,* suggesting DGAT1 plays a role in lipid mediated-tumor growth [107].

Another component of lipid droplets is CE, which is derived from the esterification of fatty acyl-CoA and cholesterol by acyl-CoA:cholesterol acyltransferase 1 and 2 (ACAT1 and ACAT2). A previous study shows higher protein expression of ACAT1 in ovarian cancer compared to untransformed cell lines [108]. The researchers also show increased concentration of CE, free cholesterol, and total cholesterol concentrations in ovarian cancer cell lines [108]. Furthermore, ACAT-1 depletion reduced cell viability, migration [108], suggesting an important role of ACAT and CE in tumor aggressiveness.

1.3.3 Fatty Acid Utilization

1.3.3.1 Catabolism of Lipid Droplets in Cancer

TAG stored in lipid droplets is sequentially hydrolyzed by cytoplasmic triglyceride lipases recruited to the lipid droplets, which may be utilized to meet cellular energy demands for cancer cell proliferation (Figure 1.1). TAG is hydrolyzed into DAG and free fatty acid by adipose triglyceride lipase (ATGL), which binds to the lipid droplet surface. ATGL is activated by cofactor comparative gene identification-58 (CGI-58), which is associated with perilipin on the lipid droplet surface in the basal state. Lipolytic stimuli promotes protein kinase A (PKA) phosphorylation of perilipin, leading to dissociation of CGI-58 from perilipin. Dissociated CGI-58 translocates to ATGL and activates ATGL, a rate-limiting enzyme for TAG hydrolysis. Evidence suggests that ATGL expression in breast tumors is related with aggressive features as higher ATGL expression is expressed in aggressive breast cancer cell lines, including MDA-MB-231 and SUM159PT, compared to non-aggressive breast cancer cell lines, such as MCF-7 and T47D [109]. In addition, activation of PKA results in phosphorylation of hormone-sensitive lipase (HSL) which hydrolyzes DAG to monoacylglycerol and free fatty acid. HSL also hydrolyzes TAG, monoacylglycerol, and CE to release free fatty acids. Monoacylglycerol lipase (MAGL) hydrolyzes monoacylglycerol into free fatty acid and glycerol. The fatty acids produced from lipolysis may serve as substrates for FAO to sustain energy, for re-esterification to TAG, for membrane synthesis or as signaling molecules. One study shows, employing proteomic analysis, that aggressive breast and ovarian cancer cell lines have high expression levels of MAGL [110]. MAGL depletion leads to a significant reduction in tumor growth by transplanting cancer cells into mice [110], suggesting the importance of MAGL on cancer cells growth.

Together, several steps of TAG hydrolysis are involved in cancer aggressiveness, indicating the role of TAG hydrolysis in breast cancer research.

In addition to the degradation of TAG by cytoplasmic lipases, recent findings indicate that autophagy is considered as an alternative pathway for lipid storage mobilization [111]. Upon engulfment of lipid droplets by a double membrane, an autophagosome is formed. Autophagosomes fuse with the lysosome to form an auto-lysosome. The engulfed lipid droplet is broken down by lysosomal acid lipase to release free fatty acids [112, 113]. Evidence suggests that autophagy may play dual roles in cancer cell progression. One study shows that the inhibition of autophagy increases the number of lipids, reduces the cell viability, and induces apoptosis in Caco-2 cell lines [114], indicating autophagic inhibition as a therapeutic target for colorectal cancer cells. On the contrary, autophagy shows an anticancer effect in other cell models. For example, increased autophagy results in free fatty acid accumulation and induces ER stress-mediated apoptosis in HeLa cell lines [115]. Taken together, further research is needed to investigate the role of autophagy in cancer cell progression and aggressiveness.

1.3.3.2 Fatty Acid Oxidation

In nutrient limiting conditions, fatty acids are an important energy substrate to sustain cell growth by FAO. Fatty acids are used to form fatty acyl-CoAs by acetyl-CoA synthetases before entry into the mitochondria. CPT1, a rate-limiting step of FAO, transports fatty acyl-CoAs through the mitochondrial outer membrane. FAO, also called β-oxidation, produces acetyl-CoA, NADH, and $FADH_2$, which results in the production ATP. There are three isoforms of CPT1, including CPT1α, CPT1β, and CPT1C [116]. While CPT1α and CPT1β are widely distributed in various tissues, CPT1C is primarily expressed in the brain. CPT1α is the primary enzyme utilized for FAO as it has a higher substrate inhibition for its substrate, malonyl-CoA, compared to CPT1β [116].

CPT1α has been reported to promote cancer cell growth and metastasis. For example, CPT1α expression is reported to be upregulated in metastatic sites compared to primary sites in clinical tissue specimens from colorectal cancer patients [117]. In addition, CPT1α-mediated FAO activation inhibits anoikis in colorectal cancer cells [117]. However, there are contradictory results that high expression of 19-genes enriched in FAO pathways is associated with increased

patient survival in METABRIC patient datasets [118]. In addition, FAO is downregulated in multiple tumor types compare to non-cancer tissues, and overexpression CPT1α decreases proliferation and migration in MDAMB-231 breast cancer cells [118]. Thus, further studies are required to understand the role of CPT1a in cancer metabolism.

1.4 Vitamin D

Vitamin D is a nutrient derived from the diet or synthesized in the skin via the energy of sunlight from the precursor 7-dehydrocholesterol [119]. Vitamin D comes in two forms: plant-derived vitamin D_2 (ergocalciferol) [120] and animal-derived vitamin D_3 (cholecalciferol), synthesized from 7-dehydrocholesterol. Thus, vitamin D_2 can be obtained by diet alone, but vitamin D_3 can also be synthesized endogenously in the skin. Although both forms of vitamin D are structurally different, the same enzymes act on both forms of vitamin D to have the similar biological effects [121]. Historically, vitamin D is known to be essential for the maintenance of health with the primary functions of calcium and phosphorus metabolism, and consequently, bone homeostasis. Emerging evidence shows that vitamin D exerts additional roles in optimizing health, including in breast cancer and in adipocytes.

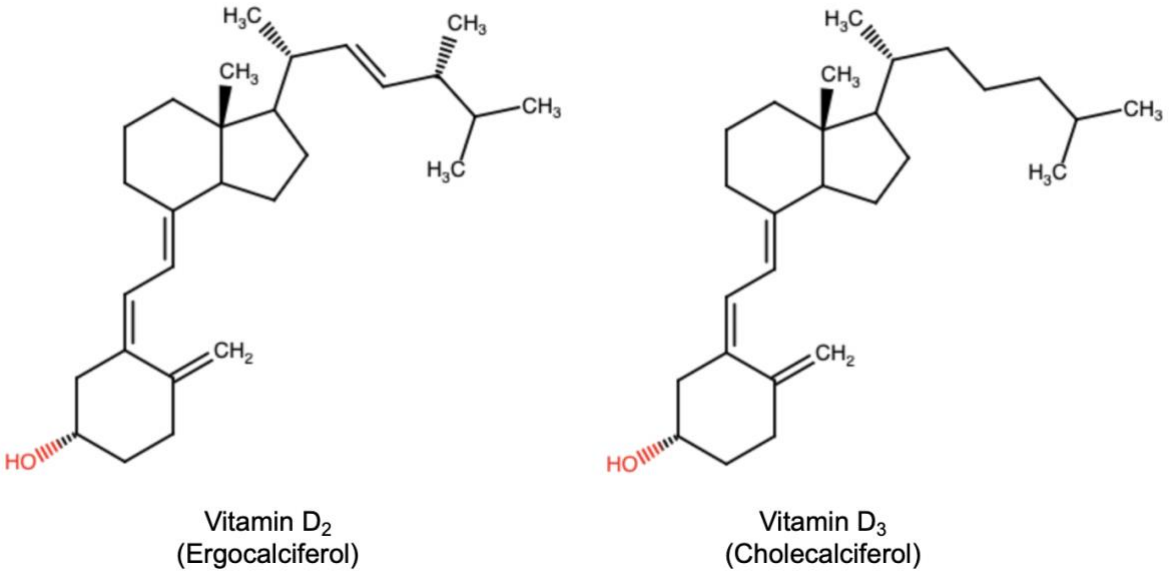

Figure 1.2 Vitamin D Structure

1.4.1 Sources of Vitamin D and Dietary Intake

Few foods naturally contain vitamin D. Fatty fish and fish liver oils are the most abundant sources of vitamin D [122]. Egg yolk and sun-dried mushrooms also contain small amounts of vitamin D. In order to meet vitamin D requirements, fortified foods such as orange juice, cheese, and milk or supplements are needed.

To synthesize vitamin D in the skin, ultraviolet (UV) B radiation is required to convert 7-dehydrocholesterol to vitamin D_3 [123]. However, adequate UV light can be limited at certain latitudes, and is influenced by seasonal variations and times of day. Latitudes above 37 degrees northern latitude or below 37 degrees southern latitude do not provide sufficient UV light for the production of vitamin D in winter [124]. In addition, skin pigment and use of sunscreen may inhibit UVB exposure to produce vitamin D_3 in the skin [123]. Due to these constraints, vitamin D deficiency is a common medical occurrence in the world as well as the United States [123]. Thus, generally, a vitamin D supplement is required to meet vitamin D requirements.

In 2010, the recommended dietary allowance for vitamin D of 600 international units (IU)/day was established for both men and women from 1 to 70 years of age by the Institute of Medicine [125]. Since vitamin D synthesis by UV exposure decreases in people over 70 years of age, 800 IU/day of vitamin D is recommended for men and women for this age group. However, vitamin D intake decreases is reported for this elderly population [126]. Even though the occurrence of vitamin D toxicity is rare, vitamin D toxicity can occur with long-term intake of high vitamin D supplementation. The current upper limit of vitamin D intake (UL, the highest daily intake of the nutrient without risk) is 4,000 IU/day for pregnant women and persons over 9 years old [127]. These recommended levels are difficult to attain through the diet thus it is important to meet the vitamin D requirement using vitamin D supplements to alleviate the vitamin D deficiency which is a common occurrence in the world.

1.4.2 Vitamin D Metabolism

Whether vitamin D is derived from the diet or synthesized in the human body, vitamin D enters the circulatory system bound to the vitamin D binding protein (DBP) [128]. The first step in vitamin D metabolism is the production of 25-hydroxyvitamin D (25(OH)D) by the 25-hydroxylase enzyme in the liver [129]. This metabolite (25(OH)D) is the primary circulating

form of vitamin D and is considered an indicator of vitamin D status as 25(OH)D has a 3-week half-life [129] and there is little regulation of the activity of the 25-hydroxylase. According to the Institute of Medicine, serum 25(OH)D at concentrations below than 30 nmol/L (12 ng/mL) is considered vitamin D deficient, which may lead to rickets in infants and children and osteomalacia in adults. It is also reported that pituitary thyroid hormone (PTH) secretion, regulated by vitamin D status, was maximally suppressed with 30 nmol/L (12 ng/mL) of 25(OH)D [130]. A level of 50-125 nmol/L (20-50 ng/mL) is considered adequate for bone and overall health. The recommended serum 25(OH)D concentrations are shown in Table 1.1.

Table 1.1 Serum 25(OH)D Levels and Health

Source: Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: National Academy Press, 2010.

25(OH)D Levels (nmol/L)	25(OH)D Levels (ng/mL)	Health Status
>125	> 50	Linked to potential adverse effects, particularly at >150 nmol/L (>60 ng/mL)
50-125	20-50	Generally considered adequate for bone and overall health in healthy individuals
30-50	12-20	Generally considered inadequate for bone and overall health in healthy individuals
< 30	< 12	Associated with vitamin D deficiency, which can lead to rickets in infants and children and osteomalacia in adults
Note: One nmol/L = 0.4 ng/mL, and 1 ng/mL = 2.5 nmol/L.		

In order for 25(OH)D to become bioactive, additional hydroxylation at the one carbon position is required. This reaction occurs through the mitochondrial cytochrome P450 enzyme 25-hydroxyvitamin D 1-α hydroxylase (CYP27B1), resulting in the production of the active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). The CYP27B1 enzyme is under tight regulation in the kidney as it is activated by serum PTH and suppressed by $1,25(OH)_2D$, calcium, and phosphorus [131]. The 1α-hydroxylase enzyme is known to be highly expressed in the kidneys, but several studies have revealed that it is also expressed in various other tissues, including the pancreas [132], adipose tissue [133], and breast [134]. Thus, the presence of the

1α-hydroxylase enzyme may lead to local production of $1,25(OH)_2D$ under conditions of high vitamin D status. Degradation of 25(OH)D and $1,25(OH)_2D$ is accelerated by 24-hydroxylase, which converts 25(OH)D or $1,25(OH)_2D$ into $24,25(OH)_2D$ or $1,24,25(OH)_3D$, respectively, leading to excretion of vitamin D metabolites. The 24-hydroxylase enzyme is also expressed in various tissues including breast [135] and kidney [136], and is regulated by $1,25(OH)_2D$, functioning as a negative feedback loop Overall, vitamin D metabolism contributes to regulation of overall body calcium homeostasis (Figure 1.3).

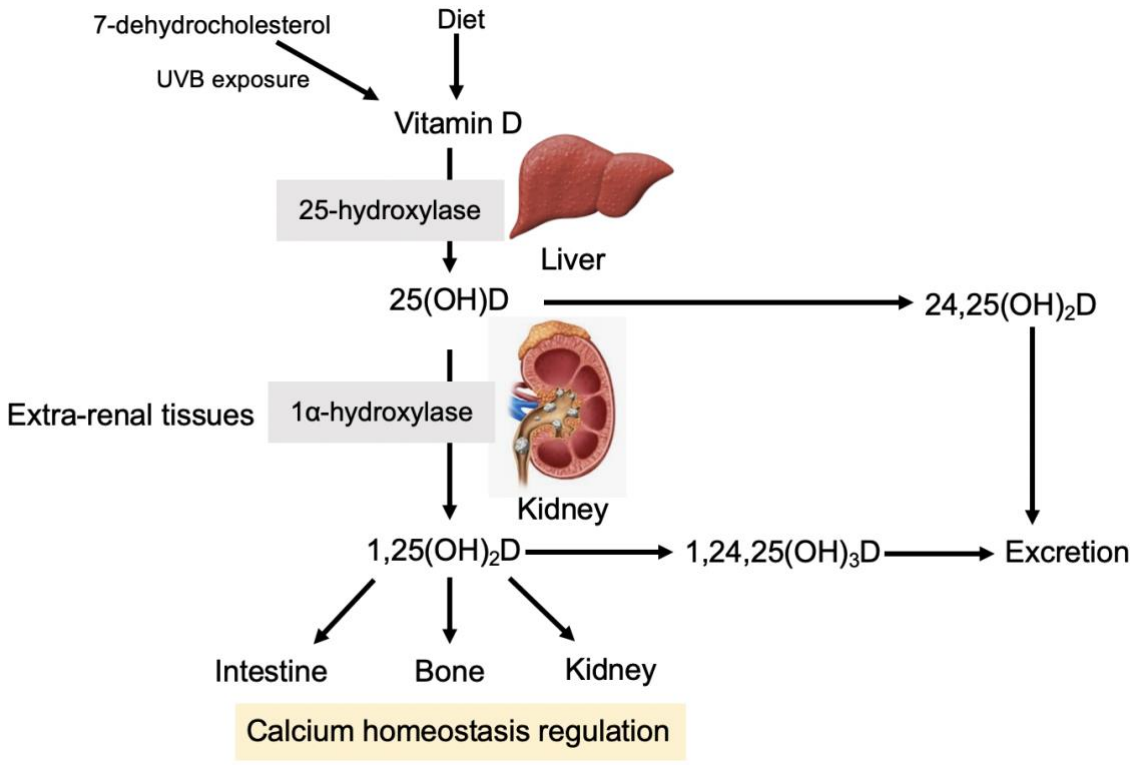

Figure 1.3 Vitamin D Metabolism in Humans

1.4.3 Vitamin D Receptor and Vitamin D Signaling

1.4.3.1 Vitamin D Receptor

The nuclear vitamin D receptor (VDR) was first discovered by Brumbaugh and Haussler in chick intestinal mucosal cells in 1974 [137]. Following this discovery, VDR was shown to be expressed in many human tissues, including the kidney, pancreas, parathyroid, pituitary gland, placenta [138], and mammary glands [139]. The affinity of $1,25(OH)_2D$ to the VDR is

approximately 100 times greater than that of 25(OH)D [140]. The expression of VDR is regulated by many factors, including PTH, retinoic acid, glucocorticoids, and 1,25$(OH)_2$D [141, 142]. For example, 1,25$(OH)_2$D induces VDR protein expression in MCF-7 cells [143], suggesting an increase in the genomic action of 1,25$(OH)_2$D by upregulation of VDR mediated transcriptional activity. On the other hand, contradictory findings have been reported that 1,25$(OH)_2$D decreases VDR protein expression in T47D breast cancer cells [144]. Therefore, further investigation is needed to determine how VDR is regulated by 1,25$(OH)_2$D in breast cancer cells to further elucidate the role of vitamin D and the VDR during breast cancer progression.

Growing evidence suggests that VDR expression is related to cancer progression. In an epidemiological study, VDR expression is inversely associated with more aggressive breast cancer [145], which suggests a reduced responsiveness to 1,25$(OH)_2$D at this stage of breast cancer. Another study by Cross *et al.*, also shows that VDR gene expression is lower in late stage colorectal cancer compared to early phase of carcinoma [146]. Conversely, a higher VDR expression is correlated with better prognosis in breast cancer patients [147]. Thus, the expression and activity of VDR are important factors in elucidating how to develop strategies for the prevention or treatment of breast cancer with vitamin D. However, little information is available on how 1,25$(OH)_2$D regulates VDR expression or transcriptional activity during breast cancer progression.

1.4.3.2 Classical Signaling of 1,25$(OH)_2$D through the Vitamin D Receptor

The main mechanism of action of 1,25$(OH)_2$D is the transcriptional regulation of genes by binding to the VDR. The vitamin D metabolite, 1,25$(OH)_2$D, is bound to serum DBP in circulation and enters the target cell to bind to VDR. The VDR-1,25$(OH)_2$D complex forms a heterodimer with the retinoid X receptor (RXR), and this complex acts as a transcription factor. The heterodimer binds to the VDR response elements (VDRE) of gene promoters [148] and The Vitamin D receptor interaction protein complex are co-activators that bind to the VDR-RXR complex [149], recruiting the basal transcription unit and RNA polymerase II to the TATA box of target genes to regulate transcription. Evidence shows that more than 200 genes have been identified as targets of 1,25$(OH)_2$D, including VDR, CYP24A1, PTH, transient receptor potential vanilloid (TRPV)5/6, and receptor activator of nuclear factor kappa-B ligand (RANKL) [150].

These genes, regulated by vitamin D metabolism, are involved in important processes such as vitamin metabolism, calcium and phosphorus metabolism, bone metabolism, and cell cycle regulation [150].

1.4.3.3 Rapid signaling of $1,25(OH)_2D$

In addition to the genomic effect through the VDR, evidence suggests that $1,25(OH)_2D$ also exerts a non-genomic effect. The membrane-associated rapid response steroid-binding proteins (MARRS) is proposed to be a membrane receptor for $1,25(OH)_2D$ and initiates various signal transduction pathways, leading to rapid responses in the target cells [151]. MARRS is also known as ER proteins 57 and 60 (ERp57, ERp60), and glucose-regulated protein 58 [151]. MARRS has been shown to be associated with scaffold proteins, such as caveolin [152]. Several studies have shown that MARRS is involved in calcium absorption in intestinal cells. For example, when MARRS is deleted in murine intestinal epithelial cells, $1,25(OH)_2D$-mediated rapid response to calcium absorption and PKA signaling is eliminated [153]. Another study tests the effect MARRS on the growth plate chondrocytes in rats [154]. In the absence of MARRS, a rapid $1,25(OH)_2D$-mediated increase in protein kinase C activity is diminished [154]. These results suggest a potential role of MARRS as a $1,25(OH)_2D$ receptor mediating non-genomic action. However, the role of MARRS receptor is currently not fully understood and needs to be further be elucidated.

In addition to the MARRS receptor in $1,25(OH)_2D$-mediated rapid signaling pathways, it is reported that VDR may also be involved in rapid $1,25(OH)_2D$ signaling. Nguyen *et al.*, demonstrate that when VDR expression is reduced the rapid response to $1,25(OH)_2D$ to increase intracellular calcium concentration in human skin fibroblasts is eliminated [155], suggesting that the VDR is required for the rapid effects of $1,25(OH)_2D$. Additionally, Buitrago *et al.*, also shows that inhibiting VDR expression significantly decreased (-94%) $1,25(OH)_2D$-mediated rapid protein tyrosine phosphorylation in skeletal muscle cells [156], indicating a role of VDR in non-genomic action of $1,25(OH)_2D$. Collectively, these results provide the evidence that both MARRS receptor and VDR may be involved in regulation of rapid signaling of $1,25(OH)_2D$.

1.4.4 Vitamin D and Obesity

It is well established vitamin D status and adiposity are inversely associated, and several reasons have been proposed to explain this relationship. A variety of studies support the inverse relationship with obesity as serum 25(OH)D levels are inversely proportional to adiposity, including fat mass, BMI, and waist circumference [157-159]. One explanation for this inverse association may be due to lifestyle factors, such as lack of vitamin D in the diet or low vitamin D endogenous production due to little outdoor activity [129]. In addition, another proposed explanation for this relationship is sequestration of vitamin D in adipose tissues, since vitamin D is a fat-soluble vitamin and may be stored in fat tissue [160]. This explanation is generally accepted to explain the association between 25(OH)D level and adiposity, although the effect of $1,25(OH)_2D$ on adipocyte metabolism to reduce lipid accumulation may also play a role. For example, it is reported that $1,25(OH)_2D$ regulates adipocyte differentiation with conflicting results. While Hida *et al.*, show that $1,25(OH)_2D$ reduces differentiation in 3T1-L1 with decreased peroxisome proliferator-activated receptor γ (PPARγ) expression [161], another study demonstrates that $1,25(OH)_2D$ induces adipocyte differentiation in rat calvaria cells [162]. These results suggest the various roles of $1,25(OH)_2D$ on adipocyte tissue physiology.

Thus, this inverse relationship provokes an interest in the relationship between vitamin D status and obesity with the hypothesis that vitamin D supplementation improves weight and reduces fat accumulation in obese individuals. To test this hypothesis, a randomized double-blind clinical trial was conducted for 12 months with placebo or a weekly dosage of 20,000-40,000 IU vitamin D group in overweight or obese men and women [163]. There was no significant weight reduction with vitamin D supplementation in overweight and obese adults even though the vitamin D group achieved serum average 25(OH)D level >85 nmol/L. Similar to the results of this study, another clinical trial shows no effect on weight loss in overweight subjects with vitamin D supplementation of 3332 IU/day for 12 months [164]. However, the vitamin D supplement group significantly decreased the level of serum triglycerides and the inflammation marker TNFα, indicating the potential beneficial effect of vitamin D to improve cardiovascular risk in overweight subjects. Taken together, these studies show that vitamin D supplementation alone is not effective for weight loss but may have a beneficial effect on other health consequences of overweight or obese individuals.

1.4.4.1 Regulation of Inflammatory Adipokines by 1,25(OH)$_2$D

During obesity, fat tissue expands, resulting in insufficient blood flow, which stimulates inflammation with hypoxia and macrophage infiltration. At this time, anti-inflammatory adipokine secretion decreases, while cytokine such as IL-6, TNFα, resistin, and MCP-1 secretion increases [165]. However, several studies in cells and animals have shown that vitamin D is involved in regulating inflammation in adipose tissues or in the whole body. One *in vitro* study by Ding *et al.* [166] shows that 1,25(OH)$_2$D treatment of human adipocytes decreases the protein expression of inflammatory cytokines including TNFα, IL-6, and MCP-1 by inhibiting NF-kB, p38 MAPK and phosphorylated ERK1/2 signaling pathways. Similarly, Mutt *et al.,* show that 1,25(OH)$_2$D decreases lipopolysaccharides-stimulated IL-6 secretion in human mesenchymal stromal cell derived adipocytes via NF-kB pathway [167], suggesting that the anti-inflammatory effect of 1,25(OH)$_2$D in adipose tissue is through inhibition of the NF-kB pathway. Similar results have been shown in animal studies. For example, Chang *et al.,* randomized rats to a control group (1000 IU vitamin D/kg), diet-induced obese sufficient vitamin D group (1000 IU vitamin D/kg), and diet-induced obese diet with low vitamin D (25 IU vitamin D/kg) group [168]. High fat diet-induced obese mice show increased IL-6 and TNFα expression and the expression of these genes was further increased in adipose tissue in the vitamin D deficient group [168], indicating a potential anti-inflammatory effect of vitamin D in obese models. Bastic *et al.,* also investigate the effects of high vitamin D and calcium intake on inflammation in mice fed a Western diet to induce obesity compared to those fed a control diet with and without vitamin D (1000 IU/day) and calcium (3000 mg/day) supplementation [169]. Similar to the previous results, in these studies the serum IL-1β and MCP-1 inflammatory markers are reduced with higher dietary vitamin D and calcium intake. Together, these results support that vitamin D decreases inflammatory markers in both *in vitro* and *in vivo* models.

However, epidemiological and clinical evidence show inconsistent results of the effect of vitamin D on inflammation. A meta-analysis using 13 randomized controlled trials shows that there is no beneficial effect of vitamin D supplementation on inflammation markers [170]. In addition, a double-blinded intervention study conducted with 52 obese subjects given vitamin D (7000 IU/day) or placebo for 26 weeks [171] showed that even though 25(OH)D levels are increased with vitamin D supplements, there is no change in inflammatory markers, including IL-6, MCP-1, leptin, and adiponectin. Similarly, Carrillo *et al.,* showed that vitamin D

supplementation (4000 IU/day) during exercise training does not affect inflammatory biomarkers, including circulating C-reactive protein (CRP), TNFα, and IL-6, in healthy overweight and obese individuals [172]. In this study, despite of no significant effect of vitamin D on CRP after vitamin D supplementation, a significant correlation between serum 25(OH)D and CRP (r=0.49, P=0.03) was shown. On the other hand, another double-blind randomized clinical trial with obese individuals with vitamin D deficiency on a weight reduction diet [173], showeda significantly greater decrease in weight, serum MCP-1 and increase in serum 25(OH)D in the vitamin D supplemented group. These results indicate a potential beneficial role of vitamin D on inflammatory markers in obese people. In summary, the results remain controversial and further controlled clinical studies, as well as further exploration of the mechanisms, are needed to investigate the role of vitamin D on weight loss as well as inflammatory markers in obese individuals.

1.4.5 Vitamin D and Breast Cancer

Although vitamin D was originally shown to solely function as a regulator of calcium homeostasis and bone health, evidence from epidemiology, clinical and pre-clinical studies demonstrate a protective effect of vitamin D in cancer. In 1980, Garland first proposed the association between vitamin D produced through UVB exposure and reduced colon cancer mortality rates [174]. Subsequently, Grant *et al.,* shows the association between UVB exposure with a reduced risk of mortality from breast, ovarian, and prostate cancers, suggesting higher vitamin D status may prevent cancers [175]. Other research also supports that serum 25(OH)D concentrations correlate with reduced risk of breast cancer [176], thus a more direct connection than UVB alone. In addition to epidemiological studies, clinical and preclinical studies have demonstrated the anti-cancer effect of vitamin D. These functions include pro-apoptosis, anti-proliferation, and anti-angiogenic effects of the vitamin and its metabolites, supporting the protective effect of vitamin D on cancer progression.

1.4.5.1 Vitamin D and Breast Cancer Prevention from Epidemiology and Clinical Studies

As described above, Garland *et al.,* first showed the association between UVB exposure and lower breast cancer risk [177], suggesting a protective effect of vitamin D on breast cancer. However, several prospective studies have shown inconsistent results. Shin *et al.,* show that higher vitamin D intake (>500 IU/day) is associated with reduced risk of breast cancer only in the premenopausal women, but not postmenopausal women [178]. Another meta-analysis that studies the relationship between vitamin D intake and breast cancer mortality by Kim *et al.,* shows that better survival among woman diagnosed with breast cancer was associated with higher vitamin D intake and higher levels of serum 25(OH)D [179]. On the other hand, a meta-analysis of observational studies from 6175 cases in 10 studies shows that there is no association between 25(OH)D level and breast cancer [180]. In addition, *Gissel et al.,* conducted a meta-analysis from 6 studies and found no association between vitamin D intake and breast cancer risk [181]. However, when they restrict the analysis comparing high vitamin D intake (>400 IU/day) versus low vitamin D intake (<150 IU/day), there is a trend towards less breast cancer with high vitamin D intake (>400 IU/day). Different subpopulations and different study designs, such as different age and previous supplement users, may lead to these inconsistent results. It is also possible that higher doses or vitamin D status than current recommendation are required to prevent breast cancer. Therefore, clinical studies designed with these factors considered are needed to investigate the clinical association between vitamin D and breast cancer risk.

Several clinical trials have also been conducted to elucidate the effect of vitamin D supplementation on breast cancer risk. The Women's Health Initiative randomized controlled trial examined the effect of calcium and vitamin D on benign proliferative breast disease in postmenopausal women after an average 6.8 years of follow-up [182]. Daily use of calcium and vitamin D (400 IU/day) did not change the risk of benign proliferative breast disease. The dose of 400 IU/day used in this clinical study may not be sufficient to impact breast cancer since the current recommended dietary allowance suggested by the Dietary Guidelines for Americans is 600 IU/day, which is based on maximizing bone health. It is possible that even higher doses than the current recommendations may be required to achieve a significant impact on other health outcomes than bone health. Another randomized placebo-controlled trial study was conducted to determine the effect of vitamin D and calcium supplementation on the risk of all types of cancers [183]. A total of 1179 subjects are randomly assigned to placebo, calcium only

(1400-1500 mg/day), or vitamin D (1100 IU/day) plus calcium group for four years. When the subjects who developed breast cancer in the first year were excluded from the analysis, vitamin D and calcium reduced the risk of all cancers in postmenopausal women compared to the placebo group. On the other hand, calcium only group does not have a significant reduced the risk of cancer risk, suggesting the effect is due to the vitamin D supplementation. Moreover, an interesting finding emerged from a retrospective review of patients with $HER2^{+ve}$ breast cancer who have received chemotherapy with trastuzumab with or without vitamin D supplementation [184]. Those who consumed the vitamin D supplements experienced significant improvement in disease-free survival. Together, evidence from these studies suggests vitamin D may have a preventive role in breast cancer, and that an understanding the molecular mechanisms is important to discern when and in what population vitamin D may be effective to prevent breast cancer.

1.4.5.2 Molecular Mechanisms of $1,25(OH)_2D$ in Cancer Prevention

There are several mechanisms implicated in the anti-cancer effects of the vitamin D metabolite, $1,25(OH)_2D$. First, $1,25(OH)_2D$ inhibits the growth of many malignant cell types by inducing cell cycle arrest and also by stimulating apoptosis [185]. For example, $1,25(OH)_2D$ inhibits the proliferation of prostate cancer cells through cell cycle arrest in the G1/G0 phase in a p53-dependent manner by increasing the expression of the cyclin-dependent kinase inhibitors p21 and p27 [186] and decreasing cyclin-dependent kinase 2 activity [187]. In addition, $1,25(OH)_2D$ induces apoptosis in several breast cancer cells by mitochondrial disruption and by suppressing the expression of anti-apoptotic genes, such as B-cell lymphoma 2, and increasing the expression of the pro-apoptotic gene Bax [188, 189]. Further, $1,25(OH)_2D$ inhibits breast cancer cell growth and induces morphological and biochemical markers of apoptosis in MCF-7 cells [190]. Thus, *in vitro* evidence supports that $1,25(OH)_2D$ plays a role in inhibition of breast cancer potentially by inducing cell cycle arrest and apoptosis.

In addition to cell cycle arrest and apoptosis, $1,25(OH)_2D$ also exhibits an anti-inflammatory effect that may also contribute to inhibition of cancer. NF-kB activity is downregulated by $1,25(OH)_2D$ in breast MCF-7 cells by repression of NF-kB p65 transactivation subunit through the VDR [191]. Also, $1,25(OH)_2D$ induces MAP kinase phosphatase 5 (MKP5) mRNA expression, the enzyme that inactivates MAPKs by binding to and activating the VDR,

with subsequent interaction with a VDRE identified in the MKP5 promoter in prostate cancer cells [192]. This action leads to a decrease in the production of pro-inflammatory cytokines IL-6 that participates in inflammation-associated carcinogenesis by inhibiting p38 signaling [192]. These results support $1,25(OH)_2D$ anti-inflammatory effects in cancer cells which may inhibit carcinogenesis.

Additionally, $1,25(OH)_2D$ regulates ER signaling pathway that is important in breast cancer proliferation. Since estrogen is known to promote mammary tumorigenesis, estrogen antagonists are therapeutic agents for breast cancer patients [193]. Transcriptional repression of aromatase, the enzyme that catalyzes estrogen synthesis from androgenic precursors, is one mechanism by which ER signaling is regulated by $1,25(OH)_2D$ [194, 195]. In addition, Stoika *et al.*, show that $1,25(OH)_2D$ reduces expression of ER transcriptionally through VDRE in ER promoter in MCF-7 cells [196]. These reduced level of estrogen as well as ER by $1,25(OH)_2D$ contribute to decreased proliferation of ER positive breast cancer cells [197]. Further, suppression of cyclo-oxygenase-2 (*Cox2*) expression by $1,25(OH)_2D$ leads to the reduction in the synthesis of pro-inflammatory mediators such as prostaglandin E2 [198], which are major stimulators of aromatase transcription in breast cancer cells [199, 200]. Taken together, this evidence supports that the active metabolite of $1,25(OH)_2D$ may inhibit cancer cell proliferation by regulating various ER and inflammatory signaling pathways involved in cancer progression.

1.4.5.3 Vitamin D and Breast Cancer Metastasis

Several studies investigate the preventive role of vitamin D on breast cancer metastasis. A meta-analysis demonstrating the association between serum 25(OH)D concentration and breast cancer mortality shows that increased 25(OH)D level is related with reduced risk of cancer associated mortality [201]. This indicates that vitamin D may have an important role in inhibiting breast cancer metastasis as breast cancer mortality is mainly due to metastasis.

There is also evidence in pre-clinical studies that vitamin D metabolites may inhibit breast cancer metastasis. One *in vivo* study demonstrates the role of the vitamin D analog, EB1089, on breast cancer metastasis. MDA-MB-231 breast cancer cells were injected into the cardiac ventricle of female mice and number and bone metastasis were measured [202]. EB1089-treated mice showed an increase in survival as well as fewer bone lesions compared to control mice. Another study by Ooi *et al.*, investigated the effect of vitamin D deficiency diet (vitamin D

free diet) on breast cancer metastasis to the bone in BALB/c mice [203]. Vitamin D deficiency promoted tumor growth in the bone, suggesting vitamin D may have a preventive role in breast cancer metastasis.

In addition to *in vivo* studies, several *in vitro* studies also provide evidence that supports an important role of vitamin D in prevention of breast cancer metastasis. Wilmanski *et al.*, demonstrated that $1,25(OH)_2D$ significantly inhibits breast to bone metastasis using a 3D reconstructed metastatic model [204]. Additionally, $1,25(OH)_2D$ inhibits EMT, an important step in metastasis, with an increase in E-cadherin and decreased in N-cadherin. Furthermore, another *in vitro* study shows that $1,25(OH)_2D$ significantly decreases cell migration and invasion compared to vehicle in SUM149 breast cancer cells [205]. These results highlight the importance of understanding the molecular mechanisms of vitamin D on breast cancer metastasis.

1.5 Leptin

Leptin is a regulator of energy balance and appetite through actions in the hypothalamus and is considered both a hormone and adipokine produced mainly by adipose tissue, also by gastric mucosa [206]. Leptin is a pleiotropic molecule that is related to food intake, inflammation, cell differentiation, and proliferation of different cell types, including breast cancer cells [207].

1.5.1 Leptin and Leptin Receptor

Before leptin was identified in 1994, Jackson laboratory identified two spontaneous obese mouse strains, *ob/ob* mice in 1950, and *db/db* mice in 1965 [208]. The *ob/ob* mice were spontaneously obese but lose weight when parabiotically paired to wild-type or *db/db* mice, suggesting that the *ob/ob* mutation occurred in a hormone that regulates hunger. The *ob* gene product was cloned in 1994 and termed leptin [208]. In contrast, the *db/db* mice are deficient in leptin receptor but overexpress leptin, leading to *db/db* as another obese model associated with leptin.

Leptin is 16kD peptide comprised of 167 amino acids produced by adipocytes, but also expressed in various tissues such as placenta, stomach, pituitary, and mammary tissues [209]. As leptin is mainly secreted from adipocytes, serum leptin level is proportional to adipose size and

mass and correlated with BMI. In addition, females produce more leptin than their male counterparts [210]. Thus, the role of leptin in the obese females is of particular interest.

The product of the diabetic (*db*) gene was identified as ObR or LEPR, termed the leptin receptor [211]. ObR is a single transmembrane protein that belongs to the class I cytokine receptor superfamily. ObR is expressed in tissues including brain, adipose tissue, pancreas, stomach, liver, lung, and placenta as well as breast tissues [212], indicating that leptin may exert pleiotropic roles in various tissues. Six isoforms of ObR are identified, Ob-Ra, b, c, d, e, and f. There are four isoforms with short cytoplasmic domains (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf), a long form Ob-Rb and a soluble for Ob-Re [213]. Among all ObR isoforms, the long isoform Ob-Rb is mainly expressed in breast tissues, thus the role of Ob-Rb and leptin continues to be an area of investigation in breast cancer.

1.5.2 Leptin in Breast Cancer Progression and Metastasis

Evidence suggests that leptin may be involved with breast cancer progression as both leptin and ObR are overexpressed in breast cancer compared to non-cancer breast epithelium [214]. Ishikawa *et al.,* also show that distant breast cancer metastasis is correlated with leptin and ObR expression [215]. In this study, 34% of ObR positive tumors show distant metastasis and all tumors are categorized as high leptin expressing group. However, no tumors that lacked ObR expression or leptin overexpression were associated with distant breast cancer metastases. Further, emerging evidence from a meta-analysis suggests that leptin may have a pivotal role in the pathogenesis and metastasis of breast cancer as the breast cancer cases with lymph node metastases show significantly higher serum leptin concentration [216]. Taken together, evidence from human and meta-analysis shows the important role of leptin on breast cancer progression and metastasis, supporting substantial additional research *in vivo* and *in vitro*.

1.5.3 Leptin Signaling Pathway in Breast Cancer

Leptin regulates various signaling pathways in breast cancers. Upon leptin binding to its receptor, leptin induces a cascade of signaling events. Leptin regulates canonical signaling pathways including JAK/STAT3, MAPK family of proteins, PI3K/AkT whose downstream effects are related to cancer cell proliferation and metastasis [217]. Furthermore, non-canonical

signaling pathways such as AMPK and Jun N-terminal kinase (JNK) are regulated by leptin in different cells [218, 219].

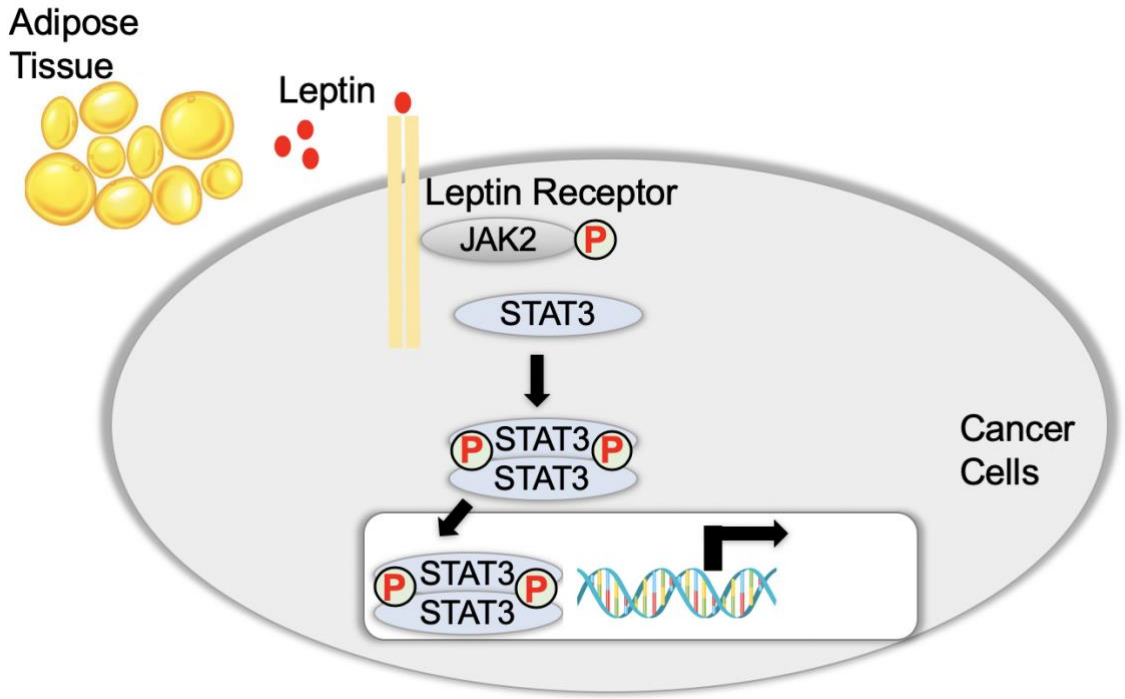

Figure 1.4 Leptin Signaling Pathway

The most commonly studied pathway mediated by leptin is JAK/STAT3 (Figure 1.4). When leptin binds to its receptor, an Ob-Rb/JAK2 complex is formed, resulting in activation of JAK2 by autophosphorylation. Subsequently, activated JAK2 phosphorylates the tyrosine 1138 residue in the intracellular domain of the leptin receptor. Tyr 1138 on Ob-Rb is important for STAT3 activation, which stimulates suppressor of cytokine signaling 3 expression. Once STAT3 is bound to the leptin receptor, STAT3 is phosphorylated by activated JAK2 followed by dimerization of the STAT3 proteins. Dimerized STAT3 translocates to the nucleus, binds to the promoter of various genes, and regulates their transcription. Wang *et al.*, show that leptin-JAK/STAT3 regulates FAO, promoting breast cancer stemness and chemoresistance [220], highlighting one of the potential roles on leptin-mediated regulation of the JAK/STAT3 pathway in breast cancer.

Leptin also regulates MAPK signaling pathway, which is important to activate ERK1/2, p38 isoforms, and JNK [221]. Phosphorylation on the tyrosine 985 residue on Ob-Rb recruits Src homology phosphatase 2, leading to activation of ERK signaling pathway [222]. Yuan *et al.*, show that leptin induces the migration of MCF-7 cells through activation of the ERK pathway [223], indicating this pathway may be a potential target to prevent breast cancer cell migration.

In addition, leptin activates PI3K/AkT pathway upon leptin binding to its receptor. Evidence suggests that PI3K/AkT is involved in leptin-mediated EMT in breast cancer MCF-7 and SK-BR-3 cells [224]. Furthermore, leptin enhances migration and invasion of MCF-7 and T47D breast cancer cells via upregulating ACAT2 through the PI3K/AkT/SREBP2 signaling pathway [225]. Collectively, leptin regulates several pathways involved in breast cancer progression and metastasis, and further investigation is needed to better understand the mechanisms of leptin signaling in order to develop strategies to inhibit breast cancer progression and metastasis.

Several results from *in vivo* studies also provided evidence for an impact of leptin in breast cancer proliferation and metastasis. For example, one study shows that mammary tumor growth is inhibited in leptin deficient mice [226]. In addition, Park *et al.,* show that [227] deletion of LEPR reduces breast tumor progression and metastasis to the lung via ERK and JAK/STAT3 pathways using mouse mammary tumor virus-polyoma virus middle T antigen model (MMTV-PyMT). In addition to *in vivo* studies, leptin is shown to promote breast cancer cell proliferation and metastasis *in vitro* at various stages and in different types of breast cancer. Leptin has a growth-stimulating effect on breast cancer cells of both ER-positive (MCF7, T47D, MDA-MB-361) and ER-negative (MDA-MB-231, SKBR3) cell lines [228]. Another *in vitro* study shows that leptin is involved in metastasis and recurrence through inhibition of ACC1 in human MCF-7 and murine 4T1 breast cancer cells [94]. Cao *et al.*, also show that leptin-related M2 macrophages promote the migration and invasion of human breast cancer MCF7 and MDA-MB- 231 cells [229]. Another study shows that leptin promotes the proliferation, invasion, and migration of MCF-7 and T47D breast cancer cells via up-regulation of ACAT2 [225]. Together, the evidence from clinical, *in vitro* and *in vivo* studies demonstrate that leptin may affect aggressiveness of breast cancer.

1.5.4 The Role of Leptin in Energy Metabolism

Evidence also suggests that leptin alters energy metabolism in various tissues. For example, Tanaka *et al.*, show that transgenic 'skinny' mice, which overexpress leptin in the liver, increase oxygen consumption (VO_2), with an increase in the AMP to ATP ratio, and decrease in plasma triglyceride and free FA level compared to non-transgenic mice [230]. Leptin also increases oxygen consumption and lipolytic activity in white adipose tissue in ob/ob mice. [231]. These results indicate that leptin may have an important role on altering energy metabolism, however, little is known about the role of leptin in regulation of energy metabolism, including lipids and glucose, in breast cancer cells.

1.5.4.1 The Role of Leptin in Glucose Metabolism

Previous evidence demonstrates that leptin regulates glucose metabolism in both *in vitro* and *in vivo* studies. Blanquer-Rosselló *et al.*, showed that leptin increases oxygen consumption rate [218], but found that glucose uptake and glucose transporter expression are not affected by leptin treatment in MCF-7 breast cancer cells. However, they show that leptin treatment decreases lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH) protein expression and increases pyruvate carboxylase (PC) protein expression [218] (Figure 1.1), suggesting leptin induces a greater use of pyruvate derived from glucose to replenish the tricarboxylic acid cycle for biosynthesis in MCF-7 cells. Another study reveals that leptin does not alter glucose uptake and glucose transporters, GLUT1 and GLUT3, protein levels in human normal sertoli cells (hSCs) [232]. However, leptin upregulates GLUT2 protein levels and LDH activity [232], indicating leptin can interfere with metabolic support of spermatogenesis potentially by altering glucose metabolism. Additionally, in Caco-2 human colorectal adenocarcinoma cells, an inhibitory effect of leptin on glucose absorption was observed when cells are differentiated and acquired the Na+-dependent glucose transporters, the facilitative Na+-independent glucose transporter (GLUT) and leptin receptors [233]. Collectively, these results indicate leptin may regulate glucose metabolism differentially depending on the type of tissues and their conditions.

1.5.4.2 The Role of Leptin in Lipid Metabolism

Previous studies also suggest that leptin alters lipid metabolism in various tissues. Leptin treatment increases mitochondrial respiration, as assessed by measuring the oxygen consumption rate fueled by fatty acids in MCF-7 breast cancer cells [218]. In addition, leptin increases the levels of key genes involved in cellular fatty acid uptake (FAT/CD36) and fatty acid oxidation (CPT1) [218]. Also, leptin induces phosphorylation and activation of AMPK, which plays a role in the regulation of cellular lipid and protein metabolism. When AMPK is activated by phosphorylation, ACC is phosphorylated, leading to inactivation of ACC and decrease of fatty acid synthesis. In the *ob/ob* mice model, Finocchietto *et al.*, show leptin increases mitochondrial respiration and lipid utilization potentially by inhibiting nitric oxide release [234], providing a potential link in its actions, AMPK, which is activated by leptin, is a negative modulator of neuronal nitric oxide synthase [234]. In addition, leptin decreases the production of acetate which is the reliable carbon source for lipid synthesis in human Sertoli cells [232]. Similarly, leptin inhibits insulin-induced glucose incorporation into lipids in adipocytes [235]. In other tissues such as skeletal muscle, leptin induces energy dissipation and prevents fatty acid accumulation by increasing FAO [236]. Taken together, these results support that leptin affects lipid metabolism to alter energy metabolism in various tissues, and that this topic requires further investigation to fully understand the impact of leptin on energy metabolism.

1.6 Research Questions

Leptin is mainly produced from adipose tissues and is a hormone that regulates appetite and energy balance. Evidence suggests that leptin increases metastasis in breast cancer. However, the impact of leptin on breast cancer migration at physiological levels of glucose concentration is not clear. In addition, leptin alters glucose and lipid metabolism in various studies, but the mechanisms by which leptin induces migration through energy metabolism is not clear. Therefore, the effect of leptin on energy metabolism may be a potential mechanism contributing to increase in breast cancer cell migration. Thus, the impact of leptin on glucose and lipid metabolism was assessed in Chapter 2.

Excess adiposity is associated with increased risk of breast cancer metastasis and mortality. Previous evidence supports that dietary or supplemental vitamin D may reduce breast cancer

metastasis. However, the link between vitamin D regulation of adipocyte metabolism and breast cancer metastasis has not been studied and continues to be a topic of investigation. The results of studies to investigate the impact of $1,25(OH)_2D$ on regulation of adipocyte release of adipokines to alter breast cancer cell migration is investigated in Chapter 3.

Vitamin D shows promise as a preventive compound in primary and metastatic breast cancer. However, loss of VDR has been reported during breast cancer progression, with the implication that vitamin D may not be effective in inhibiting breast cancer metastasis. The results of experiments investigating the impact of $1,25(OH)_2D$ on the expression and activity of the VDR in breast cancer cells at different stages of progression, are described in Chapter 4.

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2. LEPTIN REGULATION OF MIGRATION IN BREAST CANCER CELLS

2.1 Abstract

Leptin is a central regulator of energy balance. The concentration of leptin in serum is higher in obese individuals than non-obese as leptin is mainly secreted by adipose tissue in proportion to adipose mass. Previous evidence suggests that leptin increases metastasis in breast cancer cells, however, there is only a small literature base on role of energy metabolism (lipid and glucose metabolism) in leptin-induced metastasis. Although there is evidence that leptin regulates energy metabolism, much of the research on leptin impact on breast cancer cells *in vitro* has been conducted in high (25 mM) glucose medium. The role of leptin in inducing breast cancer migration in a physiological glucose level (5mM) has not been examined. We hypothesized that leptin increases migration when metastatic breast cancer cells are culture in 5 mM glucose medium through regulation of glucose metabolism. Our results showed that leptin treatment (4 days) increased migration in murine metastatic 4T1 and metM-wnt^{lung} breast cancer cells when cultured in 5 mM glucose medium. There was no effect of leptin on triacylglycerol (TAG) level or fatty acid uptake in either cell line. While leptin did not affect glucose incorporation into palmitate in 4T1 cells, leptin increased palmitate synthesis from glucose in metM-wnt^{lung} cells, suggesting differential regulation of lipid synthesis, potentially due to glucose metabolism, between the two metastatic cell lines. However, leptin decreased fatty acid synthase (*Fasn*) and carnitine palmitoyltransferase 1α (*Cpt-1α*) mRNA level in 4T1 cells but increased *Fasn* and *Cpt-1α* mRNA level in metM-wnt^{lung} cells, again supporting differential regulation of metabolism. Although leptin increased glucose transporter 1 (*Glut1*) mRNA level in both 4T1 and metM-wnt^{lung} cells, leptin-induced migration was blocked by inhibiting glycolysis in metM-wnt^{lung}, but not in 4T1 cells. Furthermore, fatty acid metabolism including fatty acid uptake, and TAG synthesis, was not altered, nor is fatty acid oxidation necessary for leptin-induced migration in either cell line. Taken together, these results demonstrated that leptin differentially regulates energy metabolism in 4T1 and metM-wnt^{lung} cells during leptin-induced migration and that glycolysis is necessary to increase leptin-mediated migratory capability only in metM-wnt^{lung} cells.

2.2 Introduction

Leptin is a hormone that regulates energy balance and appetite. Leptin is encoded by the ob gene and is related to immune response, reproduction, differentiation, and proliferation of different cell types, including breast cancer cells [1]. Molecular action of leptin is mediated through the transmembrane leptin receptor (Ob-R). Leptin binds to the leptin receptor and can activate janus kinase (JAK)/signal transducer and activator of transcription (STAT) [2] and mitogen-activated protein kinase (MAPK) signaling pathways [3, 4], whose downstream effects are linked to promoting cancer metastasis including the steps of migration and invasion.

Previous evidence suggests that leptin may also be involved with breast cancer progression and metastasis as both leptin and the leptin receptor are overexpressed in primary breast cancer as well as lymph node metastasis [5]. There are six isoforms of Ob-R are identified, Ob-Ra, b, c, d, e, and f. Among all Ob-R isoforms, the long isoform Ob-Rb activates signaling pathways in breast tissues [6], thus the role of Ob-Rb and leptin continues to be an area of investigation in breast cancer.

Reprogramming of lipid metabolism is a recognized hallmark of aggressiveness in breast cancer [7]. Lipids, either newly synthesized, taken up from the media or released from lipid droplet storage, can serve as sources of energy via fatty acid oxidation (FAO) and are involved in cellular signaling [8]. These functions of lipids may serve to support cancer progression and migration. Overexpression of fatty acid synthase (FASN) has been shown in multiple cancers [9], indicating a potential role of fatty acid synthesis in cancer metabolism. In addition, Liu *et al.*, show that leptin decreases FASN and increases FAO in MCF-7 breast cancer cells [10], suggesting leptin-mediated changes in lipid metabolism may support cancer progression.

Glucose is a nutrient that can produce ATP through glycolysis and the tricarboxylic acid cycle, as well as for carbon backbones for synthesizing other molecules needed for growth. Glucose and its metabolites serve as precursors for lipids thus glucose and lipid metabolism are linked. Additionally, previous research shows that glucose enhances leptin signaling in human fibrosarcoma cells [11], suggesting a potential role of glucose to enhance the effects of leptin. Most of the previous research conducted examining the effects of leptin in *in vitro* cell studies has been conducted in the commonly used 25 mM glucose medium. However, little information is available on how physiological levels of glucose (i.e. 5 mM) impacts the leptin regulation of breast cancer cell migration as well as the role of energy metabolism in this process.

AMP-activated protein kinase (AMPK) is a nutrient energy sensor which can regulate glucose and lipid metabolism in response to energy status [12]. When there is a deficit of energy with the increase of the AMP/ATP ratio, AMPK is activated by AMPK kinases, leading to activation of catabolic processes. Conversely, AMPK is inactivated when energy is sufficient. It is reported that decreased AMPK activation is associated with higher grades of breast cancer [13], indicating that AMPK may be important in the ability of cells to metabolically adjust to the adverse conditions which occur during metastasis.

It is critical to select the appropriate cell model to investigate cancer behavior. We employed two murine malignant metastatic cells model, 4T1 metM-wnt^{lung} cells, in order to specifically study the impact of leptin in cancer metastasis. 4T1 cells can form primary tumors when those are implanted into the mammary gland of BALB/c mice. After injection into the mammary gland, 4T1 cells can metastasize to the lung, liver, brain, and bone [14]. The murine 4T1 cells are regarded as metastatic mimicking stage IV breast cancer cell line [15]. metM-wnt^{lung} cells were developed from the non-metastatic M-Wnt cell line through serial passage, and readily form tumors and metastasize *in vivo* when transplanted into syngeneic mice [16]. Both 4T1 and metM-wnt^{lung} cells are triple negative metastatic breast cancer cells.

Although several studies have been completed to investigate the leptin regulation of breast cancer cell migration, the impact of physiological levels of glucose on leptin-induced migration has not been studied. The purpose of the current study was to investigate the energy related mechanisms by which leptin increases migration in 5 mM glucose medium in 4T1 and metM-wnt^{lung} cell lines. We hypothesized that leptin alters energy metabolism with 5 mM glucose medium and this change is involved in leptin-induced migration. This work will contribute to our understanding of leptin regulation of lipids and glucose in breast cancer cell migration, which may contribute to designing therapies to reduce obesity-associated metastasis.

2.3 Materials and Methods

2.3.1 Chemical and Reagents

Dulbecco's Modification of Eagle's Medium (DMEM) was obtained from Corning (Manassas, VA). Fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were obtained from Life Technologies, Gibco-BRL (Rockville, MD). Recombinant leptin and AICAR was

purchased from Peprotech (Rocky Hill, NJ). Aminooxyacetic acid, etomoxir, TVB-3166 were obtained from Sigma-Aldrich (St. Louis, MO).

2.3.2 Cell Culture

4T1 and metM-Wnt^{lung} cells were cultured in 5 mM glucose and 4 mM glutamine containing DMEM without sodium pyruvate, with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified environment at 37°C with 5% CO_2. Cells were treated for 4 days with either vehicle (bovine serum albumin final concentration <0.001%) or leptin (300 ng/ml).

2.3.3 Migration Assay

The cells were pretreated with 300 ng/ml of leptin or vehicle (bovine serum albumin final concentration <0.001%) for 4 days before replating in serum free medium into 8 um pore transwell inserts (Corning Inc. Corning, NY). Transwell inserts were placed into 10% fetal bovine serum and the indicated glucose containing media. After 15 or 24 hours of incubation for metM-wnt^{lung} and 4T1 cells, respectively, the bottom of the transwells were fix with 100% methanol and stained with 2% crystal violet in ethanol. The cell number was counted in 5 different fields and migration was quantified using the average sum of cells/field.

2.3.4 RNA Isolation and Analysis

RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH, USA) and reverse transcription to cDNA conducted using MMLV reverse transcriptase (Promega, Madison, WI, USA). mRNA level was determined using qPCR and data are normalized to 18S level. The mRNA abundance of target genes was determined using the threshold cycle (Ct) value. Data are expressed as fold change relative to vehicle. Primers used are shown in Table 2.1.

Table 2.1 Primers Used in the qPCR Analysis of mRNA Level

Genes	Forward Primer 5'-3'	Reverse Primer 5'-3'
Acly	ACCCTTTCACTGGGGATCACA	GACAGGGATCAGGATTTCCTTG
Cpt1a	CTGCAGACTCGGTCACCACT	ACACCCACCACCACGATAAG
Cpt1b	GCACACCAGGCAGTAGCTTT	CAGGAGTTGATTCCAGACAGGTA
Fasn	ACCACTGCATTGACGGCCGG	GGGTCAGGCGGGAGACCGAT
Glut1	GGCTTGCTTGTAGAGTGACGA	GTGAGTGTGGTGGATGGGAT
Hk2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
Ob-R	CCTCTTGTGTCCTACTGCTCG	GAAATTCAGTCCTTGTGCCCAG
18S	ATCCCTGAGAAGTTCCAGCA	CCTCTTGGTGAGGTCGATGT

2.3.5 Triacylglycerol (TAG) Level

The level of TAG was assessed using spectrophotometric assay kit (Wako Diagnostics). Results are normalized to protein assessed by the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich, St. Louis, MO).

2.3.6 Fatty Acid Synthesis

During the last 24 hours of treatment, 4T1 and metM-wnt^{lung} cells were incubated with 10 mM [$^{13}C_2$] acetate (Sigma-Aldrich, St. Louis, MO) or incubated in glucose-free DMEM containing a 1:1 mixture of D-[U-^{13}C]glucose (Sigma-Aldrich, St. Louis, MO) and non-labeled D-glucose at a final glucose concentration of 5 mM (0.9 g/L). Cells were harvested into lysis buffer, and lipid hydrolysis, extraction, derivatization, and analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed as previously described [17]. Briefly, cell lysates were heated at 90°C in a solution of acetonitrile: hydrochloric acid (37%) (4:1, vol/vol) to hydrolyze fatty acids. After cooling to room temperature, lipids were extracted with hexane and dried under nitrogen gas. Fatty acids were resuspended in acetone and derivatized with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide to form 3-acyloxymethyl-1-methylpyridinium iodide and subjected to HPLC-MS/MS analysis. The HPLC system (1100 Series LC, Agilent) with ESI source was used and separations were performed on C18 column (4.5 mm, 250 mm, 8 μM). The condition was to begin by maintaining 50% mobile

phase A (H_20 + 0.1% formic acid) and 50% mobile phase B (50% isopropyl alcohol + 50% acetonitrile + 0.1% formic acid) for 0-1 min and then increase in solvent B from 50% to 100% in 5 min with a 6-min hold at 100% solvent B. Solvent B was decreased to 50% in 12 min with 4 min hold at 100% solvent B. The flow rate was set at 0.3 mL/min and the injection volume was 8 μL. Substrate incorporation into palmitate (16:0) and stearate (18:0) fatty acids was assessed from the chromatograms as area under the curve (AUC). Weighted analysis was conducted to calculate the number of ^{13}C-labeled carbons for each peak. The data are calculated by dividing the sum of weighted AUC to the sum of all AUC times 100 as percent total palmitate (or stearate) from ^{13}C-glucose (or ^{13}C-acetate).

2.3.7 Fatty Acid Uptake

To measure fatty acid uptake, cells were washed with calcium/magnesium-free phosphate- buffered saline (pH = 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), incubated with 10 μmol/L BODIPY FL C16 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene- 3-hexadecanoic acid; Life Technologies, Carlsbad, CA, USA) in 0.1% fatty acid-free bovine serum albumin (BSA) in Hanks balanced salt solution (Gibco–Life Technologies, Carlsbad, CA, USA) for 5 minutes, and then washed twice with an ice-cold solution of 0.2% FA-free BSA in Hanks balanced salt solution. Fluorescence was measured using a Synergy H1 Multi-Mode Reader (BioTek Instruments, Inc, Winoo- ski, VT, USA) and analyzed using Gen5 Data Analysis Software (BioTek Instruments, Inc). The relative BODIPY FL C16 uptake is expressed as fluorescence intensity per well normalized to protein.

2.3.8 Western Blotting

After treatment, cells were washed with 1X phosphate buffered saline (pH=7.4) and harvested on ice into radioimmunoprecipitation assay buffer containing 1% each of phenylmethylsulfonyl fluoride (protease inhibitor) and phosphatase inhibitor cocktails (Sigma-Aldrich, St Lois, MO, USA). Cells were sonicated and centrifugated at 12,000 rpm for 15 minutes at 4°C to get the supernatant. Protein concentration was measured by BCA protein assay, loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto

nitrocellulose membranes (Bio-Rad Laboratories, Inc). After blocking, membrane was incubated with rabbit anti-AMPKα, rabbit anti-phospho-AMPKα (Thr172), or rabbit anti-β-actin (Cell Signaling, Danvers, MA, USA) overnight. The membrane is incubated with IRDye 680RD Donkey secondary antibody System (Li-Cor Biosciences, Lincoln, NE) and imaged with Li-Cor Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE).

2.3.9 Statistical Analysis

Values are presented as mean ± SEM. Results are expressed compared to the vehicle, by Student's t-tests. Where letters are provided, analysis was conducted using analysis of variance (ANOVA) with $P<0.05$ considered statistically significant.

2.4 Results

Cultured cells are often maintained in 25 mM glucose medium, which may affect the regulation of energy metabolism. However, little information is available on how physiological glucose levels (5 mM) affects breast cancer cells, particularly in their ability to adapt to changing environmental stimuli. The impact of leptin on migration in 5 mM glucose medium, was assessed in murine triple negative metastatic 4T1 and metM-wnt^{lung} breast cancer cell lines. Leptin pretreatment increased migration in both 4T1 and metM-wnt^{lung} cells with 5 mM glucose medium (Figure 2.1). Interestingly, leptin decreased leptin receptor (*Ob-R*) mRNA level in 4T1, but not in metM-wnt^{lung}, cells compared to vehicle (Figure 2.2), suggesting differential responses to leptin treatment in these cell lines.

Previous evidence suggests that leptin alters fatty acid metabolism, including neutral lipid TAG. However, leptin treatment did not change TAG level in both 4T1 and metM-wnt^{lung} cells (Figure 2.3) when cells were cultured in 5 mM glucose. In addition, leptin did not affect fatty acid uptake in either cell line, suggesting leptin is not involved in uptake of exogenous or synthesis of TAG during leptin-induced migration.

It has been estimated that 93% of triacylglycerol in cancer cells are synthesized from fatty acids *de novo* [18], thus we examined the impact of leptin on this process when the cells were cultured in 5 mM glucose. In order to determine if leptin induced differences in fatty acid synthesis, we employed [U]-^{13}C–acetate and [U]-^{13}C–glucose, respectively, in order to determine

if any impacts were due to the fatty acid synthesis or additionally through glucose supplies. In 4T1 cells, leptin did not alter palmitate and stearate synthesis from [U]-^{13}C–glucose (Figure 2.5A). The mRNA level of glucose transporter 1 (*Glut1*), which facilitates glucose transport across the membranes, was increased and fatty acid synthase (*Fasn*), which catalyzes fatty acids from acetyl-CoA and malonyl-CoA, was decreased with leptin treatment in 4T1 (Figure 2.5B). However, there was no difference on hexokinase 2 (*Hk2*), the first step in glucose metabolism, and ATP citrate lyase (*Acly*) mRNA level with treatment of leptin. Additionally, palmitate synthesis from [U]-^{13}C–acetate was not different between vehicle and leptin treatment, but stearic synthesis from [U]-^{13}C–acetate was significant lower with leptin treatment (Figure 2.5 A). In contrast, in metM-wnt^{lung} cell, palmitate and stearate synthesis from [U]-^{13}C–acetate and from [U]-^{13}C–glucose was significantly increased with leptin treatment (Figure 2.5 C). Furthermore, as leptin treatment increased mRNA level of *Glut1*, *Hk2*, and *Fasn*, leptin increased *de novo* fatty acids synthesis from glucose (Figure 2.5 D). Although *Fasn* mRNA level was increased in the metM-wnt^{lung} cell line, inhibiting fatty acid synthase by TVB3166 did not impact leptin-induced migration in metM-wnt^{lung} cells (Figure 2.9 A, B), highlighting fatty acid metabolism is not associated with increased migration following leptin treatment. Thus, these results demonstrated that, leptin differentially affects *de novo* fatty acid synthesis in 4T1 and metM-wnt^{lung} cells during leptin-induced migration.

Glucose metabolism is well known to be altered in cancer progression therefore we examined its role in leptin-induced migration in metastatic cells. The glucose transporter, *Glut1* mRNA level was increased in both 4T1 and metM-wnt^{lung} cells, suggesting a potential increase in glucose flux into the cells. However, treatment of 4T1 cells with low concentrations (0.1 and 0.3 mM) of 2DG did not affect leptin-induced migration in 4T1 cells (Figure 2.6A and C), and higher concentrations of 2.5 and 5 mM of 2DG induced cell death (data not shown), suggesting that 4T1 cells are dependent on glucose for survival, but glycolysis is not involved in leptin-induced migration in 4T1 cells. On the other hand, in metM-wnt^{lung} cells, 2DG inhibited leptin-induced migration (Figure 2.6 C), and the percent increase in migration with leptin in the presence of 2DG was significantly lower than without 2DG (Figure 2.6 D). These results support the differential regulation of energy metabolism between the 4T1 and metM-wnt^{lung} cells, with the latter, but not the former, requiring glycolysis for leptin-induced migration.

Because FAO has previously been known to involve in increase on tumor cell migration [19], we examined the impact of this pathway on leptin-induced migration when metastatic cells are cultured in 5 mM glucose. Interestingly, leptin decreased carnitine palmitoyltransferase 1α (*Cpt1α*), a rate-limiting enzyme FAO, mRNA level in 4T1 (Figure 2.7A) but increased *Cpt1α* mRNA level in metM-wnt^{lung} (Figure 2.7B). However, there was no change in mRNA level of *Cpt1*ß in either 4T1 or metM-wnt^{lung}. These results suggest the impact of leptin on fatty acid metabolism is different depending on the cell line. We further assessed the impact of FAO on leptin-induced migration using etomoxir, an inhibitor of FAO. However, etomoxir did not affect leptin-induced migration (Figure 2.8 A, B, C, D) in either 4T1 or metM-wnt^{lung}, indicating that consistent to the previous literature, FAO is not involved in leptin-induced migration when the cells are cultured in 5 mM glucose conditions.

Glutamine is another nutrient that provides energy to increase migration in human dental pulp cells, thus we assessed the role of glutamine metabolism on migration by utilizing glutamate-dependent transaminase inhibitor aminooxyacetate (AOA). Inhibiting aminotransferase did not alter leptin-induced migration in metM-wnt^{lung} cells (Figure 2.10 A, B). This indicates that glutamine metabolism is not linked to increased migration with leptin in this metastatic cell line.

Since our results support that glycolysis is required for leptin-induced migration in the metM-wnt^{lung} cells, we explored the potential that AMP-activated protein kinase (AMPK), a central nutrient sensor of energy balance, may mediate the leptin-induced migration in both cell lines. Interestingly, p-AMPK/AMPK was increased in 4T1 cells with leptin treatment (Figure 2.11 A, B), but in contrast was decreased metM-wnt^{lung} cells (Figure 2.11 C, D), indicating a differential response to leptin in terms of energy metabolism in these two cell lines. To address whether increase on p-AMPK/AMPK impacts leptin-induced migration in 4T1 cells, AMPK inhibitor Compound C was utilized. However, there is no effect of the AMPK inhibitor, Compound C, on leptin-induced migration in 4T1 cells (Figure 2.12), where AMPK activity was increased. Additionally, AMPK activator AICAR did not affect leptin-induced migration in in metM-wnt^{lung} cells (Figure 2.13) in which AMPK activity was reduced. These results suggest that AMPK signaling is not involved in the leptin-induced increase in migration in either 4T1 or metM-wnt^{lung}.

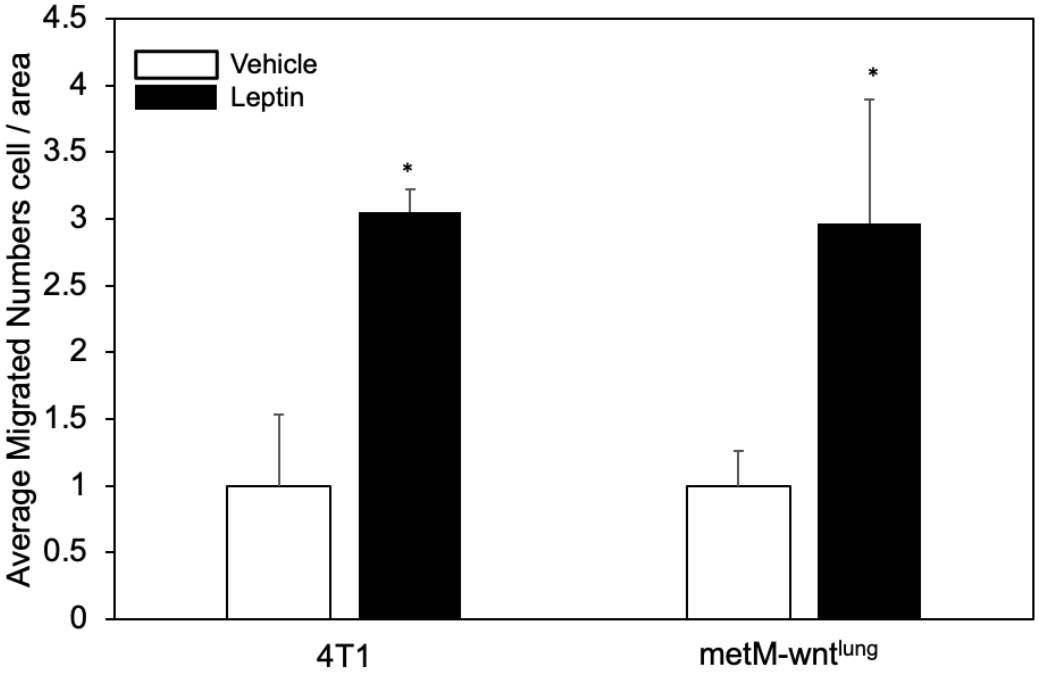

Figure 2.1 Leptin Effect on Migration of Metastatic Breast Cancer Cells in 5 mM Glucose Media.

Both 4T1 and metM-wnt^{lung} cells were pretreated with leptin (300 ng/ml) for 4 days and replated in serum free medium into 8 um pore transwell inserts. Transwell inserts were placed into 10% fetal bovine serum containing medium and migration assessed. *$p<0.05$ compared to each vehicle at the same time point. Values are means ± SEM.

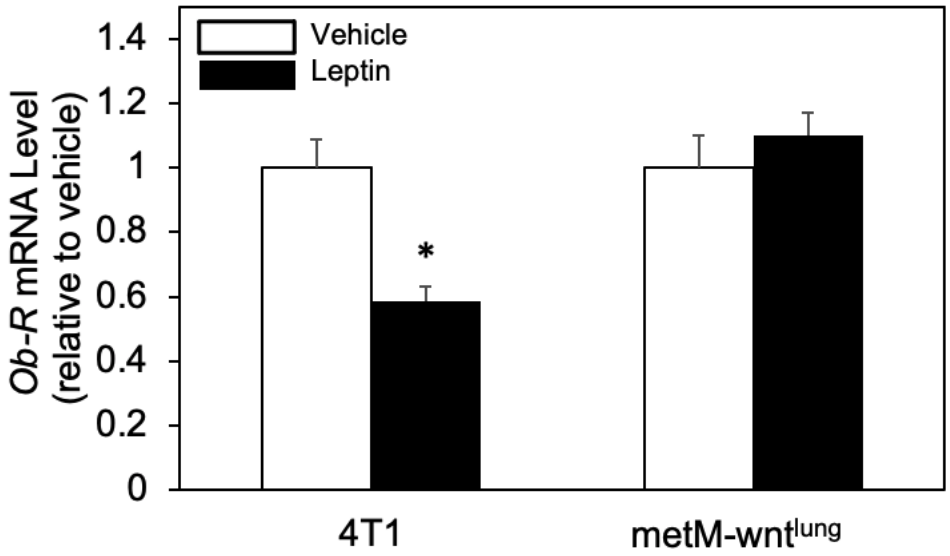

Figure 2.2 Leptin Regulation of *Ob-R* mRNA Level.

Following four days of leptin treatment (300 ng/ml) or vehicle, mRNA level was measured in (A) 4T1 and (B) metM-wnt^{lung} cells by reverse-transcription PCR and normalized to 18S. Values are expressed per vehicle and are means ± SEM. Asterisk indicates a significant difference relative to vehicle ($p<0.05$) of the same cell type.

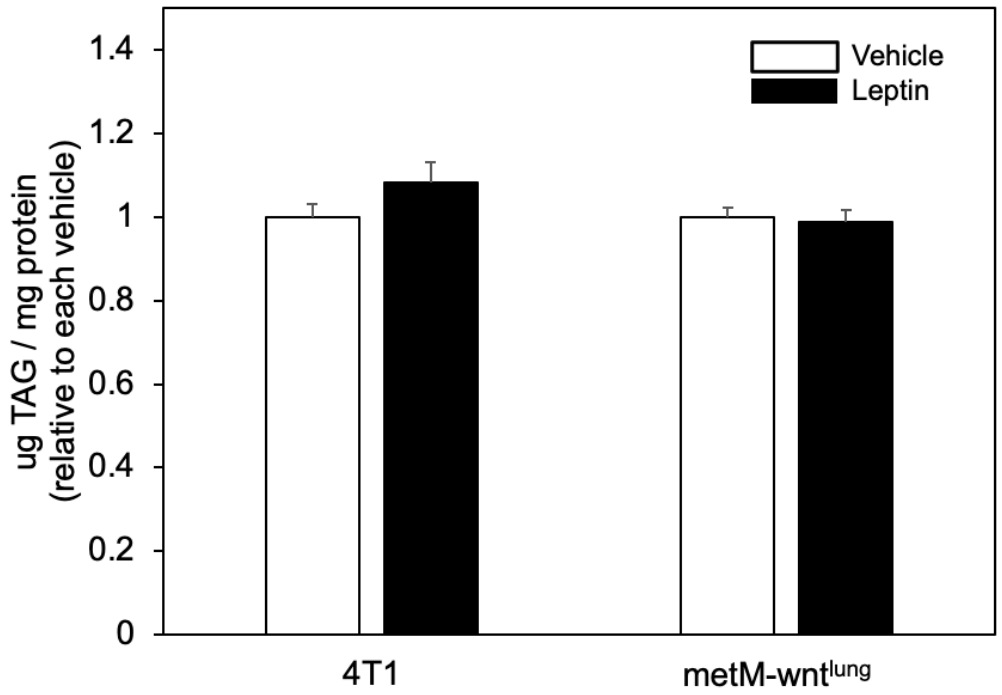

Figure 2.3 Leptin Regulation of TAG Level in 4T1 and metM-wnt^{lung} Cells with 5 mM Glucose Media.

4T1 and metM-wnt^{lung} cells with 5 mM glucose media were treated with vehicle or leptin (300 ng/ml) for four days and TAG level assessed. Results are normalized to total protein per well. No significant differences were noted, and values are means ± SEM.

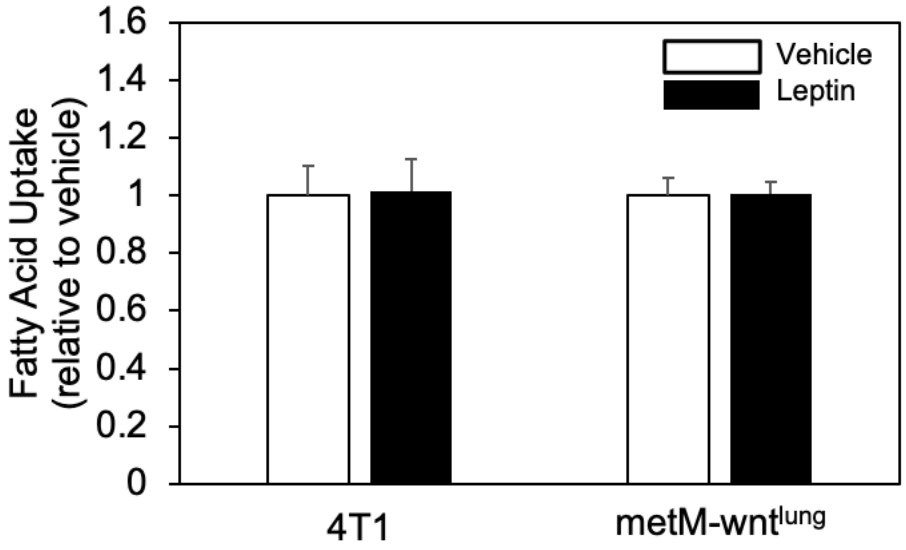

Figure 2.4 Leptin Effect on Fatty Acid Uptake in Metastatic Breast Cancer Cells in 5 mM Glucose Media.

Cells (4T1 and metM-wnt^{lung}) were treated for four days with leptin (300 ng/ml) or vehicle. Fatty acid uptake was determined following a 3-minute incubation with BODIPY FL-palmitate (10 umol/L). The relative BODIPY FL-palmitate uptake is expressed as fluorescence intensity normalized to protein. No significant differences were noted and values are means ± SEM.

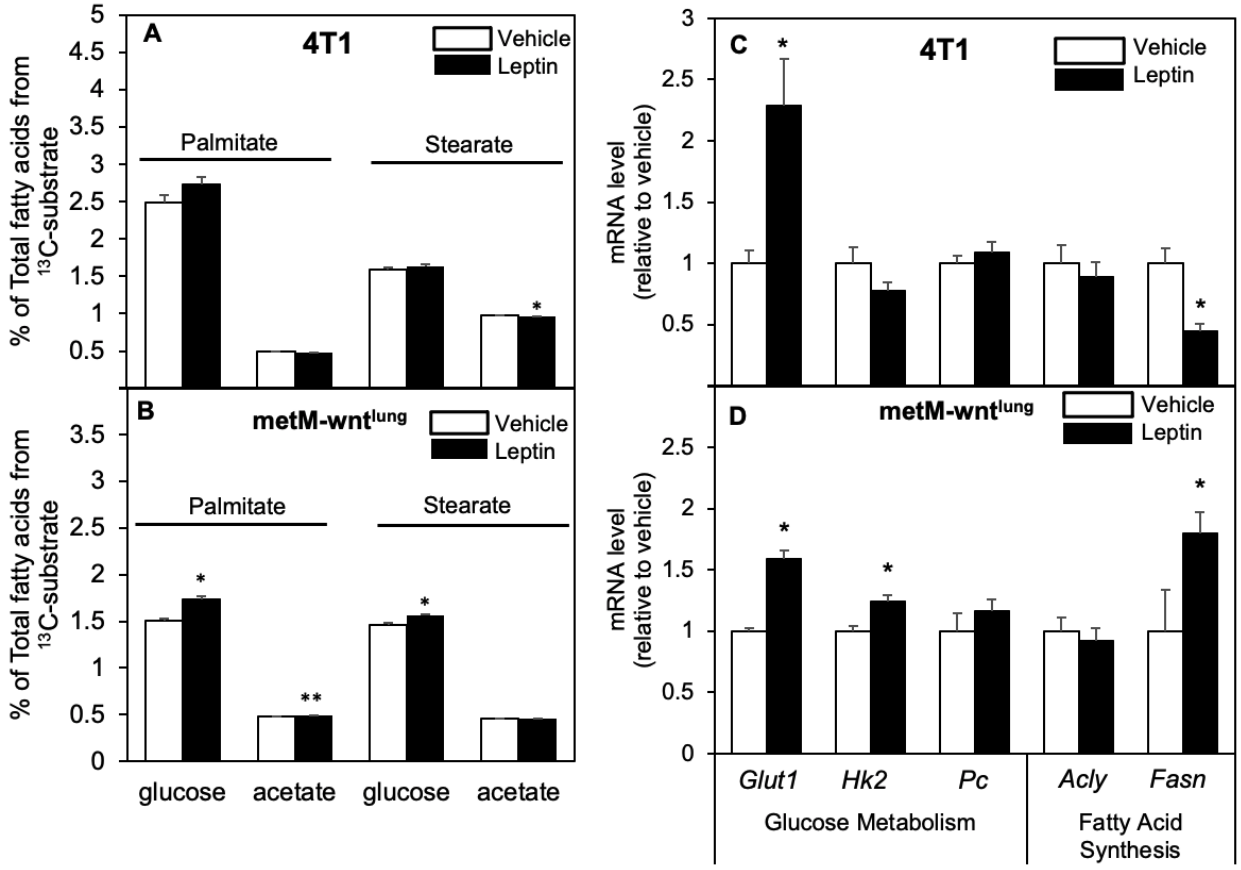

Figure 2.5 Leptin Effects on *de novo* Fatty Acid Synthesis.

(A, B) Conversion of glucose or acetate to palmitate or stearate in either vehicle or leptin-treated cells is represented as percent of total palmitate or stearate from each ^{13}C substrate. Cells (4T1 and metM-wnt^{lung}) were treated with either vehicle or leptin for four days. In separate experiments, either [U]-^{13}C-glucose or [U]-^{13}C-acetate (24 h) incorporation into palmitate or stearate was analyzed using LC-MS/MS. Values are means ± SEM. (C, D) mRNA level of genes whose protein products are involved in fatty acid synthesis were measured in (C) 4T1 and (D) metM-wnt^{lung} cells after 4 days of treatment of leptin (300 ng/ml) or vehicle by reverse-transcription PCR and normalized to 18S, and values are expressed per vehicle. Values are means ± SEM. Asterisk (two tailed t-test) and ** (one tailed t-test) indicate a significant difference relative to vehicle ($p<0.05$).

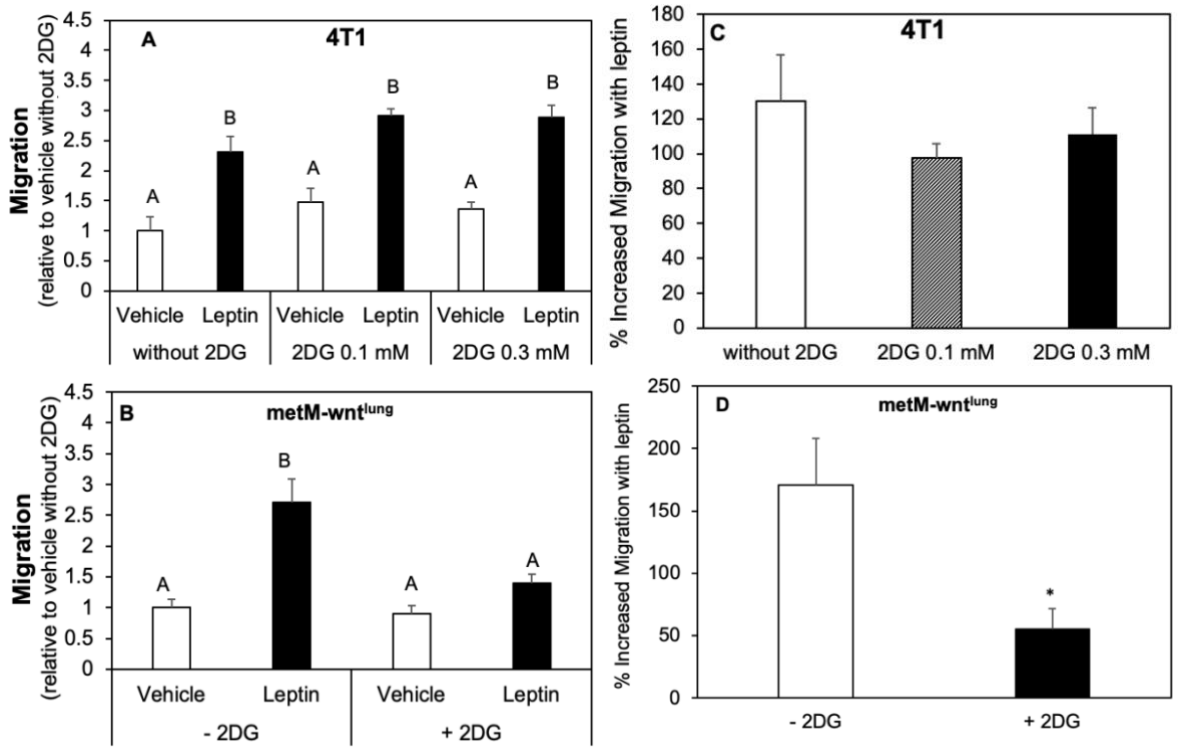

Figure 2.6 Role of Glycolysis in Leptin-Induced Migration.

(A, B) Cells (4T1 and metM-wnt^{lung}) were pretreated with of leptin (300 ng/ml) for four days before replating in serum free medium with or without 2-deoxyglucose (2DG) into 8 um pore transwell inserts. The dose of 2DG was 0.1 mM and 0.3 mM was employed for 4T1 and 5 mM of 2DG was used for metM-wnt^{lung}. After 24 hours (4T1) or 15 hours (metM-wnt^{lung}) and migration assessed with the transwell assay. In each figure, groups with different letters are significantly different ($p < 0.05$) assessed by ANOVA. (C, D) In each group, the percent increase in migration with leptin treatment was calculated by the difference on migration per average of migration in vehicle. *$p<0.05$ compared to group of without 2DG. Values are means ± SEM.

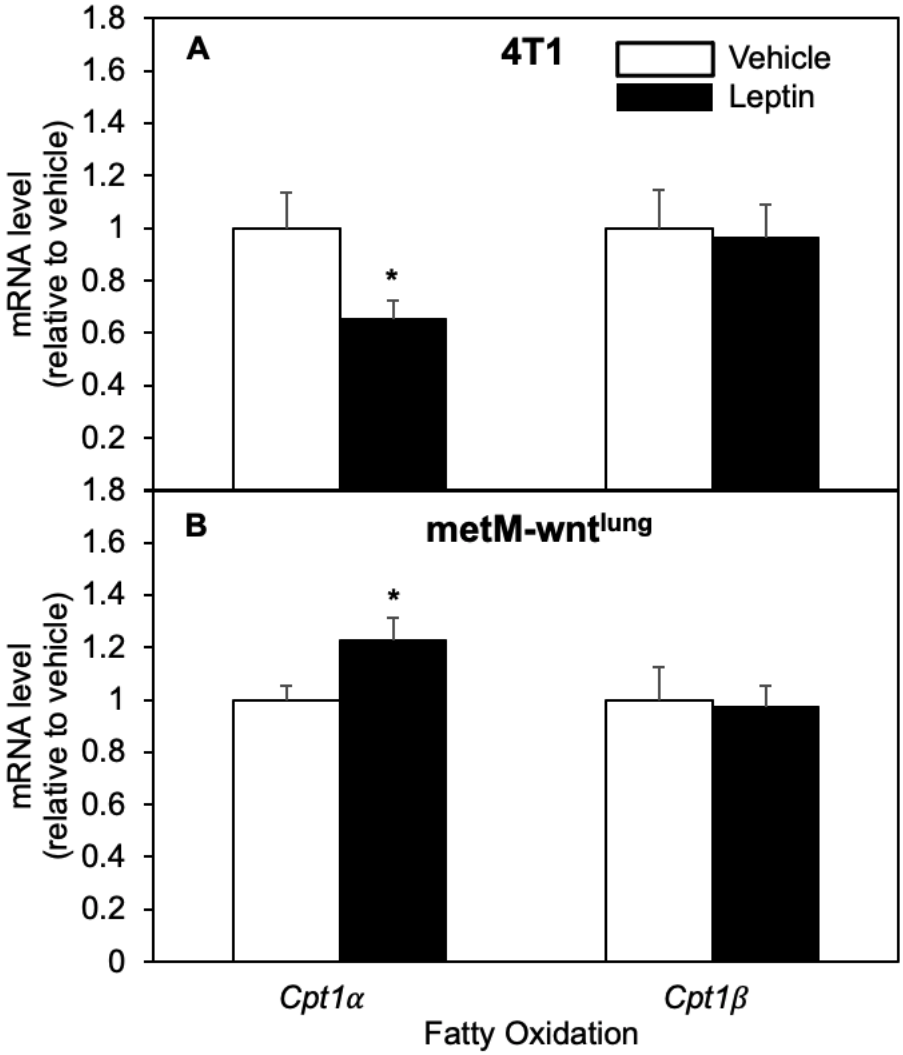

Figure 2.7 Leptin Effects on the Level of Genes Involved in Fatty Acid Oxidation in 4T1 and metM-wnt^{lung} Cells.

mRNA level was measured in (A) 4T1 and (B) metM-wnt^{lung} cells after four days of treatment with leptin (300 ng/ml) or vehicle by reverse-transcription PCR and normalized to 18S. Values are expressed per vehicle of the same cell type and are means ± SEM. Asterisk indicates a significant difference relative to vehicle ($p<0.05$).

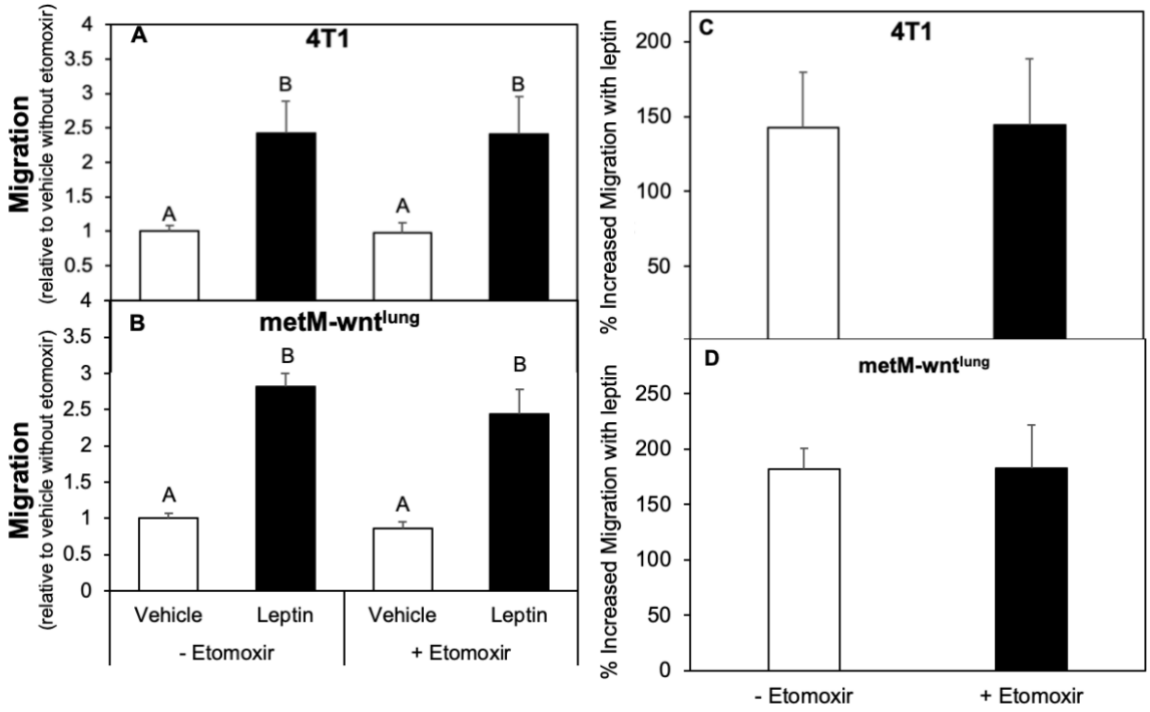

Figure 2.8 Role of Fatty Acid Oxidation on Leptin-Induced Migration in metM-wnt^{lung} and 4T1 Maintained in 5 mM Glucose Media.

(A, B) Cells (4T1 and metM-wnt^{lung}) were pretreated with of leptin (300 ng/ml) for four days before replating in serum free medium with or without etomoxir (75 μM) into 8 um pore transwell inserts. After 15 hours (metM-wnt^{lung}) or 24 hours (4T1) of incubation, migration was assessed. In each figure, bars with different letters are significantly different ($p < 0.05$) as assessed by ANOVA. (C, D) In each group, the percent increased migration with leptin was calculated by the difference on migration per average of migration in vehicle. Values are means ± SEM, and no significant differences were noted.

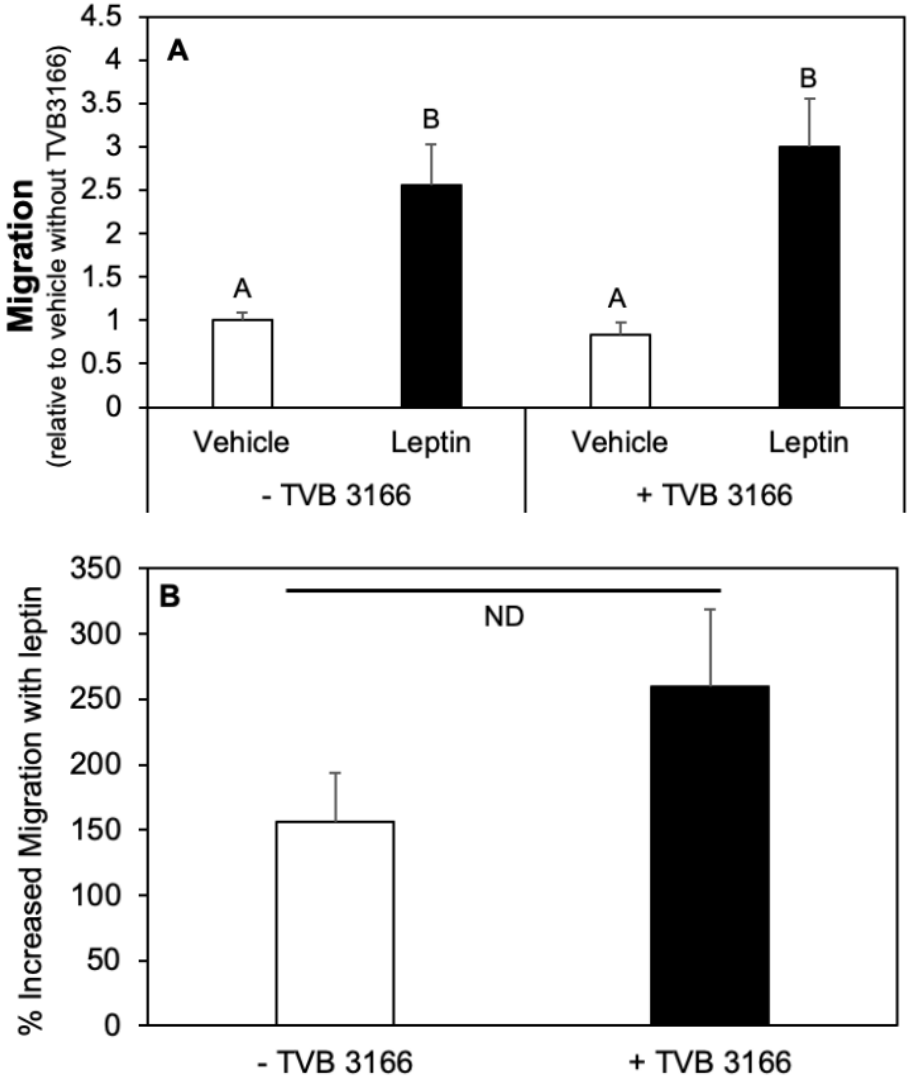

Figure 2.9 Effect of Inhibiting Fatty Acid Synthase on Leptin-Induced Migration in metM-wnt^{lung} Cells Maintained in 5 mM Glucose Media.

(A) metM-wnt^{lung} cells were pretreated with of leptin (300 ng/ml) for four days before replating in serum free medium with or without the fatty acid synthase inhibitor TVB3166 (42 nM) into 8 um pore transwell inserts. After 15 hours of incubation, migration was assessed. In each figure, bars with different letters are significantly different ($p < 0.05$) as assessed by ANOVA. (B) In each group, the percent increased migration with leptin was calculated by the difference in migration per average of migration in vehicle. Values are means ± SEM. ND indicates no significant difference.

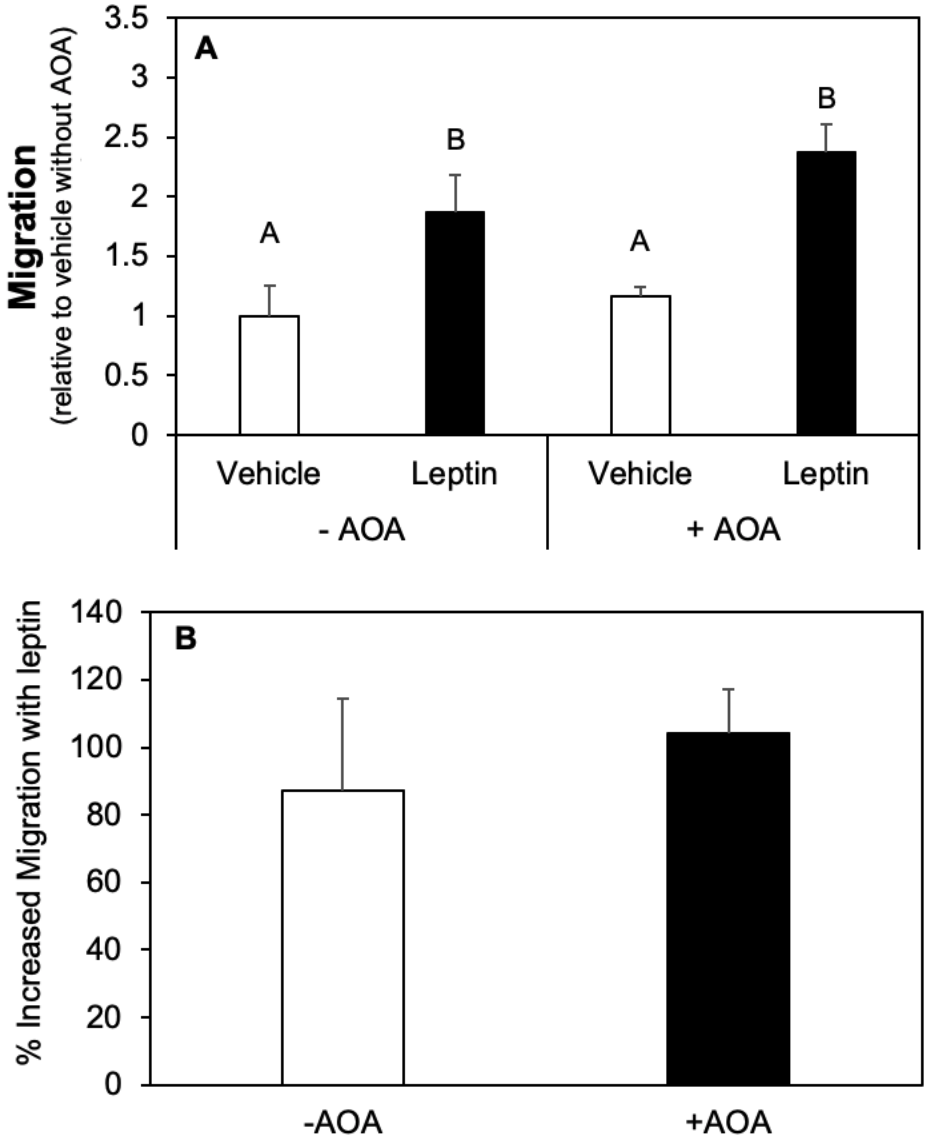

Figure 2.10 Effect of Inhibiting Glutamate-Dependent Transaminase on Leptin-Induced Migration in metM-wnt^{lung} Cells in 5 mM Glucose Media.

(A) metM-wnt^{lung} cells were pretreated with leptin (300 ng/ml) for four days before replating in serum free medium with or without the glutamate-dependent transaminase inhibitor AOA (200 uM) into 8 um pore transwell inserts and migration assessed. In each figure, bars with different letters are significantly different ($p < 0.05$) assessed by ANOVA. (B) In each group, the percent increased migration with leptin was calculated by the difference on migration per average of migration in vehicle. Values are means ± SEM and no significant difference is noted.

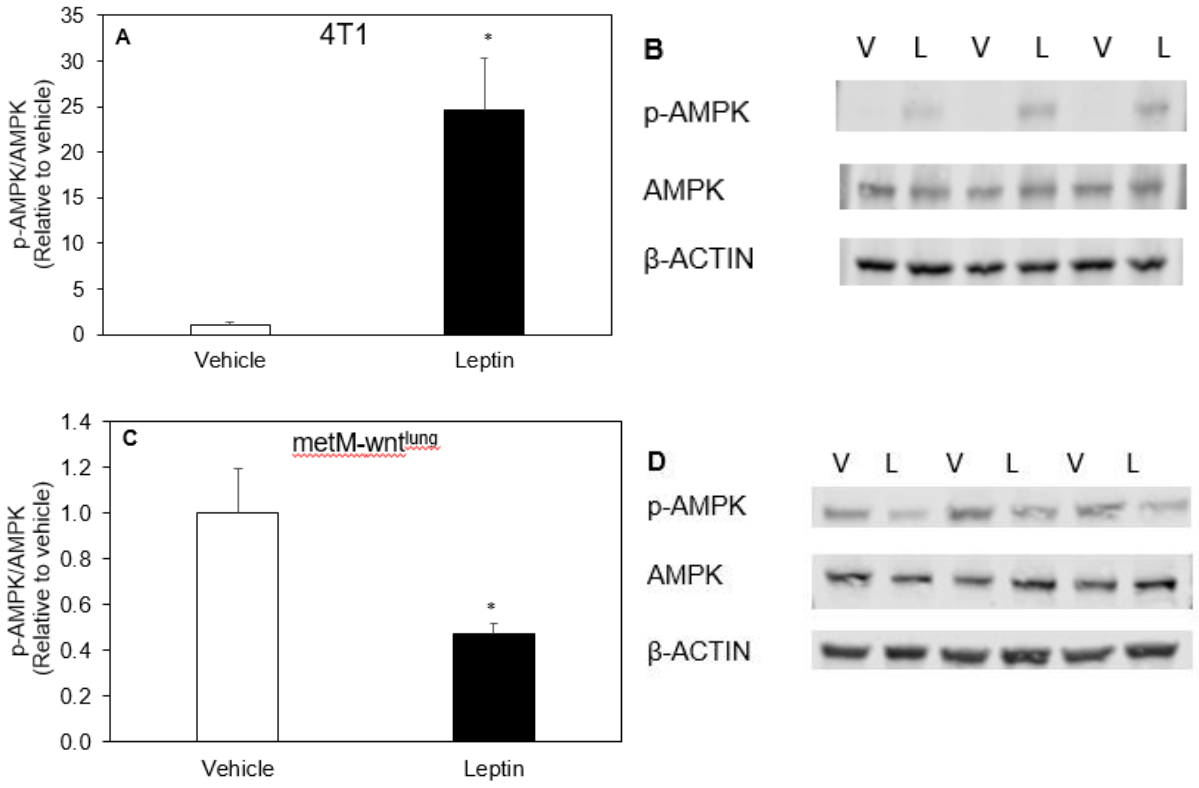

Figure 2.11 Leptin Regulation of AMPK Activation in 4T1 and metM-wnt^{lung} Maintained in 5 mM Glucose Media.

Cells were treated with leptin (300 ng/ml) or vehicle for four days. (A, C) Representative blots of p-AMPK and AMPK protein level with vehicle (V) or Leptin (L) treatment by Western blot analysis with β-actin as a loading control. (B, D) Quantification of p-AMPK/AMPK protein level relative to vehicle in each cell line. Values are means ± SEM. Asterisk indicates a significant difference relative to vehicle ($p<0.05$).

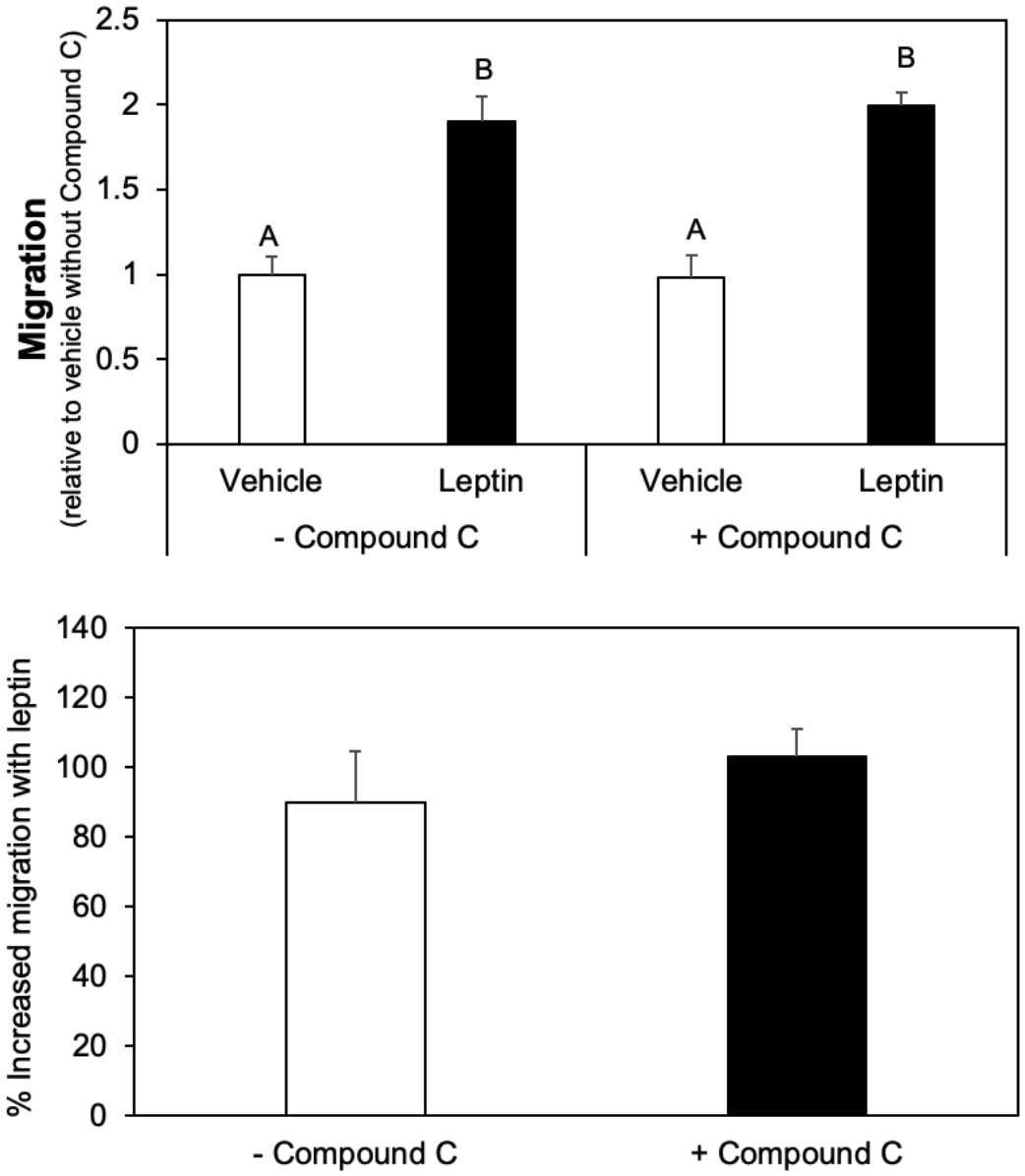

Figure 2.12 Effect of AMPK Inactivation in Leptin-Induced Migration in 4T1 Maintained in 5 mM Glucose Media.

(A) The 4T1 cells were pretreated with leptin (300 ng/ml) for four days before replating in serum free medium with or without the AMPK inhibitor compound C (1 uM) into 8 um pore transwell inserts and migration assessed. In each figure, bars with different letters are significantly different ($p < 0.05$) assessed by ANOVA. (B) In each group, the percent increased migration with leptin was calculated by the difference on migration per average of migration in vehicle. Values are means ± SEM and no significant difference is noted.

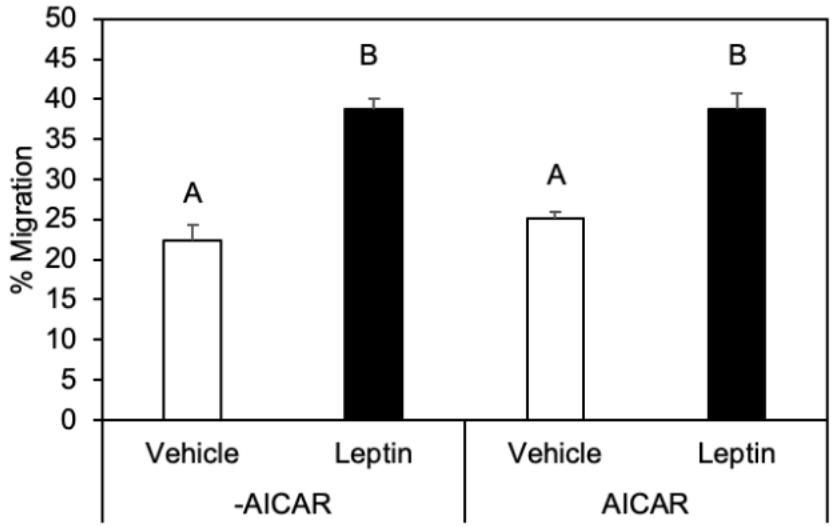

Figure 2.13 Effect of AMPK Activation in Leptin-Induced Migration in metM-wnt^{lung} Maintained in 5 mM Glucose Media.

The cells were pretreated with leptin (300 ng/ml) or vehicle for four days. During the last 24 hours of treatment the cells were incubated with AICAR (1 mM) to activate AMPK followed by replating in serum free medium with or without AICAR (1 mM) into 8 um pore transwell inserts and migration assessed. Percent migration was normalized to the number of cells in the upper well. In each figure, groups with different letters are significantly different ($p < 0.05$) assessed by ANOVA. Values are means ± SEM.

2.5 Discussion

It has been reported that high glucose levels promote both the proliferation and migration of cancer cells [20]. In addition, Su *et al.,* show that high glucose level increases leptin signaling in fibrosarcoma cells through AMPK [11], suggesting an important role of glucose on the effect of leptin. However, the impact of physiological levels of glucose on leptin-mediated migration has not yet been elucidated. Therefore, here we investigated the mechanism by which leptin increases migration in both 4T1 and metM-wnt^{lung} cells when maintained in 5 mM glucose medium.

Leptin exerts its effect via the leptin receptor. We show that *Ob-R* mRNA level was decreased with leptin treatment in 4T1, but leptin does not change *Ob-R* mRNA level in metM-wnt^{lung} cells. This is consistent with other research which shows that leptin does not upregulate level of OB-R in MCF7 and SK-BR-3 breast cancer cell lines while leptin increases invasive capability [21]. This suggests that regulation of leptin receptor level may be cell type specific.

Changes in lipid metabolism leads to cancer cell metabolism such as cell growth, apoptosis, and migration [22]. In particular, fatty acids may provide substrates for energy via FAO as well as serving as signaling molecules. Fatty acids may be available from cellular lipid stores, taken up into the cell or synthesized *de novo.* Several studies show that leptin alters lipid metabolism. For example, Palhinha *et al.*, shows that leptin induces neutral lipids level assessed using BODIPY in 3T3-L1 preadipocytes [23]. On the other hand, leptin increases lipolysis in rat adipose tissues [24]. However, the impact of leptin on fatty acid metabolism in breast cancer cells has not been investigated, particularly when cells are maintained in physiological concentrations of glucose. In our study, leptin treatment did not change TAG level or fatty acid uptake in either 4T1 or metM-wnt^{lung} cells (Figure 2.3, Figure 2.4). This latter result contrasts with the previous work by Blanquer-Rosselló *et al.,* that shows leptin increases FAT/CD36 protein level in MCF-7. This discrepancy with our results may be due to different cell types or that 4T1 and metM-wnt^{lung} cells do not rely on fatty acid metabolism to provide energy following leptin treatment. The results of our study demonstrate that inhibiting FAO does not impact leptin-induced migration in 4T1 and metM-wnt^{lung} cells, showing that FAO is not necessary in increased migratory capability with leptin treatment. This is consistent with other research that demonstrates that inhibiting FAO by etomoxir does not show any impact on invasion in e0771 breast cancer cells [25].

We also examined *de novo* fatty acid synthesis and utilized of [U]-^{13}C–acetate and [U]-^{13}C–glucose incorporation into fatty acids to determine the pathway by which leptin may induce synthesis. In 4T1 cells, leptin did not change glucose incorporation into either palmitate or stearate. However, it is of interest that *Glut1* mRNA level was increased with leptin treatment, but no difference was observed on *Hk2,* whose activity is the first step in glucose utilization in the cell. However, *Fasn* mRNA level was decreased with leptin in 4T1. These altered gene levels, increased *Glut1* and decreased *Fasn,* support the lack of difference in *de novo* fatty acid synthesis from glucose with leptin treatment. Additionally, leptin significantly decreased acetate incorporation, which bypasses glycolysis by supplying a substrate to produce acetyl-CoA, into stearate. This result is consistent with the decrease in *Fasn* mRNA level noted with leptin treatment. On the other hand, in metM-wnt^{lung} cells, leptin increased glucose incorporation into both palmitate and stearate. Leptin also increased acetate incorporation into palmitate. In addition, mRNA level of *Glut1*, *Hk2* and *Fasn* were increased with leptin treatment, suggesting

these changes in gene level may lead to the leptin-induced increase on *de novo* fatty acid synthesis from both glucose and acetate. Thus, although leptin does not affect fatty acid synthesis in 4T1 cells when maintained in 5 mM glucose, in contrast, leptin increases *de novo* fatty acid synthesis both upstream and downstream of the lipogenic pathway in metM-wnt^{lung} cells likely via both up regulation of glucose metabolism and fatty acid synthase activity.

As *Glut1* mRNA level was increased in both 4T1 and metM-wnt^{lung} cells, it was hypothesized that glycolysis is required for leptin-induced migratory behavior. Interestingly, inhibiting glycolysis by 2DG blunts leptin-induced migration in metM-Wnt^{lung}, which support the hypothesis, but does not have any effects on migration with leptin treatment in 4T1 cells. The results in the metM-wnt^{lung} cells are not consistent with the results of Blanquer-Rosselló *et al.*, that show leptin increases the use of fatty acids as fuel for energy instead of glycolysis. This discrepancy may be due to different cell type, similar to our differential results between the 4T1 and metM-wnt^{lung} cell types. Thus, glucose metabolism is involved differentially with a reliance shown in the metM-wnt^{lung}, but not the 4T1, cells in leptin-induced migration.

AMPK is a nutrient sensor which regulates energy metabolism. In our study, while leptin increases AMPK activation by phosphorylation in 4T1 cells, leptin inhibits AMPK activity in metM-wnt^{lung}. This difference is consistent with different mRNA level of genes related to glucose metabolism as well as *de novo* fatty acids synthesis from glucose following leptin treatment. In 4T1 cells, while *Glut1* mRNA level was increased, *Fasn* mRNA level was decreased. These changes in gene level may cumulatively eliminate changes in glucose incorporation into palmitate in 4T1 cells as well as AMPK activation. When AMPK is activated, downstream acetyl-CoA carboxylase (ACC) is inactivated by phosphorylation, leading to the inhibition of acetyl-CoA conversion to malonyl-CoA for fatty acid synthesis. This is consistent with research that shows that inactivation of ACC by leptin leads to invasion of breast cancer cells [25]. On the other hand, in metM-wnt^{lung} cells, mRNA level of FASN and GLUT1 were both increased. In addition, palmitate synthesis from glucose was also increased with leptin treatment. This increase may lead to a sufficient energy status and inactivation of AMPK in metM-wnt^{lung} cells. Together, these results suggest leptin-mediated AMPK activation/inactivation is likely cell type specific depending on regulation of energy metabolism.

Our study is the first to investigate the impact of leptin on migration with metastatic breast cancer cells maintained in a physiological level of glucose, particularly with a focus on

energy metabolism. The results of the current study not only confirm increased migratory capability of breast cancer cells, but also demonstrate that leptin affects energy metabolism differentially in 4T1 and metM-wnt^{lung} cells, an interesting result not reported previously. Overall, FAO is not involved in leptin-induced migration, although fatty acid synthesis is increased only in the metM-wnt^{lung} cells. Further evidence that leptin-mediated changes in energy metabolism is differentially regulated in 4T1 and metM-wnt^{lung} cells is shown with increased glycolysis involved in leptin-induced migration in metM-wnt^{lung}, but not in 4T1 cells. Further studies are necessary to demonstrate the mechanism in each cell type underlying this effect of leptin.

2.6 Acknowledgements

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3. 1A,25-DIHYDROXYVITAMIN D_3 ALTERS SECRETIONS ON ADIPOCYTES AND INHIBITS THE METASTATIC CAPABILITY OF BREAST CANCER CELLS

3.1 Abstract

Excess adiposity is associated with a higher risk of breast cancer metastasis and mortality. Evidence suggests that dietary vitamin D inhibits breast cancer metastasis, however, the mechanistic link between vitamin D's regulation of adipocyte metabolism and metastasis has not been previously investigated. Therefore, the purpose of these studies was to investigate the effect of the active form of vitamin D, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$), on adipose tissue release of bioactive compounds, and whether this regulation inhibits breast cancer cell migration, an important step of metastasis. Differentiated 3T3-L1 adipocytes were treated with $1,25(OH)_2D$ for 2 days, followed by harvesting the adipocytes and collection of adipocyte conditioned media. Conditioned media from $1,25(OH)_2D$-treated adipocytes inhibited the migration of metastatic MDA-MB-231 breast cancer cells. In order to explore the mechanism underlying these effects on breast cancer metastatic capability, the mRNA level of leptin (*Lep*), adiponectin (*Adipoq*), insulin-like growth factor (*Igf-1*), interleukin-6 (*Il-6*) and monocyte chemoattractant protein-1 (*Mcp-1*) was measured in adipocytes treated with either vehicle or $1,25(OH)_2D$. Treatment of adipocytes with $1,25(OH)_2D$ decreased mRNA level of *Lep*, *Adipoq*, *Igf-1*, *Il-6*, and *Mcp-1*. Consistent with mRNA level, concentrations of leptin, adiponectin, IGF-1, and IL-6 in adipocyte conditioned media were decreased with $1,25(OH)_2D$, except MCP-1. Although adipocyte *Lep* mRNA level and release were decreased with $1,25(OH)_2D$, leptin alone did not induce migration as a chemoattractant, suggesting there might be other factors that underly the impact of $1,25(OH)_2D$ on adipocytes to decrease migration of breast cancer cells. Further studies are needed to elucidate the mechanism underlying the impact of $1,25(OH)_2D$ on adipocytes to inhibit their capability of promoting breast cancer metastasis. In summary, these results suggest that $1,25(OH)_2D$ alters adipocyte secretions to inhibit breast cancer metastasis.

3.2 Introduction

Obesity is a growing medical problem worldwide as more than 66% of US women are overweight or obese. It is reported that high BMI is associated with lower survival rate of pre- and post-menopausal breast cancer patients [1]. Obesity is proposed to increase the risk of breast cancer via multiple mechanisms, including increasing proliferation and inflammation, inhibiting differentiation and apoptosis and altering specific cell signaling to modify cancer cell behavior. According to GLOBOCAN 2018, breast cancer is the leading cancer most frequently diagnosed worldwide, followed by colorectal cancer among females, as well as the leading cause of cancer death globally [2]. Breast cancer has a survival rate of 99% when metastasis has not occurred [3]. However, when it is metastasized to other organs, the 5-year survival rate falls to 26% [3]. Therefore, preventing metastasis is very important in breast cancer in obese individuals.

While adipocytes are previously considered solely as energy storage depots, they are now known to be organs that secrete various adipokines and cytokines [4]. Breast cells can be affected not only by an endocrine signal from whole-body adipose tissue but also by a paracrine signal from surrounding adipose tissue [4], since the breast epithelial cells are surrounded by adipocytes. Obesity-associated adipose tissue dysfunction leads to the aberrant production of adipokines that are proposed to be involved in obesity-associated mammary carcinogenesis [5]. Examples of adipokines include leptin, adiponectin, insulin-like growth factor (IGF-1), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1). These factors are known to increase migratory capability in breast cancer cells [6]. However, the association between adipocytes and breast cancer is not fully understood. Therefore, understanding these interactions between adipocytes and breast cancer risk is important in preventing obesity-associated breast cancer.

Vitamin D is derived from the diet or synthesized in the human body [7]. In the liver, 25-hydroxylase converts vitamin D to 25-hydroxyvitamin D (25(OH)D), a circulating form of vitamin D, and is considered an indicator of vitamin D status. A study conducted meta-analysis shows that serum 25(OH)D is inversely related with risk of death from breast cancers [8]. Similarly, in prospective data, African American women who have lower 25(OH)D level show increased risk of breast cancer compared to those with high level of 25(OH)D [9]. It is also reported that vitamin D level and adiposity are inversely associated, as serum 25(OH)D levels are inversely proportional to adiposity [10-12], indicating that vitamin D may have a potential

role on obese breast cancer individuals. In addition, adipose tissues and breast tissues express not only vitamin D receptor but also express 1α-hydroxylase which synthesizes 1,25$(OH)_2$D from 25(OH)D locally [13, 14]. Finally, evidence suggests that 1,25$(OH)_2$D regulates various aspects of adipocytes including differentiation, adipogenesis [15], and inflammation [16], highlighting that vitamin D and its metabolites might exert its role on both breast and adipose tissue to inhibit breast cancer.

Although several studies have investigated the interaction between adipocytes and breast cancers [9, 17], regulation of 1,25$(OH)_2$D on adipocytes in breast cancer cell migration, an important step of metastasis, has not been studied. The purpose of the current study was to investigate the impact of 1,25$(OH)_2$D on mature adipocytes to determine if factors released alter the migratory capability of metastatic breast cancer cells. We hypothesized that 1,25$(OH)_2$D inhibits gene mRNA level and release of adipokines and inflammatory factors that leads to the inhibition metastatic breast cancer cell migration. These results will provide insights into the role of vitamin D on adipocytes in regulating breast cancer metastasis.

3.3 Material and Methods

3.3.1 Chemical and Reagents

Dulbecco's Modification of Eagle's Medium (DMEM) was obtained from Corning (Manassas, VA). Fetal calf serum was obtained from Thermo Fisher Scientific (Pittsburgh, PA). The bioactive vitamin D metabolite, 1,25$(OH)_2$D, was purchased from Biomol (Plymouth Meeting, PA). Fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were obtained from Life Technologies, Gibco-BRL (Rockville, MD). Insulin (bovine), dexamethasone, 3-Isobutyl-1-methylxanthine were obtained from Sigma-Aldrich (St. Louis, MO). Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI). Recombinant leptin was purchased from Peprotech (Rocky Hill, NJ).

3.3.2 Cell Culture

MDA-MB-231 cells, regarded as highly aggressive metastatic human cell lines, are derived from pleural effusion with metastatic breast cancer. Murine metastatic breast cancer 4T1 cells were a gift from Dr. Michael Wendt. MDA-MB-231 and 4T1 cells were cultured in 25 mM

glucose containing DMEM, with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified environment at 37°C with 5% CO_2. Murine 3T3-L1 fibroblasts were obtained from American Type Culture Collection (ATCC CL-173; Manassas, VA), and cultured in DMEM containing 10% fetal calf serum with 100 units/mL penicillin and 100 µg/mL streptomycin at 37ºC with 5% CO_2.

At confluence, 3T3-L1 adipocytes that day was set to as day -2. On day 0, cells were treated with the differentiation cocktail (1.0 µg/mL insulin, 0.5 mM isobutylmethylxanthine, 1.0 µM dexamethasone, and 2.0 µM rosiglitazone) in 10% FBS containing DMEM. On day 2, the medium was replaced with DMEM containing 1.0 µg/mL insulin and 10% FCS. On day 4, the medium was changedto10% FCS containing DMEM and the medium replaced every 2 days. On day 9, differentiated mature adipocytes were treated with vehicle (final ethanol concentration of < 0.1%) or 1,25$(OH)_2$D (10 nM) for 48 hours. After 48 hours of treatment, the medium was washed with phosphate-buffered saline (pH = 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) to remove any 1,25$(OH)_2$D followed by incubation in 10% FBS containing DMEM for 24 hours. After 24 hours, the media was collected, sterile-filtered to remove cellular debris, and stored at −20°C until use. The adipocytes were lysed using TriReagent (Molecular Research Center, Cincinnati, OH) for RNA isolation.

3.3.3 Migration Assay

Differentiated mature adipocytes were treated with vehicle or 1,25$(OH)_2$D (10 nM) for two days. After 2 days of treatment, the medium was replaced with 10% FBS-containing DMEM for 24 hours. After 24 hours, the medium was collected as adipocyte conditioned media. MDA-MB-231 cells were plated in the serum-free medium into 8 um pore Fluoblock™ coated Boyden chamber transwell inserts. Transwell inserts were placed into adipocyte conditioned media from cells either treated with vehicle or 1,25$(OH)_2$D with serum free media in the upper well. After 15 hours of incubation, transwell inserts were removed and placed in dark-coated 24-well plates containing 2.5 ug/ml Calcein AM in 1X clear PBS. Migration was quantified after incubation using a bottom fluorescent plate reader (Ex./Em. 495/525). Migration was calculated by division of value in assay with cells using serum-free media in the bottom well.

For migration assay with leptin, transwell inserts were placed into 10% fetal bovine serum containing DMEM with vehicle (0.1% bovine serum albumin > 0.1% final volume) or

leptin. After 24 hours incubation, the bottom of transwells were fix with methanol and stained with crystal violet. The cell numbers were counted in 5 different fields and migration was quantified with average sum of cells.

3.3.4 Analysis of Concentration of Adipokines

The leptin and IGF-1 levels of conditioned media were measured by mouse enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, GA). The level of adiponectin in conditioned was assessed using mouse ELISA kit (R&D Systems, MN). The level of IL-6 in conditioned media was assessed using mouse V-plex Assay (Meso Scale Diagnostics LLC, MD). The level of MCP in conditioned media was measured by Mouse CCL2 MCP-1 ELISA (Invitrogen, CA).

3.3.5 RNA Isolation and Analysis

RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH, USA), and reverse transcription to cDNA was conducted using MMLV reverse transcriptase (Promega, Madison, WI, USA). mRNA level was determined using qPCR and data are normalized to 18S ribosomal RNA (18S) level. The mRNA abundance of target genes was determined using the threshold cycle (Ct) value. Data are expressed as fold change relative to the vehicle. Primers used are shown in Table 3.1.

Table 3.1 Primers Used in the qPCR Analysis of mRNA Level

Genes	Forward Primer 5'-3'	Reverse Primer 5'-3'
Adipoq	ACCAAAAGGGCTCAGGATGC	GAGCGATACACATAAGCGGC
Igf-1	TGGATGCTCTTCAGTTCGTG	TTTTGTAGGCTTCAGTGGGG
Il-6	AGTGGCTAAGGACCAAGACC	TCTGACCACAGTGAGGAATG
Lep	GCAAGAAGAAGAAGATCCCAGG	CAGATAGGACCAAAGCCACAG
Mcp-1	GCAGCAGGTGTCCCAAAGAA	ATTTACGGGTCAACTTCACATTCAA
18S	ATCCCTGAGAAGTTCCAGCA	CCTCTTGGTGAGGTCGATGT

3.3.6 Glycerol Release Assay

Conditioned media samples from adipocytes were collected after treatment and used for the analysis of glycerol. Glycerol was quantified using spectrophotometric assay kits from Sigma-Aldrich (St. Louis, MO)

3.3.7 Statistical Analysis

Values are presented as mean ± SEM. Results are expressed compared to the vehicle, by Student's t-tests, with $P<0.05$ considered statistically significant

3.4 Results

To evaluate the impact of $1,25(OH)_2D$-mediated regulation of differentiated adipocytes on migration in breast cancer cells, differentiated mature 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 2 days, followed by incubation in 10% FBS containing DMEM for 24 hours, and then conditioned media was collected from adipocytes. Conditioned media from adipocytes treated with $1,25(OH)_2D$ significantly inhibited cell migration in MDA-MB-231 cells using Boyden chamber assay (Figure 3.1), suggesting that $1,25(OH)_2D$ reduces the chemoattractive effect of adipocytes. Therefore, it is important to identify factors released from adipocytes that may impact chemoattraction and are altered with $1,25(OH)_2D$ treatment.

Leptin and adiponectin are adipokines secreted from adipocytes under different stimuli, including increased different levels of adiposity. [18]. In order to assess the effect of $1,25(OH)_2D$ on gene level of adipokines, we measured mRNA level of *Lep* and *Adipoq* in differentiated 3T3-L1 adipocytes. Treatment of $1,25(OH)_2D$ decreases *Lep* and *Adipoq* mRNA level in 3T3-L1 adipocytes (Figure 3.2A). The concentration of leptin and adiponectin was measured in the conditioned mediate to examine if the decrease in gene mRNA level is associated with a similar decreased level of adipokines in the media. Consistent with the mRNA results, $1,25(OH)_2D$ decreases protein concentration of leptin and adiponectin in conditioned media from 3T3L1 compared to vehicle treatment (Figure 3.2B). Because the leptin: adiponectin ratio is suggested as a marker of adipose tissue dysfunction [19], we determined the leptin:adiponectin ratio in adipocyte conditioned media. Interestingly, $1,25(OH)_2D$ decreased leptin: adiponectin ratio in conditioned media compared to vehicle treatment (Figure 3.2C), indicating $1,25(OH)_2D$

treatment may alleviate adipose dysfunction. To test if the decreased level of leptin in the conditioned media from 1,25(OH)2D treated adipocytes mediated the decrease in MBA-231 cell migration, we added leptin into the bottom transwell with 10% FBS containing media to determine if it acted as a chemoattractant and migration were measured. However, the addition of leptin at the bottom transwell did not induce migration (Figure 3.3), suggesting that the decrease in leptin concentration in the conditioned media does not play a role in the $1,25(OH)_2D$ impact on adipocytes to reduce migration.

To explore other potential factors that are altered in adipocytes following $1,25(OH)_2D$ treatment, we measured mRNA level of *Il-6*, *Igf-1*, and the chemokine *Mcp-1*. Treatment of adipocytes with $1,25(OH)_2D$ decreased mRNA level of *Il-6*, *Igf-1*, and *Mcp-1* (Figure 3.4A), indicating the decrease in these factors in conditioned media from $1,25(OH)_2D$ treated adipocytes may mediate the reduced migration in breast cancer cells. To further explore if the decreased mRNA concentration of *Il-6* and *Igf-1* altered the release of these factors, the media concentration was assessed. $1,25(OH)_2D$ treatment of adipocytes reduced the media concentration of IL-6 and IGF-1, but MCP-1 concentration in conditioned media was not changed by $1,25(OH)_2D$ treatment (Figure 3.4B), suggesting MCP-1 is not a factor for the reduction of migration of MDA-MB 231 cells by conditioned media.

It is reported that adipocyte-derived lipids contribute to breast cancer migratory characteristics [20]. To address whether $1,25(OH)_2D$ changes hydrolysis of triacylglycerol, we measured glycerol, a product of lipolysis of triacylglycerol, concentration in conditioned media. Treatment of $1,25(OH)_2D$ for two days increases glycerol release in mature adipocyte, indicating $1,25(OH)_2D$ may increase triacylglycerol hydrolysis or increase glyceroneogenesis to synthesis glycerol from pyruvate or lactate. These results show that $1,25(OH)_2D$ might increase free fatty acids in conditioned media, and further studies are necessary to confirm this.

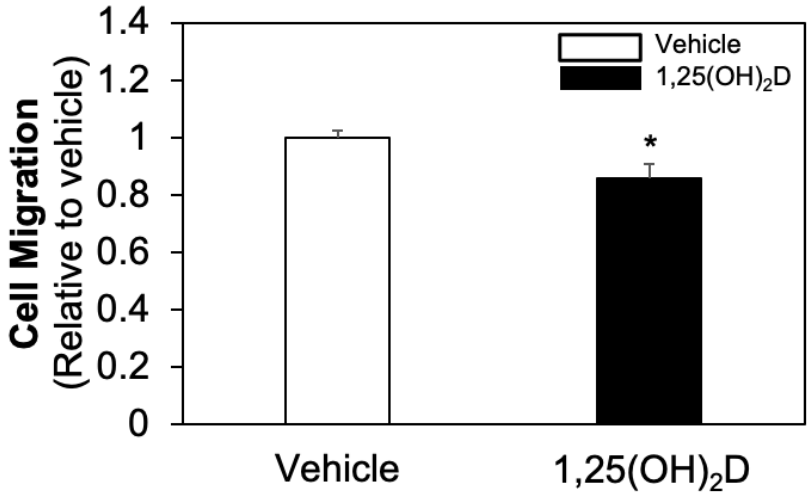

Figure 3.1 The Effect of Conditioned Media from 1,25$(OH)_2$D-treated adipocytes on Migration in MDA-MB-231 Breast Cancer Cells.

Mature 3T3-L1 adipocytes were treated with vehicle or 1,25$(OH)_2$D (10 nM) for 2 days. After 2 days of treatment, the medium was replaced with 10% FBS-containing DMEM. After 24 hours, the medium was collected as adipocyte conditioned media. MDA-MB-231 cells were plated in the serum-free medium into 8 um pore Fluoblock™ coated transwell inserts. Transwell inserts were placed into adipocyte conditioned media treated either with vehicle or 1,25$(OH)_2$D. After 15 hours of incubation, transwell inserts were removed and placed in dark-coated 24-well plates containing 2.5 ug/ml Calcein AM in 1X clear PBS. Migration was quantified after incubation using a bottom fluorescent plate reader (Ex./Em. 495/525). Migration was calculated by division with cells with serum-free media. An asterisk indicates a significant difference relative to vehicle ($P < 0.05$).

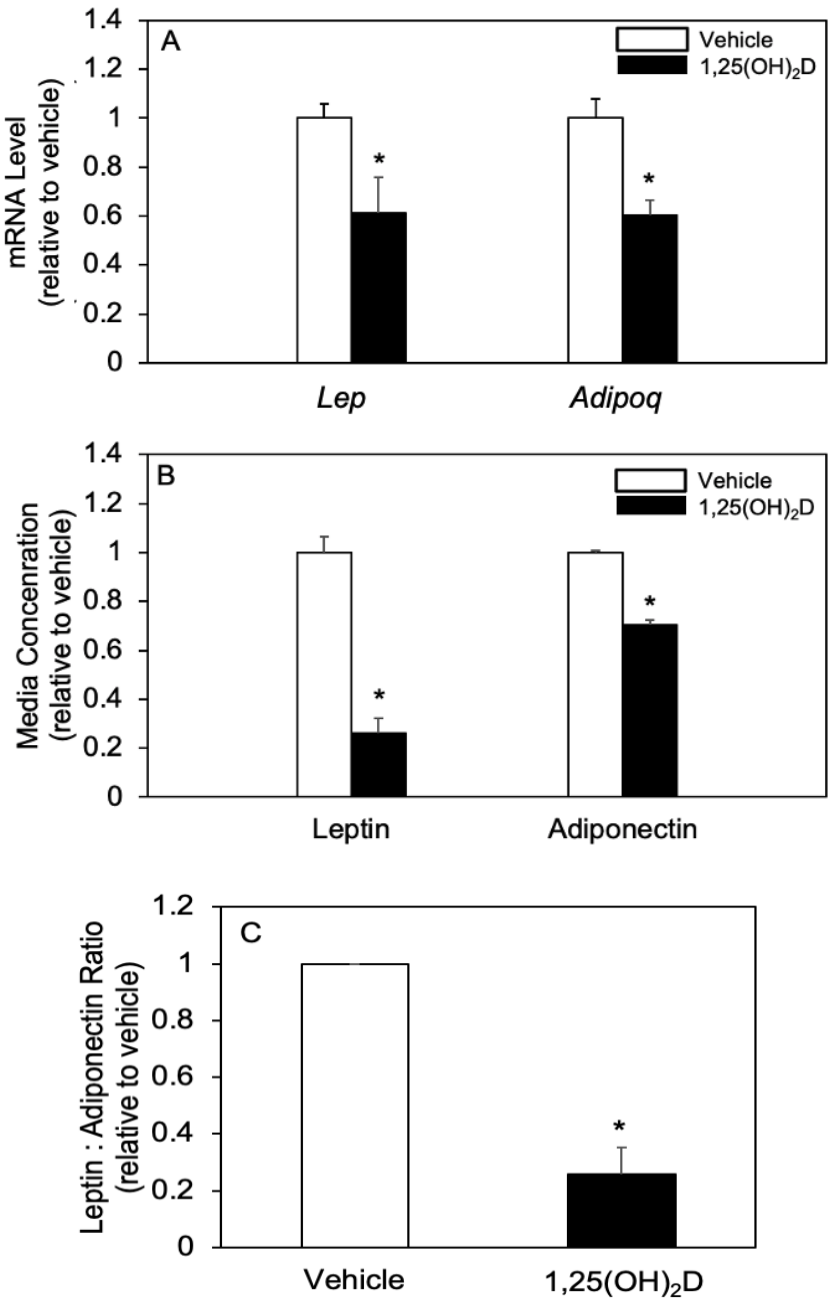

Figure 3.2 mRNA Level and Release of Adipokines, Leptin, and Adiponectin Following Vehicle or 1,25(OH)$_2$D Treatment of Mature 3T3-L1 Adipocytes.

(A) *Lep* and *Adipoq* mRNA level was measured following vehicle or 1,25(OH)$_2$D treatment in adipocytes by reverse-transcription PCR and normalized to 18S. (B) The concentration of leptin and adiponectin in conditioned media from 3T3-L1 adipocytes following treatment of vehicle or 1,25(OH)$_2$D was measured. (C) Leptin:adiponectin ratio was calculated from results shown in (B). The values are expressed per vehicle and as mean ± SEM. An asterisk indicates a significant difference relative to vehicle ($P < 0.05$).

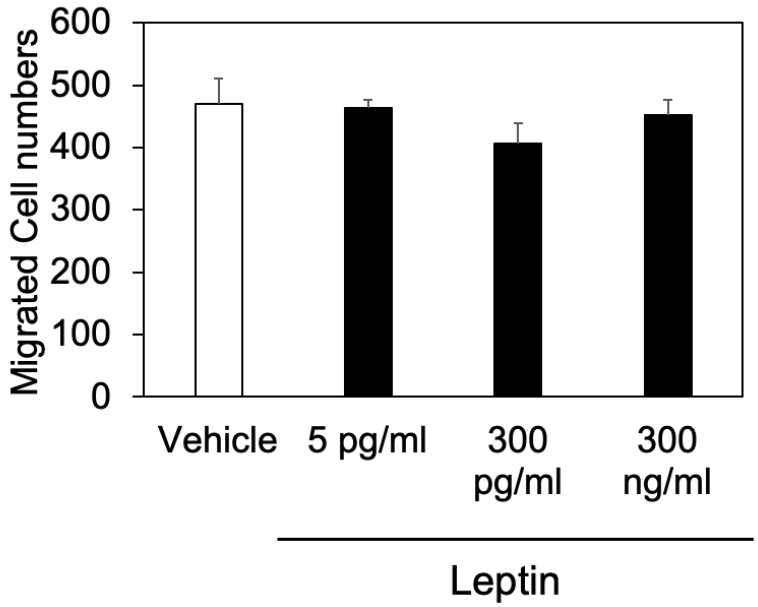

Figure 3.3 Addition of Leptin as Chemoattractant on Migration in Breast Cancer Cells.

4T1 murine breast cancer cells were plated in serum-free DMEM into 8 μm pore transwell inserts. Transwell inserts were placed 10% FBS containing DMEM with either vehicle or different concentrations of leptin. After 24 hours of incubation, the number of cells on the bottom of the transwells were stained with crystal violet. The cell numbers were counted in 5 different fields and migration was quantified with the average sum of cells. The values are expressed as mean ± SEM.

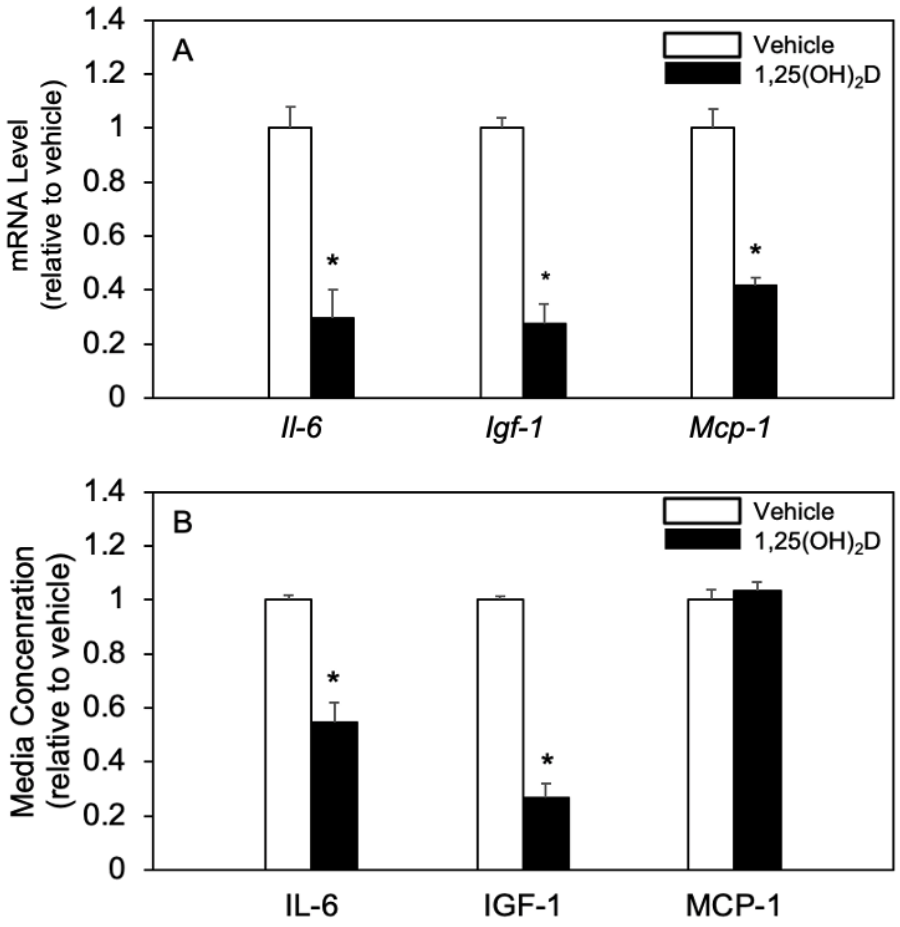

Figure 3.4 mRNA Level and Release of Adipokines, IL-6, and IGF-1 Following Vehicle or $1,25(OH)_2D$ Treatment of Mature 3T3-L1 Adipocytes.

(A) *Il-6*, *Igf-1*, and *Mcp-1* mRNA level was measured following vehicle or $1,25(OH)_2D$ treatment in adipocytes by reverse-transcription PCR and normalized to 18S. (B) The concentration of IL-6, IGF-1, and MCP-1 in conditioned media from 3T3-L1 with treatment of vehicle or $1,25(OH)_2D$ was measured. The values are expressed per vehicle and as mean ± SEM. An asterisk indicates a significant difference relative to vehicle ($P < 0.05$).

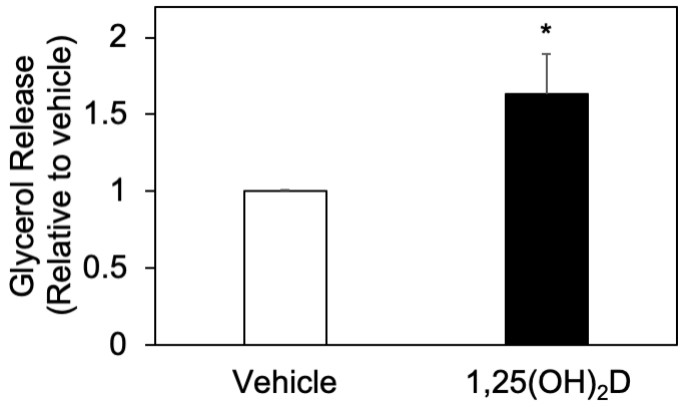

Figure 3.5 Glycerol Release Following in 1,25$(OH)_2$D Treatment in Mature Adipocyte 3T3-L1. Differentiated 3T3-L1 adipocytes were treated with vehicle or 1,25$(OH)_2$D (10 nM) for 48 hours. Glycerol was measured in conditioned media by enzymatic determination of free glycerol. The values are expressed per vehicle and as mean ± SEM. An asterisk indicates a significant difference relative to vehicle ($P < 0.05$).

3.5 Discussion

Obesity, specifically excess body weight, has been linked to an increased risk of breast cancer [21]. Similarly, a meta-analysis shows that high BMI is associated with lower survival in pre- and post-menopausal breast cancer patients [1]. Mohr *et al.,* show that serum 25(OH)D is inversely related with risk of death from breast cancers [8]. Evidence suggests that vitamin D status is negatively associated with obesity [10, 11], with previous literature suggests vitamin D status may change adipose tissue metabolism [22] as well as evidence suggesting the vitamin D is sequestered within the adipocyte [23]. Given that there are known impacts of vitamin D on adipocyte metabolism, these changes may mediate indirectly impact the progression of breast cancer. In this study, we demonstrated that conditioned media from 1,25$(OH)_2$D treated adipocytes significantly inhibited the migration of MDA-MB-231 breast cancer cells. We confirmed that 1,25$(OH)_2$D inhibited the release of adipokines including proinflammatory cytokines and chemokines from adipocytes involved in increased migratory capability in breast cancer cells. These results suggest there may be multiple mechanisms by which 1,25$(OH)_2$D contributes to inhibition of migration.

Adipose tissues secrete adipokines, leptin, and adiponectin. Leptin is secreted from adipose tissues in proportion to the size of adipocytes [24]. Although adiponectin is released

from adipocytes and serum concentration is generally negatively associated with adiposity, a decreased level of adiponectin has been observed in obese human subjects compared to non-obese individuals [25]. Emerging evidence suggests that leptin is involved in breast cancer development, such as treatment of breast cancer cells increases migration [26]. In our study, $1,25(OH)_2D$ inhibited the release of leptin in conditioned media from adipocytes, suggesting that leptin may mediate the impact of $1,25(OH)_2D$ on adipocytes to reduce breast cancer cell migration. Even though leptin increased breast cancer migration when cells were treated with leptin directly (data not shown), leptin did not induce migration when leptin was added to the bottom well as a chemoattractant. Leptin: adiponectin ratio has been suggested as a marker of adipose tissue dysfunction [19], and leptin: adiponectin ratio was also reduced with $1,25(OH)_2D$ treatment, suggesting that vitamin D's protective role to reduce breast cancer migration by other mechanisms for obese individuals.

Adipocytes act as endocrine organs that secrete inflammatory cytokines, and obesity-associated inflammation is linked to an increased risk of breast cancer in obese. Proinflammatory cytokine IL-6 is upregulated in obese people [27] and is also shown to increase migration in breast cancer cells [28]. In our study, $1,25(OH)_2D$ treatment decreased IL-6 release as well as mRNA level. Consistent with our finding, Chang *et al.,* also showed that vitamin D insufficiency significantly increased IL-6 levels in both adipose tissue and serum in high-fat induced rats [29], suggesting the role of $1,25(OH)_2D$ on obese-associated inflammation. Furthermore, treatment of $1,25(OH)_2D$ decreases both release and mRNA level of IGF-1 in mature 3T3-L1 adipocytes. It is known that increased level of IGF-1 is associated with obesity [30]. Additionally, IGF-1 is associated with increased risk in breast cancer [31], indicating decrease in IGF-1 may play a role on decrease in breast cancer cell migration. However, Trummer *et al.,* show that vitamin D supplementation does not alter IGF-1 concentration in randomized controlled trial with hypertensive patients with low 25(OH)D concentration [32]. Another randomized controlled trial by Kamycheva *et al.,* shows that vitamin D supplement reduces IGF-1/IGF binding protein-3 ratio, a measurement of free IGF-1 level, only in a subgroup of non-severe obese individuals who have BMI < 35 kg/m^2 [33]. This discrepancy might be due to different study designs and different participants. Thus, further investigation is needed to elucidate the role of vitamin D on IGF-1 level in obese individuals as well as the role of IGF-1 on breast cancer migration. Either of these factors may mediate the effect of $1,25(OH)_2D$ on adipocytes to reduce the effect of

conditioned media on migration of MDA-MB 231 cells. Further studies are required to explore this question of the levels of IL-6 and IGF in the conditioned media, particularly as a chemoattractant, on breast cancer cell migration.

In addition, MCP-1 has been shown to increase the migration of breast cancer cells as a chemoattractant [34]. Even though $1,25(OH)_2D$ also decreases *Mcp-1* mRNA level in adipocytes, the level of MCP-1 of protein was not changed following $1,25(OH)_2D$ treatment of adipocytes, indicating MCP-1 is not a factor in the $1,25(OH)_2D$-mediated reduction of migration in breast cancer cells with conditioned media exposure as a chemoattractant.

Another potential mechanism by which $1,25(OH)_2D$ regulation of adipocytes decreases breast cancer cell migration may be the altered hydrolysis of triacylglycerol, potentially leading to a decrease in the release of fatty acids into the media. Increased fatty acids from mammary adipocytes have been shown to promote tumor progression and invasion by lipolysis in breast cancer cells [17]. In our study, $1,25(OH)_2D$ treatment increased glycerol release in mature adipocytes, suggesting $1,25(OH)_2D$ may increase lipolysis to produce glycerol or increase glyceroneogenesis to synthesis glycerol from pyruvate or lactate. Larrick *et al.,* also show that $1,25(OH)_2D$ treatment increases glycerol release and did not detect non-esterified fatty acids released into the media from adipocytes [22], suggesting either an increase in glyceroneogenesis, intracellular reutilization of the fatty acids, or efficient reuptake of fatty acids into the adipocytes. These results may indicate that although $1,25(OH)_2D$ increases glycerol release, there may not be an associated with decrease in fatty acids. Further, these results are inconsistent from the previous research that show that mammary adipocytes induce breast cancer invasion through free fatty acids supplied by increased lipolysis of adipocytes [17]. Further investigation into the fatty acid concentrations, and impact on breast cancer migration is needed to clarify if the increase in glycerol release mediates the effect of $1,25(OH)_2D$ on adipocytes to reduce migration of breast cancer cells.

Our study is the first to investigate the impact of $1,25(OH)_2D$-mediated adipocytes conditioned media on breast cancer migratory capability. The results of the current study not only confirm that $1,25(OH)_2D$ inhibition of migratory potential of breast cancer cells may be indirectly regulated by alterations in adipocytes release of chemoattractants, but also reveals that $1,25(OH)_2D$ inhibits the release of various adipokines including leptin, adiponectin, IL-6, IGF-1 from mature adipocytes. As breast cancer cells were only plated in serum-free media in the upper

transwell, the only factor that might affect different metastatic capability is likely due to $1,25(OH)_2D$ mediated changes in conditioned media from adipocytes. Since conditioned media contains various adipokines as well as fatty acids and glycerol from adipocytes, it might be difficult to find a single factor that alters the migration of breast cancer cells as a chemoattractant. All of these factors may likely contribute to altering breast cancer migration. These results support the potential use of vitamin D in preventing breast cancer metastasis, particularly migration. Further studies are needed to identify the underlying mechanisms of $1,25(OH)_2D$ regulation of adipocyte to reduce migration of breast cancer cells.

3.6 Acknowledgements

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4. REGULATION OF VITAMIN D RECEPTOR BY 1,25-DIHYDROXYVITAMIN D IN BREAST CANCER

4.1 Abstract

Loss of the vitamin D receptor (VDR) is reported in later stages of tumor progression in cancer cells, including breast cancer, with the implication that vitamin D may not be effective in inhibiting metastasis. The purpose of these studies was to investigate the level and activity of the VDR in breast cancer cells at different stages of progression and the response to treatment with the active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). We hypothesized that although constitutive level of VDR is downregulated during cancer progression, $1,25(OH)_2D$ induces an increase in VDR level and transcriptional activity in metastatic breast cancer cells. We employed a series of human cells representing different stages of breast cancer, the MCF-10A series, which includes untransformed MCF10A, Harvey-*ras* oncogene transfected early progression model (MCF10A-*ras*), and metastatic MCF10CA1a cells as well as murine non-metastatic 4TO7 and metastatic 4T1 cells. Cells were treated with $1,25(OH)_2D$ (10 nM) or vehicle for 2 and 5 days. VDR mRNA level was measured by PCR and VDR transcriptional activity by mRNA level of CYP24, a gene containing a vitamin D response element. Constitutive VDR mRNA level was lower (76%) in metastatic MCF10CA1a compared to untransformed MCF10A. Similarly, *Vdr* mRNA level was significantly lower in metastatic 4T1 compared to non-metastatic 4TO7. Treatment with $1,25(OH)_2D$ for 5 days decreased VDR mRNA level in MCF10A (43%) and MCF10A-*ras* (37%) cells compared to vehicle treatment. In contrast, treatment with $1,25(OH)_2D$ for 5 days increased VDR mRNA level by 46% in metastatic MCF10CA1a cells. In addition, VDR transcriptional specific activity increased in MCF10A-*ras* cell and trended towards an increase ($p=0.06$) in MCF10CA1a cells compared to MCF10A, as assessed by measuring the ratio of CYP24/VDR mRNA level. Taken together, these results suggest that although constitutive level of VDR is reduced in metastatic cells, vitamin D may exert inhibitory effects through $1,25(OH)_2D$ upregulation of VDR level and activity in late stage of breast cancer.

4.2 Introduction

According to GLOBOCAN 2018, breast cancer is most frequently diagnosed worldwide, followed by colorectal cancer, among females as well as the leading cause of cancer death globally [1]. The 5-year survival rate for breast cancer is 99% when diagnosed early without metastasis [2]. However, when it is metastasized to secondary organs, the 5-year survival rate drastically falls to 26% [2]. Therefore, preventing the progression of breast cancer is very important in breast cancer patients to prolong their life.

Growing evidence suggests that a high vitamin D status may contribute to the prevention of breast cancer [3]. For instance, individuals with serum 25-hydroxyvitamin D (25(OH)D) of approximately 52 ng/ml had 50% lower risk of breast cancer than those with serum <13 ng/ml according to a meta-analysis [4]. In addition, Janbabai *et al.,* show that the serum level of vitamin D is significantly lower in breast cancer patients with distant metastasis compared to patients without metastasis [5]. Thus, identifying how vitamin D may be used to prevent breast cancer and metastasis may reduce deaths from this disease.

The effects of 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), the most active form of vitamin D, are mediated through the vitamin D receptor (VDR), a transcription factor which enhances or represses target gene transcription [6], although other pathways have been described [7]. In an epidemiological study, VDR level is inversely associated with more aggressive breast cancer [8], which may lead to subsequent unresponsiveness to $1,25(OH)_2D$ at late stage of breast cancer. Cross *et al.,* also show that VDR gene level is lower in late stage of colorectal cancer compared to early phase of carcinoma [9]. Furthermore, reduced VDR level induces breast tumor growth and metastasis in an *in vivo* model [10], suggesting the important role of vitamin D signaling. Therefore, the level and activity of VDR are important factors in elucidating how to develop strategies for prevention or treatment of breast cancer with vitamin D.

The transcription factor SNAIL1 encoded by Snail1 genes is reported to repress VDR level by binding to the promoter region of human VDR gene [11]. Overexpression of SNAIL1 in colon cancer cells decreases VDR level and reduces the $1,25(OH)_2D$ target genes including E-cadherin [12], and CYP24 [13]. Therefore, the relationship between SNAIL1 and VDR is of interest to elucidate the regulation of VDR level in tumor progression in breast cancers.

Although several studies have investigated the correlation between breast tumor progression and VDR level, little information is available on how $1,25(OH)_2D$ regulates VDR

level or transcriptional activity during breast cancer progression. The purpose of the current study was to investigate the level and activity of the VDR in breast cancer cells at different stages of progression and the response to treatment with $1,25(OH)_2D$. It was hypothesized that VDR level is downregulated during cancer progression, and its level and transcriptional activity are increased by $1,25(OH)_2D$ in metastatic breast cancer cells. The results of these studies will contribute to determining recommendations for vitamin D to prevent and treat breast cancer.

4.3 Materials and Methods

4.3.1 Chemical and Reagents

The bioactive vitamin D metabolite, $1,25(OH)_2D$, was purchased from Biomol (Plymouth Meeting, PA). Dulbecco's Modification of Eagle's Medium (DMEM) was obtained from Corning (Manassas, VA). Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) media, horse serum, fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were obtained from Life Technologies, Gibco-BRL (Rockville, MD).

4.3.2 Cell Culture

Human MCF10A spontaneously immortalized breast epithelial cells are derived from a patient with fibrocystic disease. MCF10A cells do not survive *in vivo* when implanted into immunodeficient mice. Harvey-*ras* oncogene transformed MCF10A (MCF10A-*ras*) is considered as early breast cancer model. MCF10A-*ras* cells establish small nodules and tumor growth sporadically when injected into immunodeficient mice. [14]. Through serial passages of MCF10A-*ras* cells into immunodeficient mice, malignant MCF10CA1a cell line was obtained [15]. Upon injected subcutaneously into immunodeficient mice, MCF10CA1a consistently form tumors.

In addition to human MCF10A series, 4TO7 and 4T1 are derived from a common parental population of 410.4 cells from BALB/c mice [16]. Upon implanted into the mammary gland of BALB/c mice, both 4TO7 and 4T1 form primary breast tumors. 4TO7 cells is regarded as a non-metastatic cell line as 4TO7 cells cannot form visible lung nodules and do not colonize in distant organs despite of disseminated 4TO7 cells into the lung [17]. On the other hand, 4T1

cells can metastasize and colonize in distant sites including lung, liver, brain, and bone [18]. Thus, 4T1 cells are considered as metastatic mimicking stage IV breast cancer cell line [19].

4.3.2.1 MCF10A Series

MCF10A human breast epithelial cells and MCF10A-*ras* cells were a gift from Dr. Michael Kinch, Purdue University. The MCF10A and MCF10A-*ras* cells were cultured in 17.5 mM glucose and 2.5 mM glutamine, 0.5 mM sodium pyruvate containing DMEM/F12 (1:1) containing 5% horse serum and supplemented with 10mg/L insulin, 20 mg/L epidermal growth factor, 50 mg/L cholera toxin, 50 mg/L hydrocortisone, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in a humidified environment at 37°C with 5% CO_2. MCF10CA1a cells were cultured in 17.5 mM glucose and 2.5 mM glutamine, 0.5 mM sodium pyruvate DMEM/F12 (1:1) media with 100 units/ml penicillin, and 0.1 mg/mL streptomycin and 5% horse serum in a humidified environment at 37°C with 5% CO_2.

4.3.2.2 4TO7 and 4T1

Cells were cultured in 25 mM glucose, 4 mM glutamine and 1 mM sodium pyruvate containing DMEM media with 100 units/ml penicillin, and 0.1 mg/mL streptomycin and 10% fetal bovine serum in a humidified environment at 37°C with 5% CO_2.

4.3.2.3 Treatment

Cells were treated for 2 or 5 days with either ethanol vehicle (final concentration <0.03%) or 1,25$(OH)_2$D (10 nM) and media changed every 24 hours.

4.3.3 RNA Isolation and Analysis

RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH, USA) and reverse transcription to cDNA was conducted using MMLV reverse transcriptase (Promega, Madison, WI, USA). mRNA level was determined using qPCR and data are normalized to 18S level. The mRNA abundance of target genes was determined using the threshold cycle (Ct) value. Data are expressed as fold change relative to vehicle. Primers used are shown in Table 4.1.

Table 4.1 Primers Used in the qPCR Analysis of mRNA Level

Genes	Forward Primer 5'-3'	Reverse Primer 5'-3'
CYP24 (human)	GTATCTGCCTCGTGTTGTATG	AGCCGATTCTGGTGATAAATG
Cyp24 (murine)	TCCATGAGGCTTACCCCAAG	CGATGCCGAATGGGAGATGA
SNAIL1 (human)	TAGCGAGTGGTTCTTCTGCG	AGGGCTGCTGGAAGGTAAAC
VDR (human)	GCTGGACGGAGAAATGGACTCT	GACACTTCAGACCCAAAGGCT
Vdr (murine)	ACATTATCGCCATCCTGCTC	CCTTGGAGAATAGCTCCCTGT
18S	ATCCCTGAGAAGTTCCAGCA	CCTCTTGGTGAGGTCGATGT

4.3.4 Statistical Analysis

Values are presented as mean ± SEM. Results are expressed compared to the vehicle, analyzed by Student's t-tests, with $P<0.05$ considered statistically significant.

4.4 Results

It is reported that VDR level is negatively correlated with the tumor aggressiveness in breast cancer patients [8]. To evaluate the basal level of VDR in early breast cancer compared to metastatic breast cancer cells, the VDR mRNA level was assessed in the human MCF10A, MCF10A-*ras* and the metastatic MCF10CA1a, murine 4TO7 and 4T1 cells. The basal VDR mRNA level was decreased in metastatic MCF10CA1a cells compared to non-metastatic MCF10A cells (Figure 4.1A). A similar pattern was observed in murine breast cancer cell lines as metastatic 4T1 cells show lower *Vdr* mRNA level compared to non-metastatic 4TO7 cell lines (Figure 4.1B). These results indicate that VDR level is inversely related with the tumor progression and metastatic status in human and murine breast cancer cells.

To investigate the impact of $1,25(OH)_2D$ on VDR mRNA level, the MCF10A series and 4TO7 and 4T1 cells were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 2 and 5 days. In MCF10A cells, $1,25(OH)_2D$ treatment for two days did not alter VDR mRNA level, but five days of treatment decreased VDR mRNA level (Figure 4.2A). In MCF10A-*ras* cells, $1,25(OH)_2D$ treatment for both two and five days decreased VDR mRNA level. However, in contrast, two days treatment of $1,25(OH)_2D$ metastatic MCF10CA1a did not change the VDR

mRNA level in MCF10CA1a cells, but five days of treatment increased VDR level, suggesting the effect of $1,25(OH)_2D$ treatment on VDR level varies depending on breast cancer progression and metastatic status. Similar to these results, in murine non-metastatic 4TO7 cells, treatment of $1,25(OH)_2D$ for two days increased *Vdr* mRNA level, but there was no difference following five days of treatment (Figure 4.2B). In contrast, in murine metastatic 4T1 cells, $1,25(OH)_2D$ treatment for two days decreased *Vdr* mRNA level, whereas five days of treatment increased *Vdr* mRNA level. Thus, five days of $1,25(OH)_2D$ treatment increased VDR mRNA level in both metastatic MCF10CA1a and 4T1 cells, suggesting longer treatment of $1,25(OH)_2D$ may increase VDR activity in metastatic breast cancer cells.

The enzyme 24-hydroxylase (CYP24) converts $1,25(OH)_2D$ to $1,24,25(OH)_3D$, targeting it to degradation of $1,25(OH)_2D$ and serves as an intracellular indicator of VDR transcriptional activity. To investigate the impact of $1,25(OH)_2D$ on VDR transcriptional activity, CYP24 mRNA level following $1,25(OH)_2D$ treatment was measured. CYP24 mRNA level was increased significantly with $1,25(OH)_2D$ treatment in all cell lines and time points (Figure 4.3A and B). Treatment of both with $1,25(OH)_2D$ induced an increase in CYP24 mRNA level in both MCFCA10CA1a and 4T1 cells, indicating a robust response to $1,25(OH)_2D$ in metastatic cell lines. Interestingly, *Cyp24* mRNA level following $1,25(OH)_2D$ treatment for 5 days was lower compared to 2 days treatment in 4TO7 and 4T1 cells, which is consistent with increased degradation of $1,25(OH)_2D$ with longer treatment as the level of *Cyp24* is increased.

To evaluate VDR transcriptional specific activity, the correlation between VDR mRNA level CYP24 mRNA level following $1,25(OH)_2D$ treatment was assessed (Figure 4.4A). Additionally, the ratios of CYP24/VDR mRNA level following $1,25(OH)_2D$ treatment in the MCF10A-*ras* and MCF10CA1a cell lines were calculated. VDR transcriptional specific activity increased in MCF10A-*ras* cell and trended towards an increase ($p=0.06$) in MCF10CA1a cells compared to MCF10A (Figure 4.4B). These results indicate that VDR transcriptional specific activity in response to $1,25(OH)_2D$ in MCF10CA1a is not lower compared to non-metastatic MCF10A even though the basal level of VDR is significant lower in MCF10CA1a.

Previous results show that the transcription factor SNAIL1 represses VDR level in human colon tumors [20]. Therefore, because our results show that VDR mRNA level is reduced during breast cancer progression, we hypothesized that SNAIL1 mRNA level increases during breast cancer progression and increased SNAIL1 level leads to decreased VDR level during breast

tumor progression. To elucidate whether SNAIL1 transcription factor is associated with VDR level and if 1,25$(OH)_2$D alters SNAIL1 level, we assessed SNAIL1 mRNA level in MCF10A series. However, SNAIL1 mRNA level was the highest in MCF10A-*ras*, and the lowest in MCF10A, which does not support our hypothesis. In addition, treatment of 1,25$(OH)_2$D for 5 days decreases SNAIL1 mRNA level in MCF10A and MCF10A-*ras* cells, and MCF10CA1a ($p=0.05$) cells by 70%, 79%, 21% respectively. Thus, SNAIL1 is unlikely to mediate the reduced mRNA level of VDR in the metastatic cell lines.

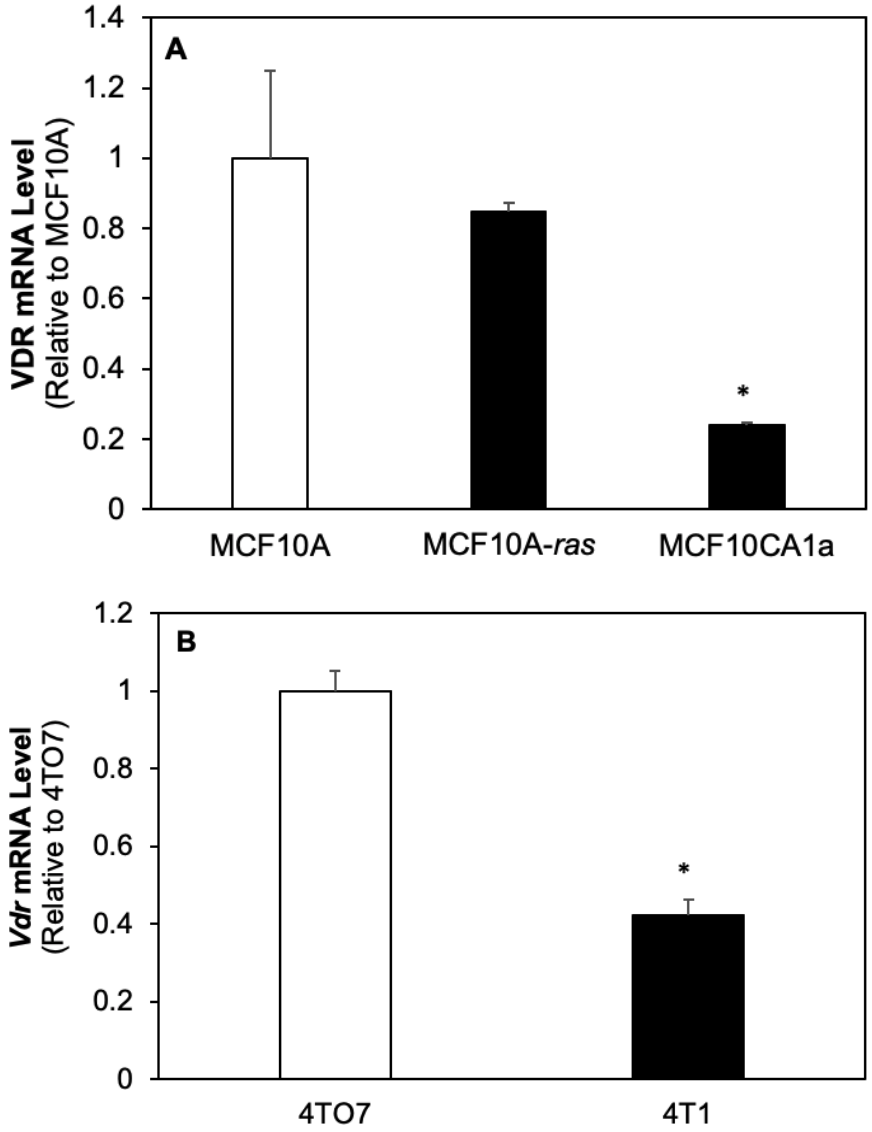

Figure 4.1 Basal Level of VDR mRNA During Cancer Progression in Human and Murine Breast Cancer Cells.

VDR mRNA level was measured in (A) human breast cancer MCF10A series and (B) murine breast cancer 4TO7 and 4T1 cells by reverse-transcription PCR and normalized to 18S. Values are expressed per vehicle and are means ± SEM. Asterisk indicates a significant difference relative to (A) MCF10A or (B) 4TO7 of $p<0.05$.

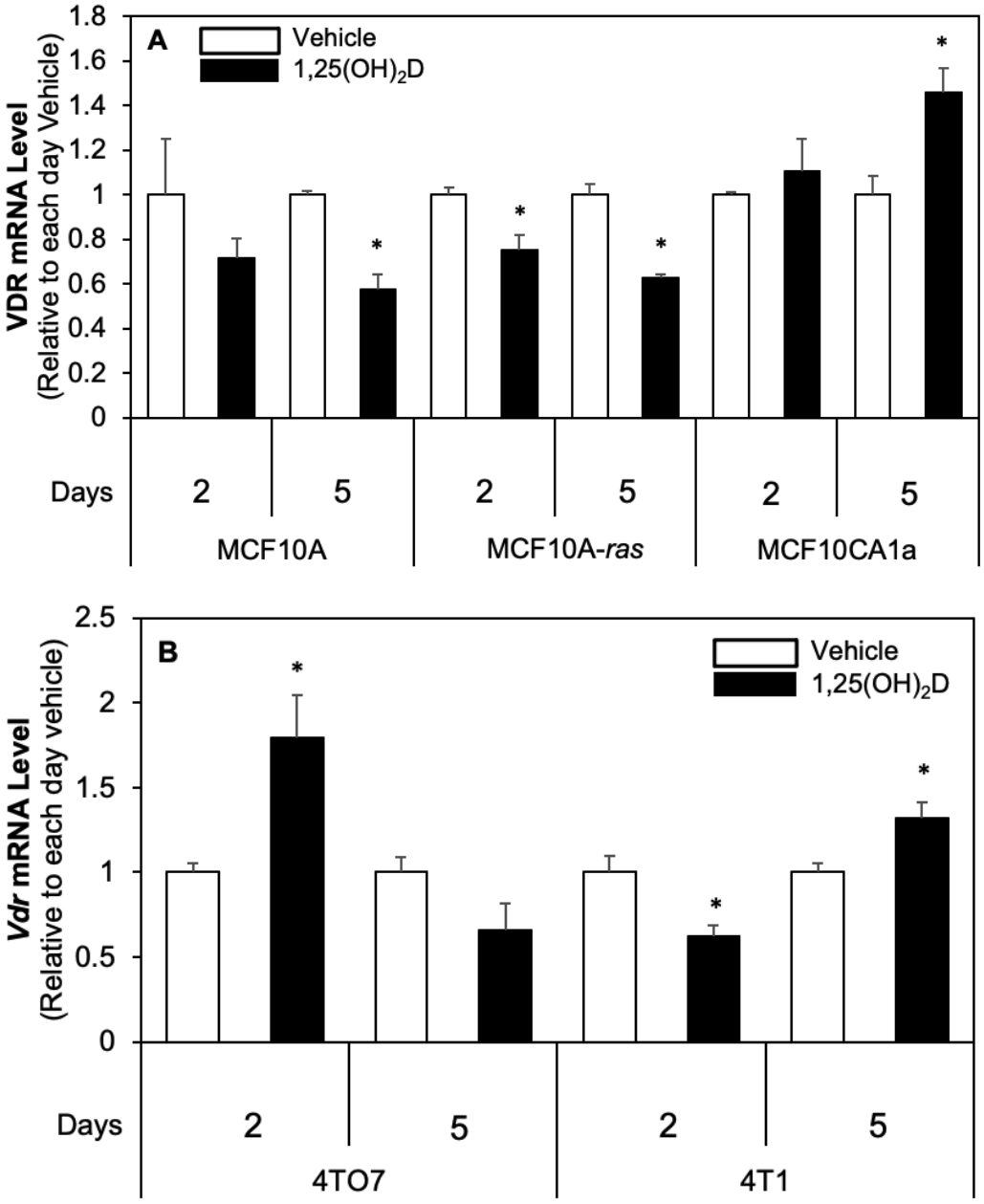

Figure 4.2 Effect of $1,25(OH)_2D$ on VDR mRNA Level in Human and Murine Breast Cancer Cells.

VDR mRNA level was measured in (A) human breast cancer cells MCF10A series and (B) murine breast cancer cells 4TO7 and 4T1 by reverse-transcription PCR and normalized to 18S. Values are expressed per vehicle and are means ± SEM. Asterisk indicates a significant difference relative to each day vehicle ($p<0.05$).

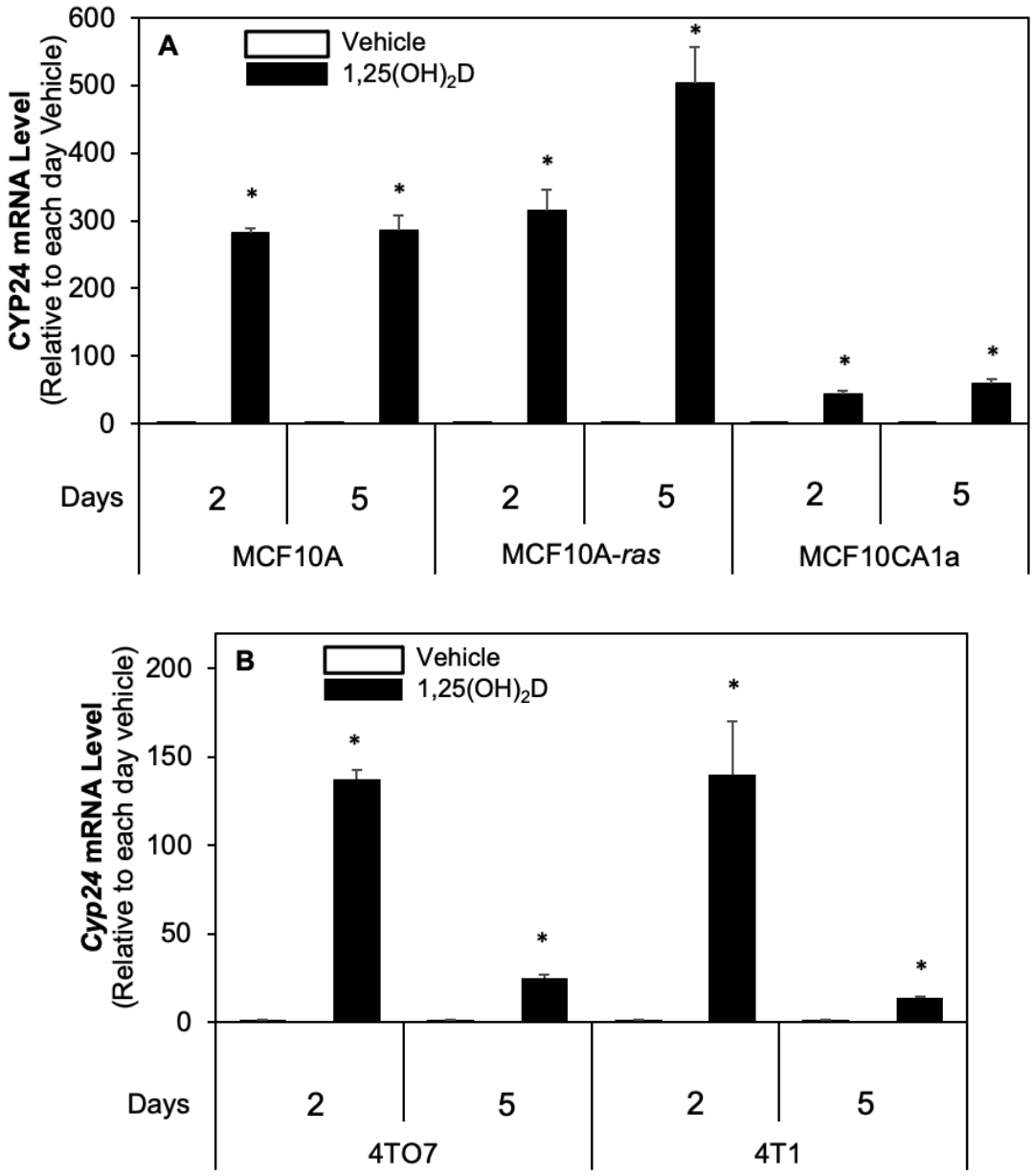

Figure 4.3 Effect of $1,25(OH)_2D$ on VDR Transcriptional Activity in Human and Murine Breast Cancer Cells.

VDR transcriptional activity was measured with CYP24 mRNA level in (A) human breast cancer cells MCF10A series and (B) murine breast cancer cells 4TO7 and 4T1 by reverse-transcription PCR and normalized to 18S. Values are expressed per vehicle and are means ± SEM. Asterisk indicates a significant difference relative to each day vehicle ($p<0.05$).

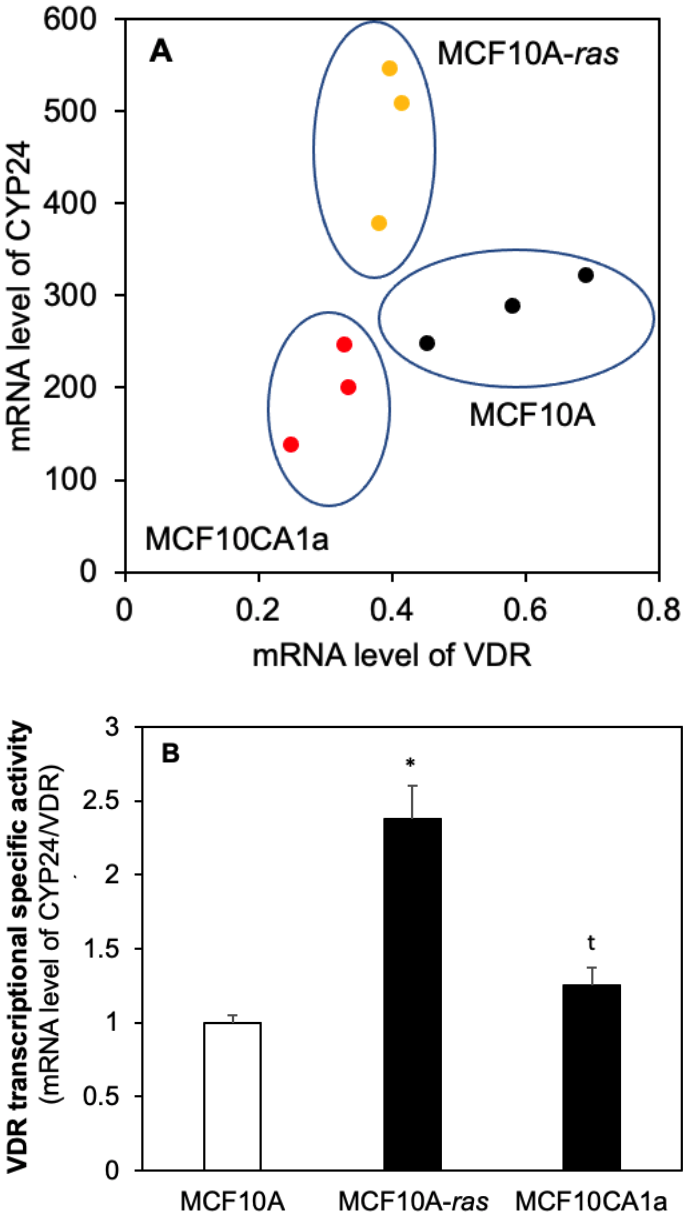

Figure 4.4 VDR Transcriptional Specific Activity During Cancer Progression in Human Breast Cancer Cell.

(A) Each sample following 1,25$(OH)_2$D treatment is plotted as VDR mRNA level versus CYP24 mRNA level. (B) The ratios of CYP24/VDR mRNA level following 1,25$(OH)_2$D treatment in each cell line were used to calculate VDR transcriptional specific activity. Asterisk indicates a significant difference relative to MCF10A. t indicates trend toward increases with p-value 0.06.

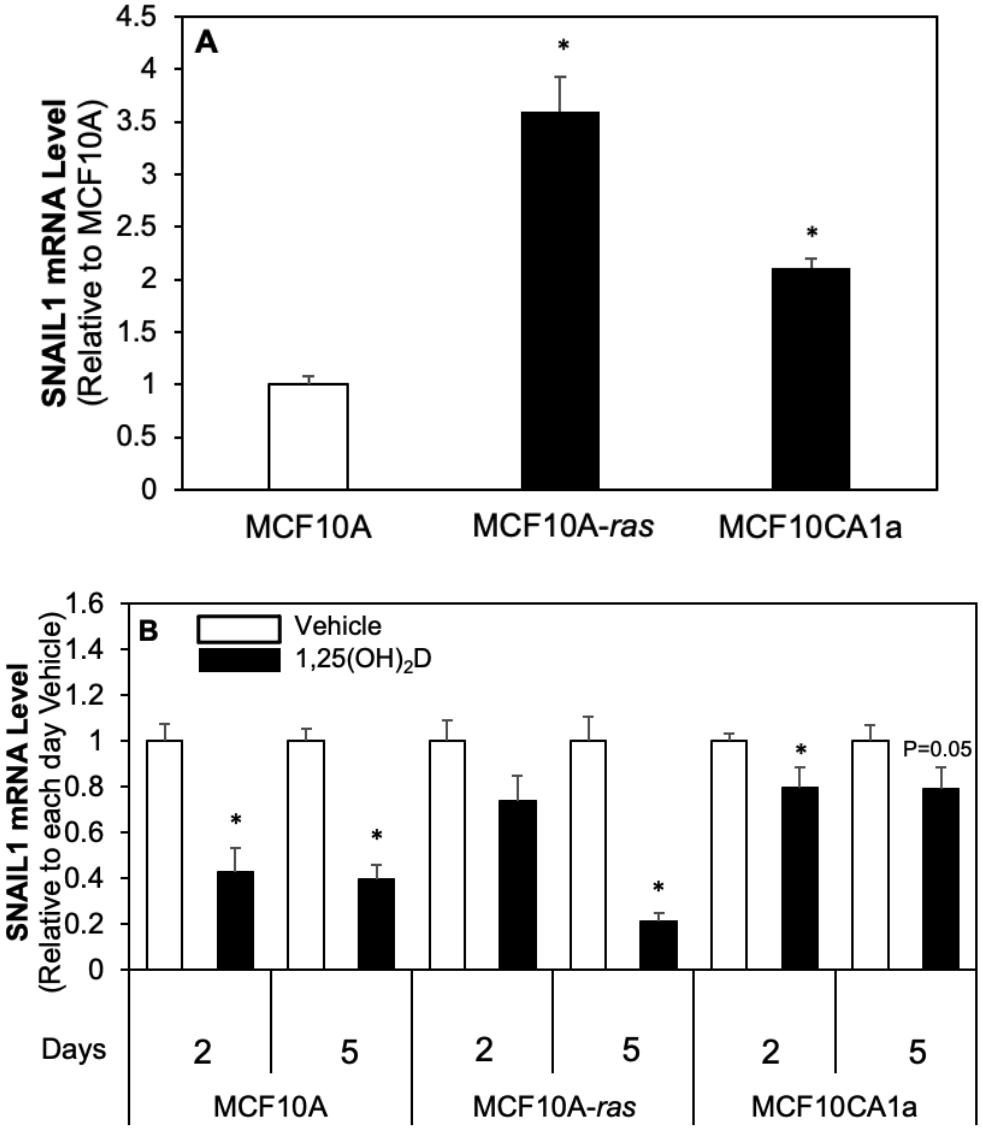

Figure 4.5 Effect of $1,25(OH)_2D$ on SNAIL1 mRNA Level in Human and Murine Breast Cancer Cells.

SNAIL1 mRNA level was measured in human breast cancer cells MCF10A series by reverse-transcription PCR and normalized to 18S. Values are expressed per MCF10A and are means ± SEM. Asterisk indicates a significant difference relative to (A) MCF10A or (B) each day vehicle ($p<0.05$).

4.5 Discussion

VDR plays crucial roles in mediating the intracellular signaling of the active metabolite of vitamin D, 1,25$(OH)_2$D. VDR is expressed in many different organs, including breast tissue [21], implicating the role of vitamin D through VDR in breast tissues. In addition, Yao *et al.,* found that 25(OH)D serum level was lower in premenopausal women with higher stage, estrogen receptor negative, and triple negative breast cancer [22], suggesting vitamin D may be a potential cancer preventive agent in premenopausal women. Furthermore, Huss *et al.,* show that high VDR level in invasive breast tumors is a prognostic factor of breast cancer as there is association between a high VDR level and a low risk of breast cancer death with favorable prognostic factors [23]. Additionally, one *in vivo* study shows that knockdown of VDR in breast cancer cells promotes bone metastasis [24], highlighting an important role of VDR in breast cancer metastasis. Previous literature suggests that VDR level is inversely related with more aggressive breast cancer in a breast cancer patient population [8], with the implication that vitamin D will therefore not have an impact to prevent metastasis. However, the impact of 1,25$(OH)_2$D on VDR level to improve responsiveness of metastatic cells has not been previously investigated.

In the current studies, we utilized MCF10A series, which are derived from a common genetic background of triple negative breast cancer as well as the murine 4TO7 and 4T1, allowing us to investigate responses to 1,25$(OH)_2$D during the progression of human breast cancer from untransformed to a late stage of breast cancer. We showed that the constitutive level of VDR was reduced in metastatic MCF10CA1a cells compared to untransformed MCF10A and in the early progression model of MCF10A-*ras* cells. In addition, metastatic murine 4T1 cells had lower *Vdr* mRNA level compared to non-metastatic 4TO7 cells [16]. Similar to the previous literature, our results demonstrate that VDR level was reduced in metastatic cells compare to non-metastatic cell lines. However, 1,25$(OH)_2$D regulation of VDR level in breast cancer cells varied across progression, with a decrease in level in early stages, and an increase in level in metastatic cells. These results suggest VDR level via 1,25$(OH)_2$D treatment varies depending on breast cancer progression and metastatic status. Our results are inconsistent with previous literature that treatment with the vitamin D gemini analog increases VDR protein level in both MCF10A-*ras* and MCF10CA1a cells [25]. This discrepancy may be due to different treatment times and the use of the analog treatment as it is reported that the gemini analog has a more potent effect compared to 1,25$(OH)_2$D and the cells were treated with gemini analog for only 24

hours. One explanation for the decrease in level in the cells representing the early stages of breast cancer may be due to a potential early increase in the level of CYP24, whose enzyme product will target $1,25(OH)_2D$ for degradation and reduce the impact of the metabolite and potentially lead to a decrease in level.

In addition, $1,25(OH)_2D$ treatment increases CYP24 mRNA level in all cell lines, indicating VDR transcriptional activity is increased in these cell lines. This is consistent with previous literature that CYP24 mRNA level is induced in colon cancer cell lines [26]. Even though there is a robust increase in CYP24 mRNA level with $1,25(OH)_2D$ treatment for five days in MCF10CA1a, the increase (44 folds) was lower than MCF10A (285 folds) and MCF10A-*ras* (502 folds), suggesting the less responsiveness to $1,25(OH)_2D$ compared to MCF10A and MCF10A-*ras* cell lines, potentially due to lower level of the VDR. The ratios of CYP24/VDR mRNA level with $1,25(OH)_2D$ treatment in MCF10A series cell lines were calculated to evaluate VDR transcriptional specific activity. Interestingly, the ratio of CYP24/VDR mRNA level in MCF10CA1a trended towards an increase ($p=0.06$) with $1,25(OH)_2$ treatment, suggesting VDR transcriptional specific activity calculated by mRNA level of CYP24 per VDR is increased even though the basal VDR mRNA level is significantly lower in MCF10CA1a. Cumulatively, our results demonstrate that $1,25(OH)_2D$ mediates an increase in VDR level, leading to a substantial increase in transcriptional activity which has important implications suggesting that vitamin D may be an effective anti-metastatic compound in breast cancer.

Emerging evidence suggests that SNAIL1 may be related to loss of VDR as SNAIL1 has been shown to repress VDR level in colon cancer [20]. Interestingly, in contrast to our hypothesis, MCF10A-*ras* cells have the highest and MCF10A cells had the lowest SNAIL1 mRNA level. Therefore, these results do not support that, in MCF10A series breast cancer cell lines, SNAIL1 downregulates VDR level during breast tumor progression. However, $1,25(OH)_2D$ decreased SNAIL1 mRNA level in MCF10A and MCF10A-*ras* cells and MCF10CA1a cells, suggesting $1,25(OH)_2D$ represses SNAIL1 transcriptionally. Thus, the MCF10A series and colon cancer cell line likely have different mechanisms that regulate VDR level.

In summary, these findings present a novel effect of $1,25(OH)_2D$ to increase VDR level in metastatic breast cancer cells. These results contribute to a growing body of evidence supporting an inhibitory role of vitamin D in metastatic breast cancer through $1,25(OH)_2D$

upregulation of VDR level and activity. Therefore, vitamin D supplementation may serve as an effective agent to prevent metastatic breast cancer.

4.6 Acknowledgements

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5. CONCLUSIONS AND FUTURE DIRECTIONS

It is well established that obesity plays an important role in breast cancer. This relationship is supported by studies showing that obesity is associated with increased risk of breast cancer and poor prognosis [1]. In addition, emerging evidence shows crosstalk between breast cancer cells and adipocytes that may enhance aggressiveness of breast tumors [2, 3]. Thus, it is of interest to investigate the release of factors from adipocytes that may regulate breast cancer cells. Interestingly, it is reported that the active form of vitamin D, $1,25(OH)_2D$, regulates adipose tissue physiology, including cell differentiation [4] and inflammation [5], suggesting an additional role of vitamin D in adipose tissue. Therefore, in the current studies, we have explored a novel role of $1,25(OH)_2D$ in the regulation of the release of factors from adipocytes which reduces migration of breast epithelial cells.

Adipocytes release many adipokines, including leptin which regulates appetite and energy balance. Leptin is reported to increase migration and invasion in breast cancer *in vitro* [6, 7] and *in vivo* [8, 9]. In addition, leptin has been shown to alter glucose and lipid metabolism in different tissues [10-12]. Thus, we are interested in elucidating the impact of leptin on metastatic breast cancer cell migration, with a focus on glucose and lipid metabolism.

Growing evidence suggests that vitamin D exerts a preventive effect on breast cancer progression and metastasis through the vitamin D receptor (VDR). However, a major obstacle for studying different stages of breast cancer is the limited breast cancer cell models that show breast cancer progression. Our MCF10CA series and the 4TO7 and 4T1 models provide an important opportunity to investigate different stages of breast cancer. Here, we investigated the level and activity of the VDR in breast cancer cells at different stages of progression and the response to treatment with $1,25(OH)_2D$.

5.1 Vitamin D Regulates Adipocytes to Inhibit Breast Cancer Migration

Obesity is a risk factor for cancer development including breast tumors. Evidence suggests that obese individuals have poorer survival after diagnosis compared to non-obese individuals breast cancer patients [1]. There are many mechanisms suggested for why excess

body fat increases breast cancer risk, including adipokines released from adipose tissues. Adipocytes secrete numerous adipokines including immunomodulatory and growth factors.

We demonstrated that conditioned media from mature adipocytes that have been treated with $1,25(OH)_2D$ reduced migration of metastatic breast cancer cells, using Boyden chamber transwells. Since the conditioned media was placed at the bottom of the transwells, $1,25(OH)_2D$-mediated decrease in migration was due to a reduced chemoattractive capability of conditioned media from $1,25(OH)_2D$-treated adipocytes. Chemotaxis is described as a phenomenon that the extracellular gradient of the chemical determines the direction of the movement of the cells [13]. Thus, $1,25(OH)_2D$-treated adipocytes secrete fewer chemoattractants or factors that increase chemoattraction into conditioned media or more factors that have inhibitory migratory capability to affect breast cancer migration compared to vehicle-treated adipocytes. However, there are other potential mechanisms besides chemoattractants that affect breast cancer migration.

One approach to determining a mechanism by which $1,25(OH)_2D$ treatment of adipocytes may alter migratory capability is to determine changes in epithelial-to-mesenchymal transition (EMT) following exposure to conditioned media. Since EMT is a crucial event in developing migratory and invasive potential, a first step in this investigation may be to determine the gene level of EMT markers or EMT transcription factors.

In addition to EMT markers, RNA-sequencing is another experiment that may be conducted. Conditioned media from adipocytes contain variety of factors including adipokines, growth factors, and inflammatory factors, leading to change breast cancer cell metabolism, such as energy metabolism. Thus, investigation of altered metabolism with associated genes in breast cancer cells is of interest. Together, these approaches will elucidate the potential factors that change the migratory capability in breast cancer cells.

Also, an *in vivo* study is required to further elucidate the effect of vitamin D on breast cancer cell metastasis in the obese model. Our lab has shown that dietary vitamin D inhibits breast to lung metastasis *in vivo* when breast cancer cells are orthotopically injected into mammary fat pad (unpublished data). Thus, we are interested in whether dietary vitamin D reduces breast cancer metastasis in the obese mouse model. Of course, it is the best to conduct the study with human subjects to investigate the impact of vitamin D in breast cancer metastasis in obesity. A study design that will elucidate whether vitamin D alleviates breast cancer metastasis in obese individuals is a randomized clinical trial with early breast cancer patients

given a placebo or supplemental vitamin D. However, this study is likely not feasible to conduct due to several reasons, such as ethics issue, too long period of study, the possibility of losing subjects during study, etc. A substitute design is a patient-derived xenograft *in vivo* model where breast cancer tissues from surgery patients are implanted into immunodeficient lean or obese mice with an endpoint of measuring metastasis to the lung with animals consuming a low or high vitamin D diet. We expect that dietary vitamin D decreases breast to lung metastasis in the obese model. This study will provide further insight into whether vitamin D alters breast cancer metastasis in the obese model.

We used a 10 nM concentration of $1,25(OH)_2D$, which is a supraphysiological concentration. In general, the concentration of $1,25(OH)_2D$ is in the pico-molar range in circulation [14]. However, adipose tissues express 1-α hydroxylase (CYP27B1) which converts 25(OH)D to $1,25(OH)_2D$, suggesting that $1,25(OH)_2D$ level in adipose tissues may be much higher than the known concentration in the circulation. With the high level of 25(OH)D in the circulation, the level of $1,25(OH)_2D$ can likely reach higher concentrations than the serum level, highlighting vitamin D might regulate tissues locally to affect breast cancer metastasis. Chang *et al.,* show that $1,25(OH)_2D$ decreases neutral lipid accumulation with a 10 nM concentration in 3T3-L1 adipocytes [15], suggesting that the $1,25(OH)_2D$-mediated regulation in adipose tissue may occur at this concentration when sufficient amount of precursor 25(OH)D exists. Further studies are needed to determine how much vitamin D intake as well as 25(OH)D levels are sufficient to achieve effects in adipocytes.

In addition to a discrepancy in the concentration of $1,25(OH)_2D$, various releasing factors could exert a combined effect to alter the migration of breast cancer cells. It is reported that leptin is induced by IGF-1 in MCF-7 breast cancer cells [16], suggesting a combination of various releasing factors from adipocytes might alter the migration of breast cancer cells. Thus, one factor alone may not modify breast cancer cell migration, complicating the investigation into determining how $1,25(OH)_2D$ regulation of adipocytes reduces the impact on migration of breast cancer cells.

In conclusion, our results provide support for a novel mechanism by which vitamin D may protect against breast cancer metastasis by regulating adipocyte physiology. This support will contribute to understanding an important role of vitamin D in preventing breast cancer metastasis.

5.2 Leptin Regulates Glucose and Lipid Metabolism in Breast Cancers

Leptin is a well-known hormone that regulates energy expenditure and satiety. Leptin is primarily produced from adipocytes and obese individuals generally have higher leptin levels in their blood compared to non-obese individuals. Previous evidence suggests that leptin may be involved with breast cancer progression since both leptin and Ob-R are overexpressed in breast tumors compared to non-tumor breast epithelium [17]. Leptin has an additional effect on regulating energy metabolism in various tissues [11, 18, 19]. In addition, glucose concentration affects leptin signaling in fibrosarcoma cells [20]. Thus, it is of interest to investigate the impact of leptin on energy metabolism as well as migration in metastatic breast cancer cells at a physiological level of glucose media.

Leptin exerts its effect through the leptin receptor. Previous literature reports that the leptin receptor is expressed in many breast cancer cell lines [16, 21], suggesting that leptin may have actions on breast tissues. Kumar *et al.*, show an important role of leptin receptor in ovarian cancer [22]. While leptin increases cell proliferation in HEY3 and SKOV3 ovarian cancer cells which express leptin receptor level, leptin does not alter cell proliferation in A2780 and ES2 cells, which do not express leptin receptors [22]. These results indicate that the leptin receptor is essential for cell proliferation with leptin treatment. Thus, it is of interest to test whether the leptin receptor is expressed in our cell lines. In our study, we confirmed that both 4T1 and metM-wnt^{lung} cells express detectable levels of leptin receptors by qPCR and leptin increases migration in both cell lines when cells are maintained in 5 mM glucose medium.

In addition, following leptin treatment, we show that leptin receptor level was reduced significantly in 4T1 cells. This is consistent with previous literature that a high sugar diet for 8 weeks increases serum leptin levels but decreases leptin receptors compared to standard chow diet in male Wistar rats [23]. However, a high sugar diet for 4 weeks increases serum leptin levels as well as of leptin receptors compared to standard chow diet. These results suggest the occurrence of leptin resistance at 8 weeks of sugar diet. In our studies, treatment of leptin for four days in 4T1 cells may be sufficient to induce leptin resistance and reduce the leptin sensitivity of the cells. It may be necessary to determine if the leptin receptor mRNA level is changed in one and two days of treatment of leptin to test if leptin receptor level is decreased from short period treatment or 4 days-treatment is needed to reduce leptin receptor level. However, there was no difference on leptin mRNA level in metM-wnt^{lung} cells following leptin

treatment, indicating that there is no leptin resistance in this cell line. This differential response in leptin receptor mRNA level may be due to different cell types. We have shown that leptin differentially affects mRNA level of FASN and CPT1a as well as AMPK activation, indicating that these two cell lines may have different states and regulation of energy metabolism.

In addition to the leptin receptor, investigating the downstream effects of the leptin receptor pathway is of interest to understand how leptin regulates migration, glucose and lipid metabolism in the breast cancer cells. Upon leptin binding to leptin receptor, janus kinase (JAK) is phosphorylated which allows phosphorylation of STAT3 on tyrosine residue 705 to activate JAK/STAT3 pathway. Thus, investigating the level of JAK and p-JAK as well as STAT3 and p-STAT3 allows us to understand whether JAK/STAT3 pathway is involved in leptin-mediated signaling during leptin-induced migration. In our study, STAT3 protein level was increased with leptin treatment but phospho-STAT3 (Tyr705) was not detected in metM-wnt^{lung} cells by Western blot analysis (data not shown). It is reported that the activation of STAT3 by phosphorylation at Tyr 705 contributes STAT3 activity to regulate genes level for cancer development [24]. It is probable that our experimental condition was not optimized for detecting p-STAT3 (Tyr705), or it is possible that JAK2-mediated activation of other STAT proteins, such as STAT5, is involved in leptin-induced migration. Interestingly, our results show that leptin treatment increased total STAT3 in metM-wnt^{lung} cells. Yang *et al.*, found that total STAT3 is an important transcription factor to regulate gene level in cancer [25], suggesting that increased total STAT3 is necessary to regulate genes involved in leptin-mediated increased migration. Further investigation on other STAT proteins and total STAT3 may contribute to understanding the pathways involved in leptin-induced migration in these breast cancer cell lines.

We focused on 5 mM glucose concentration medium in this study. Normally, many researchers use a 25 mM glucose level since this concentration is commonly used and allows cells sufficient nutrients including glucose. While 5 mM glucose level represents the physiological concentration in circulation, 25 mM may represent the conditions that occur in diabetes [26]. A previous study shows that glucose alters leptin signaling in human fibrosarcoma cells [20], indicating the possible importance of medium glucose concentration in the effects of leptin. We observed that leptin increases migration in 5 mM glucose medium as well as that shown in 25 mM glucose medium (data not shown). It is of interest to compare the effect of

leptin between 5 and 25 mM glucose media on breast cancer cell migration as well as energy metabolism.

In our studies, we employed the murine 4T1 and metM-wnt^{lung} metastatic breast cancer cell lines to examine the impact of leptin on migration as well as energy metabolism in breast cancer cells. Given the possible discrepancies between mouse and human breast cancer cells, it will be important to investigate the impact of leptin in human breast cancer cells. Possible choices may be MCF10A-*ras*, MCF10CA1a and MDA-MB-231 cell lines for triple-negative breast cancer cell lines and compare the results with murine cell lines. In addition, investing the effect of leptin in MCF10A-*ras* and MCF10CA1a may lead to elucidating the response to leptin in early breast cancer compared to metastatic breast cancer cell lines. Further investigation in human cell lines provides a better understanding of the effect of leptin in breast cancer cells.

In addition to human breast cancer cell lines, the impact of leptin on breast cancer should be investigated *in vivo*. One approach is to investigate whether leptin affects breast cancer metastasis as well as total fat mass in breast tissues. In this approach, breast cancer cells are orthotopically injected into the mammary fat pad of mice and breast to lung metastasis and total fat mass in breast tissues examined to determine changes with leptin administration. In addition, we might measure how glucose and fatty acids are metabolized in *in vivo* models by assessing glucose uptake and fatty acid oxidation. However, there is a limitation on this study design since leptin is generally secreted at higher levels in obese conditions and thus this model does not represent the obese model. To examine the effect of the endocrine leptin hormone on breast cancer metastasis *in vivo*, we might employ the leptin-deficient ob/ob mice, high-fat diet mice, and control wild type C57B1/6J mice models with an injection of tumor cancer cells into mammary fat pad. It is expected that high-fat diet mice would have the highest leptin serum level and significantly higher breast to lung metastasis among all groups. Leptin-deficient ob/ob mice is expected to have lower metastasis than control mice, and leptin administration in leptin-deficient ob/ob mice would increase metastasis. These expected results would highlight the role of leptin on breast cancer metastasis *in vivo*.

In conclusion, our results support evidence for a novel mechanism by which leptin differentially regulates energy metabolism in breast cancer cells during leptin-induced migration. These results will provide new insight into understanding of leptin on breast cancer migration and energy metabolism.

5.3 Vitamin D Regulates VDR During Breast Cancer Progression

Due to the importance of vitamin D to prevent cancer progression via the VDR that is suggested by previous studies, it is essential to understand vitamin D regulation of VDR in breast cancer cells. We measured the basal level of VDR level and the effect of $1,25(OH)_2D$ on VDR level and activity in different stages of breast cancer cells. Our results demonstrated that the constitutive level of VDR was reduced in metastatic breast cancer cells. In addition, $1,25(OH)_2D$ increased VDR mRNA level only in metastatic human MCF10CA1a and murine 4T1 cell lines, suggesting that dietary vitamin D may exert inhibitory effects through upregulation of VDR level and activity in metastatic breast cancer.

We have demonstrated that VDR level was reduced in metastatic breast cells, which is consistent with other results [28]. Huss *et al.*, also show that VDR level in invasive breast tumors is associated with a prognosis or breast cancer [29]. Additionally, VDR is expressed at higher levels in benign lesions compared to invasive breast tumors [30]. Thus, we also are interested in why VDR level is reduced during tumor. If we demonstrate the causal relationship between VDR and tumor progression, it would be beneficial to open new insight into preventing breast cancer.

The limitation of our research is that we only conducted an *in vitro* study on changes in VDR level during tumor progression. Thus, *in vivo* and human studies would help us confirm our results. One approach is to conduct an *in vivo* preclinical study to investigate whether the late stage of breast cancer has lower VDR level compared to the early stage of breast cancer. An example of a study design is to employ early breast cancer model MCF10A-*ras* and metastatic breast cancer model MCF10CA1a cells which are engrafted into mammary fad pad and mice are fed with either control or a high vitamin D diet. While MCF10A-*ras* cells would form primary tumors in breast tissues, MCF10CA1a cells would form primary tumors as well as secondary tumors in the metastatic site including in the lung. It is expected that MCF10CA1a engrafted mice have more primary tumors compared to MCF10A-*ras* engrafted mice. The metastatic tumors are only shown in mice with MCF10CA1a. VDR level is expected to be reduced in the metastatic lesion with MCF10CA1a compared to primary site. Additionally, it is expected that dietary vitamin D would decrease primary tumors in both cell lines, and metastatic tumors with MCF10CA1a. These results would suggest that even though low VDR is expressed in the metastatic lesion, the tumor is responsive to dietary vitamin D, highlighting the possibility that vitamin D is capable of regulating the metastatic lesion.

In addition, it is of interest to elucidate the association between VDR level and serum 25(OH)D level, and the effect of dietary vitamin D on breast cancer prognosis depending on different VDR levels. An example of a study design is to identify breast cancer patients subjects who have low and high VDR level in breast cancer tissues and investigate whether vitamin D supplementation affects their prognosis. However, there are various hurdles to conduct a clinical study of this design since the length of the study may be long in order to observe their prognosis and retention may be difficult. Variable exposure to sunlight over seasons also makes this study unmanageable. Despite its difficulty to conduct, a clinical study in different stages of breast cancer patients with vitamin D supplementation would elucidate the effect of dietary vitamin D on breast cancer prognosis.

The responsiveness of the VDR to $1,25(OH)_2D$ is determined by measuring CYP24 mRNA level, and CYP24 level is increased in our studies with 10 nM of $1,25(OH)_2D$ treatment. As mentioned in Chapter 5.1, we used 10 nM of $1,25(OH)_2D$, which is supra-physiological levels, although intracellular concentrations may be higher than serum levels due to local production. Thus, it is of interest to measure CYP24 mRNA level with a lower concentration which may represent a physiological concentration of $1,25(OH)_2D$.

The cells are treated with the active form $1,25(OH)_2D$ in our study, however, previous studies observed the level of 1-α hydroxylase (CYP27B1) in adipocytes and breast epithelial cells [31]. There is a possibility that CYP27 level might be different during breast tumor progression, leading to the yield of different concentrations of $1,25(OH)_2D$ in breast tissues with the same amount of vitamin D and subsequent 25(OH)D serum concentrations. Therefore, it is critical to investigate how CYP27 and VDR are regulated during breast tumor progression with 25(OH)D treatment in order to determine how much vitamin D supplementation is necessary to achieve the effects noted in the cell studies.

In our study, VDR mRNA level was increased in metastatic MCF10CA1a and 4T1 cells with 5 days treatment of 10 nM of $1,25(OH)_2D$. Previous evidence shows that the VDR mRNA and protein level in response to $1,25(OH)_2D$ varies between cell types. For example, $1,25(OH)_2D$ induces VDR protein level in SUM-229PE breast cancer cells [32]. In the same study, $1,25(OH)_2D$ does not induce VDR protein level in T-47D breast cancer cells [32], indicating that $1,25(OH)_2D$ regulation on VDR level is cell type-specific.

While there are still several questions to be answered, our study provides information regarding the level and activity of VDR during breast cancer progression with $1,25(OH)_2D$ treatment. This understanding may contribute to setting dietary vitamin D recommendations for inhibiting metastatic breast cancer.

5.4 Conclusions

Several factors, including obesity, affect an increased risk of breast cancer incidence and metastasis. Many studies have shown that vitamin D is related to a decreased risk of breast cancer. In our current study, we have demonstrated that $1,25(OH)_2D$ regulated mature adipocyte release of factors that inhibit or reduce release of factors that promote cell migration in breast epithelial cells. Our results also showed that $1,25(OH)_2D$ decreased leptin release from mature adipocytes. An investigation into leptin's effect on metastatic breast cancer cells demonstrated that leptin differentially regulated energy metabolism in both murine metastatic 4T1 and metM-wnt^{lung} breast cancer cells during leptin-induced migration.

We also demonstrated that VDR level was reduced in metastatic breast cancer cells compared to non-metastatic breast cells. However, $1,25(OH)_2D$ induced an increase in VDR transcriptional activity and VDR level in metastatic breast cancer cells, highlighting that dietary vitamin D may exert inhibitory effects in metastatic breast cancers through upregulation of VDR level. In conclusion, our results provide new insights suggesting that vitamin D contributes to the prevention of breast cancer metastasis, and leptin affects an increase in breast cancer metastasis.

5.5 References

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