Dysregulation of Phospholipase D (PLD) isoforms increases breast cancer cell invasion

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By:

Kristen Fite
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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Kristen Fite ENTITLED Dysregulation of Phospholipase D (PLD) isoforms increases breast cancer cell invasion BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT:

Dysregulation of Phospholipase D (PLD) isoforms increases breast cancer cell invasion.

Breast cancer remains the second most prevalent cancer among women in the U.S. with metastatic breast cancer having the worst prognosis. A rapidly proliferating tumor under various stressors will promote phenotypic cellular changes, known as epithelial-to-mesenchymal transition (EMT), which allows cells to begin to invade surrounding tissue, enter the circulatory system, and eventually seed a distant metastatic site. The phospholipase D (PLD) enzymes are critical regulators of cell signaling pathways necessary for cell migration. While the importance of PLD enzymes in cancer cell invasion is well known, clinically applicable methods of PLD inhibition are not yet available.

The best-studied isoforms are the ‘classical’ PLD1 and PLD2 membrane lipases that hydrolyze phosphatidylcholine (PC) to free choline and phosphatidic acid (PA). PA acts as a secondary messenger activating numerous pathways leading to cell growth and proliferation, vesicle trafficking, and cell migration. PLD1 and PLD2 are upregulated in several cancers including breast and, furthermore, promote tumorigenesis and disease progression. However, the specific molecular mechanism that regulates overexpression of PLD1 and PLD2 in cancer remains unclear. I investigated post-transcriptional regulation of the classical PLD isoforms by microRNA (miRNA) in cancer cell invasion and in stress (nutrient starvation) leading to EMT. MicroRNA are currently widely investigated as potential therapeutic agents involved in a myriad of diseases. Naturally produced in the body, miRNA are attractive therapeutic molecules based on their small size, surprising stability, and potent regulatory capabilities.

A repertoire of four microRNAs present in non-cancerous breast cells that are down-regulated in invasive breast cancer cell were found and demonstration of their regulation of PLD1 or PLD2 mRNA was done by luciferase reporter assay. Exogenous addition of these microRNAs to invasive breast cancer cells reduced cell invasion. Additionally, initial nutrient starvation increased classical PLD protein levels,
concomitant with cell invasion. With prolonged starvation, PLD-targeting microRNA were upregulated and functioned in a negative feedback loop to downregulate PLD protein expression.

While much is known about the PLD1 and PLD2 isoforms, the other mammalian PLD isoforms (PLD3, PLD4, and PLD6) are vastly less studied. Interestingly, PLD6 has recently been found to be overexpressed in breast cancer tumors, however, further studies investigating the role of PLD6 in cancer progression have not been published. PLD6 localizes to the mitochondrial outer membrane and uses cardiolipin as a substrate to produce PA. This reaction promotes mitochondrial fusion, thereby inhibiting apoptosis, while increasing oxidative phosphorylation, both of which are critical to cancer progression. While PLD1 and PLD2 are ubiquitously expressed, PLD6 is expressed at lower basal levels in most tissues, making PLD6 an attractive therapeutic target, when upregulated in cancer, with potentially less adverse side effects.

The protein and gene expression of PLD6 are upregulated in invasive breast cancer cells compared to non-cancerous breast cells. PLD6 subcellular localization changed in response to invasion-promoting factors, nutrient starvation and EGF signaling. Furthermore, this is the first report showing that modulating PLD6 expression influences cell invasion of aggressive breast cancer cells.

Overall, I show that increased expression of PLD1, PLD2, and PLD6 in invasive breast cancer cells leads to higher cell invasion. A novel molecular regulation of classical PLD by a repertoire of microRNA was demonstrated. Additionally, PLD6 increases breast cancer cell invasion of certain breast cancer cell lines. This work highlights the importance that targeting PLD family members could have in breast cancer therapeutics.
# Table of Contents

## I. CHAPTER 1. INTRODUCTION

- Breast Cancer .................................................................................................................. 1
- Epithelial-To-Mesenchymal Transition and Metastasis .................................................. 2
- Phospholipase D ................................................................................................................ 3
- Phosphatidic Acid ............................................................................................................. 4
- Phospholipase D6 .............................................................................................................. 5
- Mitochondria and cancer ................................................................................................. 6
- MicroRNA .......................................................................................................................... 7

## II. CHAPTER 2. MATERIALS AND METHODS

- Reagents ............................................................................................................................ 14
- Cell Culture ....................................................................................................................... 14
- Cell Starvation .................................................................................................................. 15
- Transfection of Cells ....................................................................................................... 15
- PLD Activity Assay ......................................................................................................... 15
- Western Blotting ............................................................................................................... 15
- Subcellular Fractionation ................................................................................................. 16
- qRT-PCR ............................................................................................................................. 16
- qRT-PCR for miRNA Expression ...................................................................................... 17
- Immunofluorescence Microscopy ..................................................................................... 18
- Luciferase Reporter Assay ............................................................................................... 18
- Cell Invasion Assay .......................................................................................................... 18
- Identification of miRNA .................................................................................................... 19
- Xenotransplant of SCID mice and metastatic breast cancer models ............................ 20
- Statistical Analyses .......................................................................................................... 20

## III. CHAPTER 3. AIM 1: To investigate the molecular regulation of classical PLD isoforms by microRNA in breast cancer cell invasion

- Subaim 1A: To characterize PLD1 and PLD2 expression and signaling in breast cancer cell lines ......................................................................................................................... 22
- Subaim 1B: To identify microRNA regulation of PLD1 and PLD2 in breast cells .......... 33
Subaim 1C: To assess the effect of microRNA regulation of PLD on breast cancer cell invasion………………………………………………………….46

IV. CHAPTER 4. AIM 2: To study the molecular regulation of classical PLD isoforms by microRNA in breast cancer cells subjected to nutrient starvation….55

Subaim 2A: To evaluate PLD1 and PLD2 expression in breast cancer cells subjected to stress…………………………………………………………..56

Subaim 2B: To study microRNA regulation of PLD1 and PLD2 in breast cancer cells under nutrient starvation…………………………………61

V. CHAPTER 5. AIM 3: To determine the effect of upregulated PLD6 on breast cancer cell invasion………………………………………………………69

Subaim 3A: To establish the expression of PLD6 in breast cancer cells…………………………………………………………………………………70

Subaim 3B: To determine the effect of starvation and EGF signaling on PLD6 expression and subcellular localization on breast cancer cell invasion…………………………………………………………………78

VI. CHAPTER 6. DISCUSSION……………………………………………………………92

VII. REFERENCES……………………………………………………………………104
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Human breast cell lines</td>
<td>10</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Characteristics of mammalian phospholipase D isoforms</td>
<td>11</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Chemical reaction of PLD lipase activity</td>
<td>12</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Mechanism of action of microRNA</td>
<td>13</td>
</tr>
<tr>
<td>Figure 5</td>
<td>PLD1 and PLD2 protein expression in multiple breast cell lines</td>
<td>26</td>
</tr>
<tr>
<td>Figure 6</td>
<td>$pld1$ and $pld2$ transcript levels in multiple breast cell lines</td>
<td>27</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Basal PLD enzymatic activity in multiple breast cell lines</td>
<td>28</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Cell invasion of human breast cell lines</td>
<td>29</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Effect of EGF on $pld2$ gene expression and PLD lipase activity</td>
<td>30</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Effect of inhibiting PLD lipase activity on cell invasion</td>
<td>32</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Predicted binding sites of several miRNA on PLD 3’UTR</td>
<td>38</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Endogenous expression of mature miRNA in human breast cells</td>
<td>39</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Exogenous miRNA reduces PLD protein expression</td>
<td>40</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Immunofluorescence of PLD protein after miRNA transfection</td>
<td>41</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Exogenous miRNA decreased PLD lipase activity</td>
<td>43</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Successful transfection of miRNA in human breast cancer cells</td>
<td>44</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Luciferase reporter assay supports miRNA binding to PLD mRNA</td>
<td>45</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Exogenous miRNA decrease breast cancer cell invasion</td>
<td>49</td>
</tr>
<tr>
<td>Figure 19</td>
<td>miRNA “rescue” cell invasive phenotype after PLD overexpression</td>
<td>50</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Pre- and post-EMT markers in human breast cancer cell lines</td>
<td>51</td>
</tr>
<tr>
<td>Figure 21</td>
<td>miR-3619 expression in the presence of pre- and post-EMT markers</td>
<td>52</td>
</tr>
<tr>
<td>Figure 22</td>
<td>miR expression after silencing EMT markers in human breast cancer cells</td>
<td>53</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Proposed model of microRNA activity on PLD 3’UTR inhibiting cell invasion</td>
<td>54</td>
</tr>
<tr>
<td>Figure 24</td>
<td>$pld1$ and $pld2$ mRNA levels after starvation in multiple human breast cell lines, cancerous and non-cancerous</td>
<td>58</td>
</tr>
<tr>
<td>Figure 25</td>
<td>PLD protein expression and lipase activity after starvation</td>
<td>59</td>
</tr>
<tr>
<td>Figure 26</td>
<td>Cell invasion of MDA-MB-231 cells after increasing starvation</td>
<td>60</td>
</tr>
<tr>
<td>Figure 27</td>
<td>miRNA expression after starvation in breast cancer cells</td>
<td>64</td>
</tr>
</tbody>
</table>
Figure 28. miRNA expression after starvation in non-cancerous breast cells……..65
Figure 29. Luciferase assay shows miRNA inhibit increase in protein after starvation.................................................................66
Figure 30. miRNA expression in breast cancer cells after stimulation with PA……..67
Figure 31. Model depicting the proposed effect of starvation on microRNA in cancer cells.................................................................68
Figure 32. PLD6 expression in breast cancer cells..................................................73
Figure 33. Fluorescence microscopy of PLD6 and MitoTracker in breast cells……..74
Figure 34. PLD6 expression in xenotransplanted mammary tumors..................75
Figure 35. H&E staining of xenotransplanted mammary tumors.......................76
Figure 36. PLD6 levels in MDA-MB-231 cell lines stably transfected with control and PLD2 shRNAs......................................................77
Figure 37. PLD6 expression and subcellular localization after starvation and EGF stimulation.........................................................83
Figure 38. Human PLD6 amino acid sequence..................................................85
Figure 39. Nuclear fractionation and measurement of PLD6 protein expression.....86
Figure 40. Confocal microscopy images of endogenous PLD6 in EGF-treated MDA-MB-231 cells..........................................................87
Figure 41. PLD6 expression in MDA-MB-231 cells............................................89
Figure 42. Cell invasion of cells overexpressing or silencing PLD6...............90
Figure 43. Successful overexpression and silencing of PLD6.........................91
List of Tables

Table 1. Chemical inhibitors of PLD lipase activity........................................31
Table 2. Conserved sequences of PLD 3’UTR-targeting miRNA.....................37
<table>
<thead>
<tr>
<th>Abbreviations:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-DAG</td>
</tr>
<tr>
<td>CL</td>
</tr>
<tr>
<td>DAG</td>
</tr>
<tr>
<td>DGK</td>
</tr>
<tr>
<td>Drp 1</td>
</tr>
<tr>
<td>EMT</td>
</tr>
<tr>
<td>FIPI</td>
</tr>
<tr>
<td>GFP</td>
</tr>
<tr>
<td>H&amp;E</td>
</tr>
<tr>
<td>HMEC</td>
</tr>
<tr>
<td>IHC</td>
</tr>
<tr>
<td>LPAAT</td>
</tr>
<tr>
<td>MCF-7</td>
</tr>
<tr>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>miR, miRNA</td>
</tr>
<tr>
<td>mTOR(C)</td>
</tr>
<tr>
<td>NFOT</td>
</tr>
<tr>
<td>OXPHOS</td>
</tr>
<tr>
<td>PA</td>
</tr>
<tr>
<td>PAGE</td>
</tr>
<tr>
<td>PC</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
</tr>
<tr>
<td>PLC</td>
</tr>
<tr>
<td>PLD</td>
</tr>
<tr>
<td>RFP</td>
</tr>
<tr>
<td>qRT-PCR</td>
</tr>
<tr>
<td>SDS</td>
</tr>
<tr>
<td>Abbreviation</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>SEM</td>
</tr>
<tr>
<td>UTR</td>
</tr>
<tr>
<td>WT</td>
</tr>
</tbody>
</table>
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**CHAPTER 1. INTRODUCTION**

**Breast Cancer**

Breast cancer is a leading cause of cancer related death in women worldwide (1-3). It is estimated that one in eight women in the U.S. will be diagnosed with breast cancer during their lifetime. Each year, half a million women lose their battle with breast cancer worldwide (4). While breast cancer incidence rates are relatively stable in the U.S., rates are rising in Europe, Latin America, Asia, and Africa (5). This is likely due to an increase in Western lifestyle including consumption of processed foods, physical inactivity, as well as changes in reproductive patterns such as delayed childbearing and having fewer children (5). Breast carcinoma can be classified generally as either carcinoma *in situ* or invasive carcinoma. Carcinoma *in situ* remains contained within the tumor boundaries, whereas invasive carcinoma has invaded the surrounding tissue with increased disease spread corresponding to an increase in tumor stage (6,7). Invasive carcinoma is further classified by histology with the most common being No-special-type carcinoma (ductal). Ductal carcinomas are again further categorized by gene expression profiling and the five major types include Luminal A, Luminal B, Normal breast-like, Basal-like, and HER2 Positive. These five subtypes have clinical importance in their correlation with prognosis and response to therapy (6-8). Recent decades have produced tremendous improvements in the detection and treatment of breast cancer including increased public awareness as well as advancements in mammography (5). Remaining challenges in successful treatment of breast cancer include the treatment of triple negative breast cancer as well as drug resistance (5). Triple negative breast cancer is so named because cancer cells do not express the estrogen, progesterone, or HER2 receptors. Targeting these receptors in other forms of breast cancer has significant clinical success and the lack of these receptors on triple negative cancer cells limits the therapeutic options for these patients.

This study was primarily conducted in three different cells lines depicted in Figure 1. Two breast cancer cell lines were used including the high-invasive MDA-MB-231 and the low-invasive MCF-7 ductal breast carcinoma cell lines. MDA-MB-231 cells are also classified as triple negative and claudin-low. Triple negative breast cancers generally have the worst prognosis and are the most difficult to treat clinically (5,9).
Claudin-low refers to low levels of cell-cell junction proteins claudin and E-cadherin. This classification is usually seen in triple negative breast cancers and represents an invasive phenotype (5). In contrast, MCF-7 cells are classified as estrogen receptor positive (ER+) and luminal subtype. This class of breast cancer is typically less aggressive and is responsive to estrogen-targeted therapy (5). The non-cancerous HMEC primary cell line was used as a control for non-cancerous human mammary epithelial cells.

**Epithelial-to-Mesenchymal Transition (EMT) and Metastasis** The majority of breast cancer mortalities (>90%) are related to metastatic disease (10). Similar to other cancers, breast cancer metastasizes to distant organs including lungs (11,12), liver (12), bone (13), and brain (14). It was once thought that metastasis was a result of only prolonged disease progression, meaning a primary tumor grew to a certain size or duration of time and cells eventually metastasized. However, studies have documented cancer cell invasion and micrometastasis formation at very early stages of disease progression (15). Thus, understanding molecular mechanisms of metastasis is ever more pertinent. Metastasis begins in a growing tumor where cells are subjected to stressors, such as nutrient starvation and hypoxia if angiogenesis doesn’t match tumor growth. Tumor cells under stress enter a series of genetic and epigenetic changes called epithelial-to-mesenchymal transition (EMT) (16-18), wherein the cell adopts a less-differentiated and more invasive phenotype (19,20). These cells are able to invade the surrounding tissue eventually entering the circulatory system (blood or lymph circulation) and seed a distant site to form a metastatic tumor (19-21). Invasive cancer remains difficult to treat, fighting disseminated cells throughout the body. In addition, invasive cells have different phenotypes with a myriad of signaling pathways activated compared to the primary tumor cells (21).

EMT is a complex process, which requires a reprogramming of a cell. During development, EMT and its reverse process, mesenchymal-to-epithelial (MET), are critical in developing tissues (22). Cells undergoing EMT lose cell polarity as well as cell-cell adhesion and thus gain a more mesenchymal, or less differentiated, phenotype along with greater potential to migrate and invade surrounding tissue and additionally increased anti-apoptotic potential (10,16-18). EMT involves genetic and epigenetic changes and several
transcription factors involved include Slug, Snail, Twist, and ZEB1/2 (16,22). Characteristic changes include the downregulation of cell adhesion proteins such as E-cadherin, concomitant with the upregulation of mobility proteins such as vimentin. Thus, understanding the signaling pathways involved in invasive breast cancer cells remains critical to developing novel therapeutics.

**Phospholipase D** The phospholipase D (PLD) enzyme family contains several mammalian isoforms all with a characteristic HKD motif in their catalytic site (23-27). Figure 2 summarizes the mammalian isoforms and their features. The classical PLD mammalian isoforms, known as PLD1 and PLD2, are the best characterized. They are ubiquitously expressed and are involved in numerous signaling pathways, particularly through their catalytic byproduct phosphatidic acid (PA) (23-27). Each isoform contains two HKD-containing catalytic domains that function together to hydrolyze the membrane lipid phosphatidylcholine into free choline and phosphatidic acid. The PLD enzymatic reaction is illustrated in Figure 3A. PA, is a negatively charged lipid and accumulation of it results in negative membrane curvature (23-27). This negative curvature promotes vesicle trafficking and lamellipodia formation in mobile cells. PLD2 localizes predominantly to the plasma membrane, while PLD1 localizes to cytoplasmic membranes (26,27).

PLD1 and PLD2 are vital enzymes in many tissue types, including healthy breast tissue, and their enzymatic products exert influence over an abundance of cellular pathways, including cytoskeletal rearrangement, membrane trafficking, and cell migration (26). The role of classical PLD in these signaling events also makes it integral to cancer cell metastasis (28-30). PLD plays an important role in maintaining the integrity of cellular membranes. Furthermore, increased PLD protein expression and enzymatic activity have been reported in multiple cancer types, including breast cancer (30-33) and K-Ras driven cancers (34-36). Additionally, classical PLD has been implicated in cellular signals that suppress apoptosis and contribute to cancer cell survival (27,37,38). Elevated PLD activity leads to activation of mammalian target of rapamycin (mTOR), a survival signal often hyperactivated in cancer (39,40) and also subdues the tumor suppressors p53 and protein phosphatase 2A (38). Zheng et al. published a model for enhanced survival
and migration signals in the developing tumor (41). In a developing tumor mass, cells inside the mass were subjected to hypoxia and nutrient and growth factor deprivation. These stressed cells responded by elevating PLD protein levels and survived presumably by gaining the ability to migrate. While the classical PLD isoforms are involved in cancer progression, little is known about their protein regulation in healthy and cancerous tissues.

Non-classical mammalian PLD isoforms include PLD3, 4, and 6, and are vastly understudied (42). PLD3 and PLD4 are transmembrane proteins that localize to the endoplasmic reticulum and Golgi, respectively (42). PLD6 localizes to the outer mitochondrial membrane where it uses cardiolipin as a substrate to produce phosphatidic acid, which is discussed further below. A mammalian PLD5 isoform has not been described.

**Phosphatidic Acid** PA is a critical phospholipid within the cell. PA serves as the substrate for all membrane phospholipid biosynthesis, necessary to maintain membrane integrity as well as for cell growth and division (43,44). PA is also an important secondary messenger signal activating notable pathways including mTOR (43,45). Numerous proteins bind to PA and PA thereby functions to recruit proteins to membranes and promote cell signaling. There are three major pathways that produce PA. Lysophosphatidic acid acyltransferase (LPAAT) converts lyso-PA to PA. The PA produced from this pathway is converted to CDP-DAG (cytidine 5’-diphosphate diacylglycerol), which is used for phospholipid biosynthesis (43). Another pathway is conversion of diacylglycerol (DAG) to PA by diacylglycerol kinase (DGK). This DAG population is generated from triglyceride storage or PLC-mediated hydrolysis of PI(4,5)P₂, and thus represents a smaller proportion of PA production within the cell. Finally, PA is produced by PLD-mediated hydrolysis of PC. While the PA produced from LPAAT is predominately used for general phospholipid synthesis, the DAG and PLD pathways produce PA that becomes secondary messenger signals. PLD and PLC-DAG pathways are activated by nutrients (46,47), growth signals (48), or stress (27,49) and often produce localized PA populations, which further activate signaling within the cell.

As mentioned above, one of the major pathways activated by PA is mTOR. Both
mTORC1 and mTORC2 interact with PA, thereby stabilizing mTORC (50). mTORC activation promotes cell growth while suppression of mTORC leads to cell cycle arrest in G₁ (51,52). This is an important regulatory step within the cell cycle because mTORC senses the presence of essential amino acids and glutamine, which are necessary for protein synthesis in S phase of the cell cycle (51). Because PA stabilizes mTORC and PA is the substrate for *de novo* phospholipid biosynthesis, the PA-mTORC interaction likely functions as an additional nutrient signal (43). The availability of PA to interact with mTORC positively correlates with cell’s ability to synthesize phospholipids to generate new membranes during cell division. Furthermore, PA is also produced as a result of growth factor and nutrient signaling via PLD. Thus, PA signaling is likely important in cancer cells within a proliferating tumor.

**Phospholipase D6** Phospholipase D6 (PLD6) is also known as MitoPLD and Zucchini (Zuc). It is the smallest member of the mammalian PLD family at only 28 kDa. Along with a mitochondrial localization signal and a transmembrane domain, PLD6 contains the characteristic HKD (His-Lys-Asp) catalytic motif of the phospholipase D protein family (53-55). While PLD1 and PLD2 contain two HKD motifs, PLD6 only contains one and therefore homodimerizes with the catalytically active site at the dimer interface (53,55). The classical PLD isoforms contain PX and PH lipid-binding domains to associate with membranes while PLD6 contains a N-terminal mitochondrial localization signal, which also functions as a transmembrane domain (55). PLD6 is anchored in the outer mitochondrial membrane and functions as a lipase and endonuclease (53,55). As a lipase, it hydrolyzes the mitochondrial specific lipid cardiolipin to produce phosphatidic acid. Additionally, PLD6 is an endonuclease involved in maturation of piwi-interacting RNA (piRNA) by defining the 5’ end of piRNA and associating with the piwi-interacting protein Aubergine (53). The substrate-binding pocket is positively charged and can therefore accommodate negatively charged ssRNA or ssDNA (53,56,57). PLD6 is most abundantly expressed in the testes where its involvement in the piRNA pathway is important to spermatogenesis (53). This is where PLD6 is most studied and characterized and little is known about PLD6 in other tissues.

Some of the important cellular functions mitochondria regulate include apoptosis,
intracellular calcium signaling, and lipid synthesis and transport (55,58,59). Mitochondria are a dynamic organelle system, constantly undergoing fusion and fission. In this manner, mitochondrial size, shape, and number adjust to cellular demands, which is important for human health (55). Furthermore, mitochondrial fusion and fission allows exchange of internal RNAs, proteins, and lipids to aid in homogenizing mitochondrial content throughout the cell (60). A disruption in mitochondrial dynamics can lead to pathologies such as excess apoptosis (55), neurodegenerative disorders such as Charcot-Marie-Tooth subtype 2A (61), dominant optic atrophy (62), metabolic disorders (63), and cardiac failure (64,65). Elongated mitochondria are difficult for autophagosomes to engulf and therefore cells with many enlarged mitochondria experience severely defective mitophagy and the accumulation of damaged mitochondria (66-68). Experiments overexpressing PLD6 resulted in elongated mitochondria (54) while silencing PLD6 resulted in mitochondrial fragmentation (54,69). Furthermore, these studies implicated the catalytic activity of PLD6 in their findings. On the mitochondrial surface, PLD6 converts cardiolipin (CL) to phosphatidic acid (PA). In mitochondrial fission, the cytosolic protein dynamin-related protein 1 (Drp1) is recruited to the mitochondrial membrane where it oligomerizes and its GTPase activity leads to the constriction of the mitochondrial membrane (67,70,71). Drp1 can bind to the polar head of PA and this association inhibits the GTPase activity of Drp1 (67). In addition, Drp1 binds PLD6, which enriches the PA content in the microenvironment of mitochondrial fission machinery (67). PA is converted to DAG by the phosphatase Lipin 1 (72). PA recruits Lipin 1 to the mitochondrial membrane and thus the occurrence of several fusion events is followed by a fission event (68). A study in Drosophila showed that not only did PLD6 overexpression oppose mitochondrial fragmentation, this opposition reduced necessary apoptosis during embryogenesis (73). Furthermore, mitochondrial fission is an important upstream event of cytochrome c release during apoptosis. Figure 3B illustrates the lipase activity of PLD6 in mitochondria.

**Mitochondria and Cancer**  Beginning with Dr. Warburg’s pivotal proposal in 1956, mitochondria have been of interest in studies of cancer initiation and disease progression. Dr. Warburg believed that the primary cause of all cancers was derived from a shift of
energy production from oxidative phosphorylation to glycolysis due to dysfunctional mitochondrial activity (74). It has since been demonstrated that oxidative phosphorylation (OXPHOS) efficiency remains unchanged in cancer cells (75-77), but it is true that cancer cells rely more heavily on glycolysis than do normal cells (75,78,79). Normal cells derive approximately 70% of their ATP from oxidative phosphorylation in the inner mitochondrial membrane and the remaining 30% from the less efficient glycolysis (75,80). This switch is associated with worse clinical outcome for cancer patients (81). Glycolysis is much less efficient, producing 2 ATP molecules per cycle, while OXPHOS generates 32 ATP, however, glycolysis is quicker and better for the hypoxic environment usually associated with a rapidly proliferating tumor (81-83).

Of course, mitochondria have vital functions in the cell namely bioenergetic and apoptosis-related pathways, both of which are important to cancer progression. Mitochondria are well known for housing the machinery for oxidative phosphorylation, the major ATP producing pathway with cells. However, another bioenergetic pathway is that of glutamine metabolism with the mitochondrial enzyme glutaminase (75). Moreover, glutamine metabolism is important in highly proliferative cancer cells, with cancer cells being seemingly “addicted” to glutamine (84). Glutamine is an important amino acid metabolite for several cellular pathways including oxidative metabolism and ATP generation, serving as another energy source. In cancer cells, glutamine becomes in high demand (85,86) and glutamine metabolism falls under the regulation of both tumor suppressors and oncogenes (87,88).

Mitochondria are critical to the intrinsic apoptosis pathway and cancer cells gain the ability to avoid apoptosis. Recently, mitochondria have been investigated as potential therapeutic targets in the treatment of various cancers. For example, Skrtic et al. showed that inhibiting mitochondrial translation had promising results in treating acute myeloid leukemia (89). However, other studies have reported mixed results in the success of targeting mitochondria in cancer treatment, suggesting the complex nature of mitochondrial function in cancer and context-dependent functions (90-93).

**MicroRNA** MicroRNA (miRNA, miR) are a subset of non-coding RNA molecules that are short RNA molecules, approximately 19-24 nucleotides in length (94-96).
MicroRNA precursors undergo several steps of maturation. Primary miRNA (pri-miRNA) transcripts are synthesized by RNA Polymerase II and contain a stem loop structure. While still in the nucleus, pri-miRNAs are cleaved into precursor miRNA (pre-miRNA) by the RNase III Drosha and its coenzyme DGCR8. Pre-miRNAs are subsequently exported from the nucleus by exportin 5. In the cytoplasm, pre-miRNAs are further cleaved to double-stranded mature miRNA duplex by another RNase III, Dicer and its coenzyme TRBP (97-100). Mature miRNA duplexes in the cytoplasm associate with Argonaute 2 (Ago2) protein and the RNA Induced Silencing Complex (RISC), causing one miRNA strand to dissociate from the complex. As part of RISC, miRNA can associate with target messenger RNA (mRNA), usually at the 3’ untranslated region (3’UTR), as a loosely conserved compliment to the mRNA sequence. This association with mRNA inhibits protein translation either by inhibiting ribosomal function or by inducing mRNA degradation (97-99). A schematic of miRNA function is depicted in Figure 4. Because of the poor conservation between miRNA and mRNA binding, one miRNA can target multiple mRNA and one mRNA can be regulated by multiple miRNA. MicroRNA are widely becoming recognized as essential players in proteome regulation in development, health, and disease states (97-99).

MicroRNA involvement in cancer was first discovered in 2005. Subsequently, dysregulation of miRNAs has been documented in almost all types of human cancer. Additionally, specific cancers have unique signatures of altered miRNA profiles (101-103). One reason for this is that miRNAs are often located in genomic regions that are deleted, amplified, epigenetically modified, et cetera in cancer and can, therefore, act as tumor suppressors or oncogenes (104). In fact, several animal studies demonstrated that alterations in miRNA expression is sufficient to induce neoplasia in cells (105-107). MicroRNAs can also function as both activators and suppressors of tumor metastasis (108-111). Furthermore, individual miRNAs has been shown to serve as both an oncogene or tumor suppressor depending on the cell type (101,112,113). MicroRNAs can function cooperatively to achieve a more universal affect on cancer (114,115). This makes therapeutic targeting single specific miRNA molecules more challenging and suggests therapeutically targeting the broader effects would be a more promising avenue. Additionally, differing expression patterns of microRNA has been documented in cases
of drug resistant tumors, suggesting miRNA expression and regulation could be key to understanding the complex nature of chemotherapy resistance (5,116). This further supports the exploration of miRNAs as therapeutic targets.

Overall, PLD1 and PLD2 have been shown to be upregulated in breast cancer and their increased expression and activity was correlated with worse prognosis. Additionally, much of the human proteome is regulated by miRNA, which are also frequently dysregulated in cancer. Finally, studies on the mitochondrial PLD isoform, PLD6, suggest it may play a role in breast cancer. Thus, I hypothesize that the dysregulation of PLD isoforms in breast cancer promotes cell invasion.

The objectives of this proposal were attained by pursing the following specific aims:

**Specific Aim 1**: To investigate the molecular regulation of classical PLD isoforms by microRNAs in breast cancer cell invasion.

**Specific Aim 2**: To study the molecular regulation of classical PLD isoforms by microRNAs in breast cancer cells subjected to nutrient starvation.

**Specific Aim 3**: To determine the effect of upregulated PLD6 in breast cancer cells on cell invasion.
Figure 1. Human breast cell lines. HMEC (Human Mammary Epithelial Cells) are non-cancerous breast cells, characterized by their epithelial cell morphology and propensity to grow in clusters. MCF-7 cells are a low invasive ductal breast cancer cell line that retains some degree of cuboidal morphology and are characterized as estrogen receptor (ER) positive. MDA-MB-231 cells represent a highly invasive ductal breast carcinoma cell line that have lost epithelial morphology and have a spindle-like morphology. Additionally, MDA-MB-231 cells are classified as triple negative and therefore do not express the estrogen, progesterone, or HER2 receptors. Images were taken at 40X magnification.
Figure 2. Characteristics of the mammalian phospholipase D isoforms. All PLD isoforms contain at least one characteristic HKD catalytic motif. The classical PLD isoforms are PLD1 and PLD2 and are the most well characterized isoforms. They have lipase activity hydrolyzing phosphatidyl choline to free choline and phosphatidic acid. Their PH and PX domains anchor PLD to the membrane via interaction with lipids. PLD3 and PLD4 are transmembrane proteins localizing to the ER and Golgi respectively. PLD6, also termed mitoPLD, is the smallest isoform and localizes to the outer mitochondrial membrane. It dimerizes and functions as a lipase, hydrolyzing cardiolipin to phosphatidic acid, and also functions as an endonuclease in the maturation of piRNA.
Figure 3. Chemical reaction of PLD lipase activity. (A) The lipase activity PLD1 and PLD2 involves hydrolysis of the membrane lipid phosphatidyl choline into free choline and phosphatidic acid. The small box indicates the lipid bonds cleaved by several phospholipases. (B) PLD6 localizes to the outer mitochondrial membrane where it hydrolizes cardiolipin (CL) to produce phosphatidic acid (PA). PA can be dephosphorylated by Lipin 1 to produce diacyl glycerol (DAG). The chemical structure of CL is also shown. Panel A was adapted from a figure published by Gomez-Cambronero (117) and used with author permission.
Figure 4. Mechanism of action of microRNA. After several nuclear and cytoplasmic processing steps, mature microRNA associate with Ago2 protein in the RISC (RNA Induced Silencing Complex). The entire complex binds to target mRNA via complementarity to the microRNA seed sequence. Once bound, the RISC either inhibits protein translation of the mRNA by interfering with ribosomal function or promoting the degradation of the mRNA.
CHAPTER 2. MATERIALS AND METHODS

**Reagents.** miRNA mimics predicted to target PLD1 or PLD2 mRNA were Dharmaco
products purchased from GE Healthcare. The putative PLD1 mRNA-targeting miRNA
was 182–5p (TargetScan Human accession number MIMAT0000259). The miRNAs
predicted to target PLD2 mRNA were 203a, 3619–5p, 887–5p (accession numbers
MIMAT0000264, MIMAT0017999, and MIMAT0004951 respectively). For specific
experiments (as indicated in the text and figures) miRNA plasmids were used that also
contained a GFP coding sequence. miExpress Precursor miRNA Expression Clones were
from GeneCopoeia (Rockville, MD) and include these catalogue numbers: HmiR0249-
M04-B, HmiR0957-MR04-B, and HmiR0512-MR04-B, which are specific for these
miRNAs: hsa-mir-203, hsa-mir-3619, and hsa-mir-887, respectively. Additionally, one
Precursor miRNA Expression Clone for PLD1 was from GeneCopoeia, that of hsa-mir-
182 (catalogue# HmiR0115). Transit2020 transfection reagent was from Mirus (Houston,
TX). Chemical inhibitors of PLD lipase activity, FIPI and NFOT, were purchased from
Cayman Chemical (Ann Arbor, MI).

**Cell Culture.** MDA-MB-231, MCF-7, BT-474, and BT549 human breast cancer cells
and MCF-10A transformed human breast cells were obtained from ATCC (Manassas,
VA). Primary human mammary epithelial cells (HMEC) were obtained from Cell
Applications Inc. (San Diego, CA). MCF-7 and MDAMB-231 cells were cultured in
Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal
calf serum (FCS). BT-474 cells were cultured in Hybri-Care medium (ATCC)
supplemented with 1.5 g/liter NaHCO3 and 10% fetal bovine serum (FBS). BT-549 cells
were cultured in RPMI 1640 medium (ATCC) supplemented with 0.023U/ml insulin and
10% FBS. HMEC and MCF-10A cells were cultured in mammary epithelial cell growth
medium including bovine pituitary extract (BPE), human epidermal growth factor
(hEGF), hydrocortisone, GA-1000, and insulin. HMEC were cultured on collagen-coated
flasks. Cells were maintained at 37°C in an incubator with a humidified atmosphere of
5% CO2.
**Cell Starvation.** To starve cells, medium was aspirated from cells, which were then washed 2 times with phosphate-buffered saline (PBS) and incubated in cell starvation medium (DMEM, 0.1% bovine serum albumin [BSA]) for several lengths of time as indicated in the legends of the figures.

**Transfection of Cells.** Cells were seeded in 6-well plates with an equal number of cells per well and were then allowed to grow for 12 to 24 h prior to transfection. Plasmid transfection reaction mixtures included 1 to 2 µg of DNA plasmid and 1 µg DNA per 2-µl volume of Transit2020 transfection reagent. Mimic transfection reaction mixtures included 100 nM the appropriate miRNA mimic and the appropriate volume of DharmaFECT2 transfection reagent based on the cell line and number of cells seeded as recommended by the manufacturer. Transfection reactions were added to complete media and incubated with cells overnight. Media was replaced and samples were trypsanized and collected 36 to 48 h after initial transfection.

**PLD Activity Assays.** Samples were processed for PLD lipase activity in PC8 liposomes and n-[³H]butanol beginning with the addition of the following (final concentrations): 3.5 mM PC8 phospholipid, 45 mM HEPES (pH 7.8), and 1.0 uCi n-[³H]butanol in a liposome form. Samples were incubated for 20 min at 30°C with continuous shaking. Addition of 0.3 ml ice-cold chloroform-methanol (1:2) stopped the reactions. Lipids were then isolated and resolved by thin-layer chromatography. The amount of [³H]phosphatidylbutanol-di- 16:0 ([³H]Pbut) that co-migrated with PBut standards was measured by scintillation spectrometry.

**Western blotting.** Cells were transfected with expression plasmids as described in the figure legends, and examined after 2 days. Cells were lysed in special lysis buffer (SLB; consisting of 5 mM HEPES, 1mM leupeptin, 768 nM aprotinin, 100 uM sodium orthovanadate, and 0.4% Triton X-100). After sonication, aliquots of the supernatant were resolved by SDS-PAGE, transferred to a polyvinylidenedifluoride (PVDF) membrane, followed by immunoblot analysis with IgG antibodies as described in figure legends, and visualized using enhanced chemiluminescence (ECL) reagents.
Subcellular Fractionation. Cells were trypsanized and pelleted at 200 x g for 7 min. Pellets were resuspended in 400 µl of Buffer A (250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, and 1% protease inhibitors leupeptin and aprotinin). Samples were homogenized on ice and homogenates were incubated on ice for 30 min. Samples were vortexed at max speed for 15 sec and subsequently pelleted at 800 x g for 15 min. The resulting supernatant (S_c) contained the cytoplasmic fraction and was saved for later use. The pellet contained the nuclear fraction and was resuspended in 400 µl of Buffer A. The nuclear fraction was then vortexed at max speed for 15 sec and then centrifuged at 500 x g for 15 min. The supernatant was discarded and the pellet containing the nuclear fraction was washed twice with 400 µl of Buffer A, pelleting at 500 x g for 15 min. The final wash was pelleted at 1000 x g for 15 min. The supernatant was discarded and the pellet was resuspended in Buffer B (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-X-100, and 1% protease inhibitors leupeptin and aprotinin). The nuclear fraction was vortexed at max speed for 15 sec and incubated on ice for 30 min. Nuclei were lysed with sonication and centrifuged at 9000 x g for 30 min. The supernatant was the final nuclear fraction. The cytoplasmic fraction (S_c) was further processed to separate mitochondrial fraction. S_c is centrifuged at 800 x g for 10 min. The pellet was discarded and the supernatant was further centrifuged at 11,000 x g for 10 min. Supernatant contains cytosolic fraction and was transferred to a new tube. The pellet (P_M) contains the mitochondrial fraction and was kept on ice. The cytosolic fraction was precipitated in cold 100% acetone at -20°C for at least 1 hr. The fraction was then centrifuged at 12,000 x g for 5 min. The supernatant was discarded and the pellet was resuspended in Buffer A and was labeled final cytosolic fraction. The mitochondrial pellet (P_M) was resuspended in Buffer A and centrifuged at 11,000 x g for 10 min. The supernatant was discarded and the mitochondrial pellet was resuspended in Buffer C (50 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.5% Triton-X-100, and 1% protease inhibitors leupeptin and aprotinin). The mitochondria were lysed by sonication and this was labeled the final mitochondrial fraction. This protocol was adapted from (118).

Gene expression measurement by qRT-PCR. Total RNA was isolated from cells with the RNeasy minikit according to the manufacturer’s protocol (Qiagen, Valencia, CA).
RNA concentrations were determined using a NanoDrop, and samples were diluted to 50 ng/µl RNA. Reverse transcription was performed with 2 µg RNA, 210 ng random hexamers, 500 µM deoxynucleotide triphosphates (dNTPs), 84 units RNaseOUT, and 210 units of Superscript II reverse transcriptase, and the mixture was incubated at 42°C for 55 min. Quantitative real-time reverse transcription-PCR (qRT-PCR) assays were run with 100 ng total input RNA, 2µl of PLD1 gene expression assay mixture (6-carboxyfluorescein FAM labeled), or 2 µl of the PLD2 gene expression assay mixture (FAM labeled) or with the housekeeping gene (β-actin) (FAM labeled), with the final concentrations being 200 pmol and 400 pmol for the primers and probe, respectively. Primers and fluorescent probes were synthesized by ThermoFisher Scientific. qRT-PCR conditions for the Stratagene Cycler were 95°C for 3 min and then 50 cycles of the next 3 steps, i.e., 30 s at 95°C, 1 min at 60°C, and then 1 min at 72°C. The cycle threshold (CT) values were arbitrarily chosen from the linear part of the PCR amplification curve, where an increase in fluorescence can be detected at >10 standard errors of the means (SEM) above the background signal. ΔCT was calculated as follows: ΔCT = avg PLD CT − avg housekeeping CT (where avg is average), and the gene fold increase expression was calculated as $2^{\Delta \Delta CT} = 2^{(\text{experimental condition, } \text{CT} - \text{control, } \text{CT})}$.

**Quantitative reverse transcription-PCR for microRNAs.** Cells that were transfected with the PLD miExpress Precursor miRNA expression clones were used for RNA lysates 48 h post-transfection using the TaqMan microRNA Cells-to-CT kit according to the manufacturer’s protocol (catalog no. 4391848; Life Technologies). RNA concentrations were determined using a NanoDrop, and samples were normalized to ~66 ng/µl RNA. Reverse transcription was performed in a 15-µl reaction volume with 1 µg of RNA, 1.5 µl 10X buffer, 1 mM dNTPs, 3.8 units of RNase inhibitor, and 1 µl of Multiscribe reverse transcriptase, and the mixture was incubated in one cycle at 16°C for 30 min, 42°C for 30 min, and then 85°C for 5 min. Quantitative RT-PCRs were run in a 20-µl reaction volume using 10 µl TaqMan master mix, ~88 ng of total input RNA, 1 µl of the relevant microRNA gene expression assay (FAM labeled) multiplexed with the housekeeping gene (U6 FAM labeled). TaqMan miRNA primers and fluorescent probes were from Life Technologies. Quantitative PCR conditions for the Stratagene Cycler were 95°C for 10
min and then 40 cycles of the next 3 steps, i.e., 15 s at 95°C, 1 min at 55°C, and then 30 s at 72°C. The CT values were chosen from the linear part of the PCR amplification curve, where an increase in fluorescence can be detected at >10 standard errors (SE) above the background signal. \( \Delta C_T \) was calculated as follows: \( \Delta C_T = \text{avg PLD CT} - \text{avg housekeeping CT} \). The gene expression fold change was calculated as \( 2^{\Delta_{\Delta C_T}} = 2^{(\text{experimental condition}_{C_T} - \text{control}_{C_T})} \).

**Immunofluorescence microscopy.** Cells were fixed onto coverslips using 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, and then incubated in IF blocking buffer (10% FCS–0.1% Triton X-100 in PBS) for up to 4 h at room temperature. Primary antibodies, as described in figure legends were diluted (1:200) in IF blocking buffer overnight at 4°C. Cells were incubated with a 1:2000 dilution of secondary antibody: donkey anti-rabbit or donkey anti-mouse IgG antibody conjugated with tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein (FITC) for 1 h at room temperature. Nuclei were stained using a 1 µg/ml dilution of 4,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min at room temperature. Coverslips were mounted onto glass microscope slides using VectaShield mounting medium, and cells were visualized using a Nikon 50 Eclipse epifluorescence microscope or an Olympus FV1000 confocal microscope.

**Luciferase reporter assay.** Luciferase reporter assays were performed using the LightSwitch luciferase assay kit from ActiveMotif (catalog no.32031). The reporter vectors contain a 3’UTR region and a downstream RenSP luciferase region. Cells were co-transfected as previously described with either a miRNA mimic or plasmid and a LightSwitch 3’UTR positive control vector or PLD2 3’UTR vector in a 96-well plate. After 36 h, Light-Switch luciferase assay reagent was added to the wells, and the signal was read on a luminometer. The signal knockdown from the control was normalized to a negative-control mimic.

**Cell invasion assay.** MDA-MB-231 human breast cancer cells were serum starved for 2 h and resuspended at a concentration of 1.5 x 10^6 cells/ml in chemotaxis buffer (DMEM
+ 0.5% bovine serum albumin). Next, 3 x 10^5 cells were applied to the upper chambers of 8 µm pre-coated PET Matrigel inserts (Corning Life Sciences) with a 6.5-mm-diameter membrane, and cells were allowed to invade for 6 h at 37°C in a humidified 5%CO₂ cell culture incubator. The final concentration of chemoattractant used was 0 or 30 nM EGF in 500 µl of chemotaxis buffer placed in the lower wells of 24-well plates. Matrigel was scraped from the insert and cells were then stained for 1 h with hematoxylin. Six separate fields of cells were counted for each invasion assay, and results were expressed in terms of total number of invading cells.

**Identifying PLD-targeting miRNAs.** Knowing the 3’UTR sequences of both PLD isoforms, PLD1 and PLD2, we found five putative miRNAs that align with specific regions by using the TargetScan-Human bioinformatics analysis (http://www.targetscan.org/vert_71/) (119). Additionally, we found that the predicted binding site of these five microRNA to PLD mRNA is widely conserved among several mammalian species as shown in Table 1. We used several bioinformatic algorithms to determine the favorability of miRNA:mRNA binding pairs, which are also reported in Table 2. First we calculated the Gibbs free energy of the miRNA seed sequence:mRNA binding using mfold web server (The RNA Institute, University At Albany; http://unafold.rna.albany.edu/?q=mfold). Second, the mirSVR score (120) as obtained from the miRanda database (http://www.microrna.org/microrna/home.do) was reported. Finally, we reported the weighted context score as reported by TargetScanHuman and calculated according to Agarwal et al (119). The mirSVR score and weighted context score represent different algorithms in miRNA binding prediction that incorporate multiple miRNA:mRNA binding factors. In all, negative scores represent favorable binding, with greater favorability with increased score magnitude. DNA plasmids from GeneCopoeia were derived with the relevant miRNAs sequences cloned in or a scrambled negative control clone (catalog nos. HmiR0249-MR04-B, HmiR05120MR0-04-B, Hmi-R0957-MR04-B, and CmiR0001-MR04-B) as well as RNA mimics from GE Healthcare-Dharmacon (catalog nos. C-300605-05-0002, C-300557-07-0002, C-300562-03-0002, C-301245-01-0002, C-301515-00-0002, or CN-001000-01-05). Additionally, luciferase reporter plasmids were generated by LightSwitch (Active Motif) with the
3’UTR sequence of PLD2 downstream to a RenSP optimized luciferase gene with a constitutive promoter on a pLightSwitch_3UTR backbone plasmid.

Xenotransplant of SCID mice and metastatic breast cancer models. Mouse studies were conducted as described by Henkels et al. (28). Briefly, 4-6 x 10^6 cells were injected into the left mammary fat pad of SCID mice under isoflurane anesthesia. Tumors were allowed to grow for approximately 4 weeks at which time mice were humanely euthanized. The primary breast tumor and lung metastases were surgically excised. Tumors serial sections were fixed onto glass slides. Samples were stained with hematoxylin and eosin or processed for immunofluorescence staining. Stably transfected cell lines used in the mouse study were generated via lentiviral transfection as described in (28). Lentiviral transfection of MDA-MB-231 cells with pLenti vector (pKLO-shControl or pKLO-shPLD2 Sigma cat no. NM_002663) was conducted as described by Henkels et al (121).

Statistical analysis. The data presented in the figures as bars are means + SEM (standard deviation/√(n-1), where n = sample size). Experiments were performed in duplicates for at least 3 independent experiments. The difference between means was assessed by the student t-test, calculated using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA). A probability (P) of <0.05 indicates a significant difference.
CHAPTER 3. AIM 1.

Specific Aim 1: To investigate the molecular regulation of classical PLD isoforms by microRNA in breast cancer cell invasion.

Rationale

The classical isoforms of PLD, PLD1 and PLD2, are overexpressed in various cancers and aid tumorigenesis and metastasis. Our lab and others have demonstrated pharmacologic inhibition of classical PLD isoforms was advantageous in reducing tumor size and metastases in breast cancer mouse models (28,122). Current small molecule inhibitors are profusely hydrophobic, difficult to solubilize, and exert deleterious side effects. Thus, alternative regulation of PLD, specifically microRNA regulation, is of interest due to the growing interest in investigating miRNAs as therapeutic agents. The post-transcriptional regulation of classical PLD molecules is widely unexplored. Although it has been previously reported that miR-203 inhibits the proliferation and invasion of U251 glioblastoma cells by directly targeting PLD2 (22), this study did not provide insights into the molecular mechanisms of how this was accomplished. Likewise, it is unlikely that only one miRNA will be enough to down regulate PLD, as it appears to be common biologically that several miRNAs cooperate to impede translation of a particular protein.

Experimental Design

In the first subaim, Western blot and qRT-PCR were used to determine the protein and mRNA levels of PLD1 and PLD2 in the breast cell lines of interest. Additionally, an in vitro lipase activity assay was used to assess the enzymatic activity of PLD1 and PLD2 in the cell lines. Matrigel invasion assays were conducted to confirm the invasion phenotype of the cell lines as well as compare invasion capability in the presence or absence of PLD1 or PLD2 overexpression.

In the second subaim, miRNA predicted to target either PLD1 or PLD2 mRNA were identified using bioinformatic analysis websites including TargetScan Human and miRNA.org. Then, the miRNA expression levels in our cell lines was determined and
miRNA expression inversely correlated with PLD protein expression. To determine if the miRNA selected can regulate PLD expression, PLD protein expression was measured in breast cancer cells overexpressing miRNA by Western blot and immunofluorescence. PLD lipase activity was also assessed in the presence of overexpressed miRNA. Furthermore, PLD activity was measured in the presence of combinations of miRNA. Finally, miRNA targeting of PLD was detected using a luciferase reporter assay with a reporter plasmid containing the PLD 3’UTR that, if targeted by the miRNA, will repress the translation of luciferase.

Finally in the third subaim, the effect of exogenous miRNA on cell invasion in both cancer cell lines was evaluated. PLD and miRNA plasmids were co-overexpressed to observe if miRNA can reverse the positive effect of PLD upregulation on cell invasion. Additionally, because highly invasive breast cancer cells have presumably undergone EMT, the role of EMT effectors on miRNA expression in high and low invasive cell lines were investigated.

Subaim 1A: To characterize PLD1 and PLD2 expression and signaling in breast cancer cell lines.

Results

PLD1 and PLD2 expression was evaluated in several breast cancer cell lines to compare to previously reported results as well as establish a cell line model to further explore miRNA regulation. Endogenous protein expressions of both isoforms of “classical” PLD (PLD1 and PLD2) were elevated in MCF-7 (low-invasive luminal) and MDA-MB-231 or BT-549 (high-invasive, epidermal growth factor receptor [EGFR]-high) cancer cells compared to normal MCF-10A cells or HMEC (Figure 5). Additionally, relative endogenous pld1 and pld2 gene expression levels were increased in the four cancer cell lines tested (MCF-7, MDA-MB-231, BT-474, and BT-549) compared to the two normal breast cell lines (MCF-10A and HMEC) (Figure 6), with MDA-MB-231 and BT-549 having the highest (2.5-fold over controls) basal levels. This correlates with previously reported increases in PLD1 and PLD2 mRNA and protein expression in invasive breast cancer (28,123). As shown in Figure 7, basal lipase activity was elevated
in MDA-MB-231 and BT-474 cell lines compared to HMEC, although not in the MCF-7 cell line and only marginally in the BT-549 cell line. Taken together, these data indicate that high-invasive breast cancer cells have higher levels of PLD gene and protein expression, which correlated to increased PLD activity compared to that of the respective controls. Of note, both PLD1 and PLD2 mRNA and protein are lower in BT-474 cells than BT-549 cells (Figures 5-6). However, the PLD lipase activity in these cell lines is similar (Figure 7). One possible explanation is that a higher percentage of PLD protein found in BT-474 cells is phosphorylated and therefore active, when compared to BT-549 cells. A Western blot using an antibody specific for the phosphorylated form of PLD would aid in answering this question. Further, a consistent pattern of PLD expression and activity was more apparent when comparing MCF-7 and MDA-MB-231 cells as low and highly aggressive breast cancer cells respectively. For instance, MDA-MB-231 cells showed elevated protein and gene expression of both isoforms as well as elevated lipase activity when compared to MCF-7 cells. In contrast, the highly aggressive BT-549 cells had less measurable PLD lipase activity than the less aggressive BT-474 cells. Similarly, both BT cell lines showed similar levels of PLD2 protein expression. Thus, I chose to continue investigating PLD in MCF-7 and MDA-MB-231 cells for low and highly aggressive breast cancer cell lines respectively. Similar results in the non-cancerous HMEC and MCF-10A cell lines were observed and both were used as control cell lines.

Next, it was investigated if PLD1 and PLD2 protein expression correlated with cell invasiveness. To confirm the reported invasiveness of MCF-7 or MDA-MB-231 a Matrigel-based cell invasion assay was performed, using EGF (30 nM) as a chemoattractant. As shown in Figure 8, both low-invasive MCF-7 and high-invasive MDA-MB-231 breast cancer cells increasingly migrated through the Matrigel matrix in response to EGF as a function of time, whereas control HMEC cells did not invade to any great extent during the same timeframe. The increased cell invasion of MDA-MB-231 cells relative to MCF-7 and HMEC cells is consistent with reported literature and additionally, correlates with increased PLD expression and activity under basal conditions. Figure 8B shows photomicrographs of representative fields for invaded cells (after 6 h) in Matrigel membranes. EGF was chosen as a chemoattractant because EGF signaling activates PLD (124) and further EGF promotes cell migration (125). The levels
of \textit{pld2} gene expression in three breast cell lines were evaluated after increasing time of incubation with epidermal growth factor (EGF). Figure 9A documents significantly increased PLD gene expression in the highly aggressive MDA-MB-231 cells compared with HMEC cells after EGF stimulation, suggesting a role for PLD in breast cancer cell invasion. Further, little increase in \textit{pld2} gene expression was observed in response to EGF treatment in MCF-7 cells. In a tandem experiment, PLD lipase activity was measured in response to EGF treatment in the same three cell lines. Again, the largest increase in PLD activity was observed in MDA-MB-231 cells. However, there was a significant increase in PLD lipase activity observed in MCF-7 cells as well. This indicates that while EGF treatment doesn’t necessarily increase \textit{pld2} gene expression, there is an increase of PLD enzymatic activity in response to EGF. This is an important finding when considering the increased cell invasion observed in MCF-7 cells in response to EGF. MCF-7 cells express less copies of EGFR when compared to MDA-MB-231 cells (126). However, the observed increase in PLD lipase activity in both cell lines likely contributes to the increased cell invasion in the presence of EGF.

Small molecule inhibitors were used to further investigate PLD lipase activity in cell invasion. The two inhibitors used were FIPI and NFOT, and their characteristics are summarized in Table 1. FIPI is a dual PLD1 and PLD2 inhibitor, while NFOT is a PLD2-specific inhibitor. Wild type and PLD2-overexpressing MDA-MB-231 cells were treated with two concentrations of either FIPI or NFOT and cell invasion eas measured (Figure 10A). There was a significant increase in cell invasion in the presence of overexpressed PLD2, which was subsequently negated by treatment with either FIPI or NFOT. Wild type MDA-MB-231 cells also showed diminished cell invasion when treated with FIPI or NFOT. This experiment strongly suggests PLD, particularly PLD2, promotes breast cancer cell invasion. Figure 10B demonstrates that FIPI and NFOT are able to inhibit lipase activity of both endogenous and transiently overexpressed PLD2 in MDA-MB-231 cells, with increasing inhibitor concentrations yielding less PLD lipase activity. Data for Figure 10 were graciously provided by Ramya Ganesan and Madhu Mahankali.

In this subaim, elevated expression of PLD1 and PLD2 gene and protein as well as increased lipase activity was detected in invasive breast cancer cell lines. Furthermore, EGF signaling increased PLD activity, which correlated with increased cell invasion.
when EGF was used as a chemoattractant. Finally, chemical inhibition of PLD activity reduced breast cancer cell invasion in a Matrigel-based assay.
Figure 5. PLD1 and PLD2 protein expression in multiple breast cell lines. Western blot analysis of endogenous PLD1 and PLD2 protein expression was detected in several cancerous and non-cancerous breast cell lines. MCF-10A and HMEC cell lines are non-cancerous human breast cells. MCF7 and BT-474 are low invasive human breast cancer cell lines. MDA-MB-231 and BT-549 are highly invasive human breast cancer cell lines. The Western blot shows an increase in both PLD1 and PLD2 protein levels in breast cancer cell lines when compared to non-cancerous breast epithelial cells. β-actin was used as a loading control.
Figure 6. *pld1* and *pld2* transcript levels in multiple breast cell lines. qRT-PCR was used to measure *pld1* and *pld2* mRNA levels, (A) and (B) respectively, in cancerous and non-cancerous human breast cell lines. MCF-10A and HMEC cell lines are non-cancerous human breast cells. MCF7 and BT-474 are low invasive human breast cancer cell lines. MDA-MB-231 and BT-549 are highly invasive human breast cancer cell lines. The gene expression of both PLD isoforms was increased in cancerous cell lines when compared to non-cancerous cells. Expression was normalized to β-actin. A probability (P) of <0.05 indicates significance and was calculated with the student t-test. (*) denotes a significant increase relative to MCF-10A. (#) denotes a significant decrease relative to MCF-10A.
Figure 7. Basal PLD enzymatic activity in multiple breast cell lines. PLD lipase activity was measured in whole cell lysates of cancerous and non-cancerous human breast cells. MCF-10A and HMEC cell lines are non-cancerous human breast cells. MCF7 and BT-474 are low invasive human breast cancer cell lines. MDA-MB-231 and BT-549 are highly invasive human breast cancer cell lines. Because PLD1 and PLD2 perform the same enzymatic reaction, this assay does not distinguish between the activity of the isoforms and therefore represents total PLD activity. Basal PLD lipase activity was elevated in three of the breast cancer cell lines relative to MCF-10A. Bars represent dpm/mg protein of the cell lysate ($x10^4$). A probability (P) of <0.05 using the student t-test, indicates significance. (*) denotes a significant increase relative to MCF-10A.
Figure 8. Cell invasion of human breast cell lines. Cell invasion was measured using Matrigel inserts and 30 nM EGF was used as a chemoattractant. An equal number of cells (500,000) per condition were placed in the upper chamber of the insert and allowed to invade for 3 or 6 hours. HMEC cells are non-cancerous human breast epithelial cells. MCF-7 and MDA-MB-231 cells are human breast cancer cells with low and high invasion phenotype respectively. (A) The mean number ±S.E.M. of invading cells (x10^3) for each condition. There was no change in HMEC cell invasion up to 6 hours, whereas MCF-7 and MDA-MB-231 cells increased in number of cells invading with increasing time. Furthermore, more MDA-MB-231 cells than MCF-7 cells invaded under identical conditions. A probability (P) of <0.05 indicates significance, as calculated using the student t-test. (*) denotes a significant increase relative to 0 hr of each cell line. (B) Representative images (20x magnification) of cell invasion for each cell line after 6 hours of invasion. Green arrows point at examples of empty pores in the insert membrane. Blue arrows indicate examples of stained cells. Cell invasion assays were performed in duplicate and 6 fields were counted per insert.
Figure 9. Effect of EGF on pld2 gene expression and PLD lipase activity. pld2 gene expression (A) and PLD enzymatic activity (B) increases with longer stimulation by EGF in MDA-MB-231 cells and MCF-7 cells but not in HMEC cells. These data correlate with increased invasion seen in response to EGF as a chemoattractant (Figure 8). In (A), all samples are relative to 0 hr HMEC. In (B), 0 hr for each cell line was set as 100%. Significance was calculated using the student t-test and a probability (P) of <0.05 indicates significance and is denoted by (*).
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**Table 1.** Chemical inhibitors of PLD lipase activity. This table lists the chemical inhibitors of PLD used herein including their IUPAC name, abbreviation, chemical structure and PLD isoform specificity. Inhibitors were purchased from Cayman Chemical (Ann Arbor, MI).
Figure 10. Effect of inhibiting PLD lipase activity on cell invasion. (A) Matrigel cell invasion assay was conducted with MDA-MB-231 cells using either Mock conditions or transiently overexpressed PLD2 protein. Further, cells were treated with two concentrations of either FIPI (dual PLD inhibitor) or NFOT (PLD2 specific inhibitor). Overexpression of PLD2 increased cell invasion, which was additionally reduced by chemical inhibition or PLD and its subsequent enzymatic activity. A probability (P) of <0.05 indicates significance using the student t-test and a significant increase is denoted by (*) while a significant decrease is denoted by (#). Significant decreases are relative to 0 nM treated cells in each transfection condition. (B) PLD activity was measured in the presence of increasing concentrations of chemical inhibitors, FIPI (red lines) or NFOT (black lines). This assay was conducted in MDA-MB-231 cells either mock treated or transiently overexpressing PLD2, the same conditions used for the cell invasion assay in panel A. Activity is represented as percent of control activity, where 100% is endogenous PLD activity in untreated MDA-MB-231 cells. Data for this figure were provided by Ramya Ganesan and Madhu Mahankali.
Results

As shown in the schematic presented in Figure 11, the human pld2 gene is located on human chromosome 17. Knowing the 3’UTR sequences of both PLD1 and PLD2 mRNA, I found four putative microRNAs that target PLD by using bioinformatics software (TargetScanHuman). The 3’UTR of PLD2 mRNA spans 568 bp in which potential alignment with miR-203, miR-887, and miR-3619 can be found. Note that there are two potential regions of alignment for miR-3619 to the PLD2 3’UTR, whereas the remaining miRNAs tested herein (miR-203 and miR-887) have only one region of alignment with the PLD2 3’UTR. Additionally, miR-182 targeted PLD1 mRNA. The species conservation of the miRNA binding sites and several predictive scores (TargetScan; miRNA.org) are listed in Table 2. ΔG values represent the Gibbs free energy of the miRNA-mRNA binding pairs and were calculated using the mform web server from the RNA Institute at the University at Albany. The mirSVR and Weighted Context ++ Scores were reported on the bioinformatic sites miRNA.org and TargetScan respectively. These scores use an algorithm to incorporate the complexity of positive and negative influences or factors that determine miRNA-mRNA binding. Some of these factors include, the number of complementary nucleotides between the pair, the position of the miRNA site within the 3’UTR, the flanking mRNA sequence, and secondary mRNA structure (119).

I hypothesized that if one or several microRNA exist that could impair translation of PLD mRNA, it might thereby act as a tumor suppressor miRNA. Using miRNA primer/probe sets specific for miR-182, -203, -887, and -3619-5p, the endogenous levels of expression of each miRNA were measured in the two different human breast cancer cell lines, MCF-7 and MDA-MB-231, relative to the control cell line, HMEC. As shown in Figure 12, four miRNAs had decreased endogenous miRNA expression in the highly invasive MDA-MB-231 cancer cells compared with the low invasive MCF-7 cells. The decreased expression of these miRNA in MDA-MB-231 correlates with the increased PLD protein observed in the same cell line. Interestingly, MCF-7 cells did not show a significant decrease in expression of these miRNA relative to HMEC cells and further,
miR-887 actually increased in expression in MCF-7 cells. This suggests that levels of these miRNA vary in specific breast cancer cells, most likely downregulated in highly invasive breast cancer cells.

Next, the potential of these miRNA to bind and inhibit translation of PLD mRNA was investigated. MCF-7 and MDA-MB-231 cells were transfected with two methods of miRNA delivery. Commercially available miRNA plasmids specific encoding for miRNA or commercially available miRNA mimics, the double stranded miRNA precursor were used. The miRNA-encoding plasmids also contained the GFP gene, separate from the miRNA gene, which can be visualized with fluorescence microscopy. Confirmation of successful miRNA-overexpression in MCF-7 and MDA-MB-231 cells is shown in Figure 16 via miRNA qRT-PCR.

To assess the effect of miRNA on PLD, miRNA plasmid DNAs were overexpressed in cells for 48 h and then whole cell lysates were prepared that were then used for SDS-PAGE and subsequent Western blotting or for PLD enzymatic assays. As shown in Figure 13 A-B, there was a decrease in PLD2 protein in the presence of the PLD2 mRNA-targeting miRNA in both MCF-7 cells and MDA-MB-231 cells. A more potent decrease in PLD2 protein expression was observed in MDA-MB-231 cells. MCF-7 cells have higher endogenous levels of these miRNA than MDA-MB-231 cells and thus further overexpressing the miRNA may not have as large an impact on PLD protein expression. Similarly, a decrease in PLD1 protein expression was observed in the presence of the PLD1 mRNA-targeting miRNA, miR-182 (Figure 13C). Further, the decrease in PLD2 protein was shown to be dose-dependent on the amount of miRNA transfected (Figure 13D). Additional confirmation of miRNA-dependent downregulation of PLD protein was demonstrated by immunofluorescence microscopy (Figure 14). MDA-MB-231 cells were transfected with GFP-tagged miRNA plasmids and probed with TRITC-labeled anti-PLD2 (A) or anti-PLD1 (B) antibody. Successfully transfected cells produce GFP protein and appear green. Therefore, the expression of PLD protein can be observed in individual cells that were successfully transfected. In Figure 14, red-PLD staining was decreased in miRNA-transfected, green-labeled cells. The Mock sample was treated with only the transfection reagent and therefore represents endogenous PLD protein expression. An empty vector containing the plasmid backbone
and GFP gene only (no miRNA gene) shows no effect on PLD protein expression compared to the Mock cells. This suggests the decrease in PLD signal observed was due to the overexpression of miRNA in individual cells.

PLD enzymatic activity was evaluated in the presence of miRNA to investigate the physiological relevance of decreased PLD protein level. Overexpression of each of the four miRNAs tested significantly reduced PLD lipase activity in the highly invasive MDA-MB-231 cells compared with non-invasive/non-cancerous HMEC cells (Figure 15A). Again, the decrease was less obvious in MCF-7 cells. In the presence of miRNA, PLD protein levels were decreased to a larger extent in MDA-MB-231 cells compared to MCF-7 cells. Thus, this suggests a larger decrease in PLD protein corresponds to a larger decrease in PLD lipase activity. Further, a combination of miRNAs produced the maximum effect on enzymatic activity (Fig. 15B), with different pairing exerting varying effects on lipase activity. This would be important in determining optimal decrease in PLD activity to inhibit cell invasion, for example. Additionally, because one miRNA has numerous targets, it would be interesting to explore pathways downregulated by miRNA combinations.

Thus far, the data show a correlation between miRNA overexpression and decrease in PLD protein expression but direct targeting of miRNA on PLD mRNA has not been established. A LightSwitch reporter plasmid containing the 3’UTR sequence of PLD2 downstream to a RenSP luciferase gene and microRNA mimics that putatively targeted PLD were cotransfected in breast cancer cells (Figure 17A). The 3’UTR of PLD was inserted into the LightSwitch plasmid within the RenSP luciferase gene directly upstream of the poly-A encoding region. This creates a fusion transcript mRNA attaching the PLD 3’UTR to the luciferase mRNA. Presumably, binding of the miRNA to the PLD 3’UTR would result in diminished luciferase translation, measurable by a luminometer. All three miRNAs tested significantly decreased luciferase signal when cotransfected with the reporter plasmid in COS-7 cells compared with mock-transfected or scramble mimic cells (Figure 17B). This suggests the miRNAs targeting PLD2 mRNA at the 3’UTR. A further experiment necessary to confirm miRNA binding would have been to mutate the predicted miRNA-binding site on the reporter plasmid and demonstrate the subsequent loss of miRNA regulation. Unfortunately, this experiment has not been
completed at this time. COS-7 cells are non-cancerous fibroblasts and were used in this experiment due to their high transfection efficiency. To help ensure co-transfection of both the reporter plasmid and miRNA mimic into the same cells and thus to minimize erroneous signal, a cell line with a high transfection efficiency was preferred. The breast cancer cell lines are much more difficult to transfect and the non-cancerous breast cells are difficult to grow. Because the cotransfection of the luciferase plasmid and miRNA mimic creates an artificial system, COS-7 cells are an acceptable alternative to the breast cell lines for this assay.

In this subaim, a set of four miRNA were identified that were predicted to bind to either PLD2 or PLD1 mRNA. The expression of these miRNA was decreased in highly aggressive breast cancer cells. Further, the exogenous addition of these miRNA decreases PLD protein levels and PLD lipase activity in breast cancer cells. A luciferase reporter assay suggested the downregulation of PLD protein by miRNA was due to miRNA binding to the 3’UTR of PLD mRNA.
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**Table 2.** Conserved sequences of PLD 3’UTR-targeting miRNA. 3’UTR sequences aligning with miR-182 represent PLD1 3’UTR, while sequences aligning with miR-203, -887, and -3619 represent PLD2 3’UTR. miR-3619 has two predicted binding sites on PLD2 mRNA and the site better predictive score is listed in this table. MicroRNA sequences listed are the seed sequence of each miRNA, which represents the miRNA region that associates with mRNA. Species conservation information was taken from TargetScanHuman. ΔG values were calculated for the human binding pair of the miRNA seed sequence and corresponding PLD mRNA sequence using mform web server software (The RNA Institute, University at Albany). mirSVR scores are as reported in the miRanda-mirSVR database. A negative score denotes a favorable match with scores ≤ -1.0 representing the top 7% of predictions. Data for miR-3619–5p were not yet available on miRanda-mirSVR at time of publication. Weighted context ++ scores were obtained from TargetScanHuman based on the algorithm described Agarwal et al (119). Again, a negative score denotes a favorable reaction with greater magnitude indicating higher favorability.
Figure 11. Predicted binding sites of several miRNA on PLD 3’UTR. This schematic depicts the binding sites of the miRNA studied herein, as predicted using bioinformatic analysis (TargetScan). There are three miRNA predicted to target PLD2 mRNA (miR-203, -887, -3619) and one miRNA predicted to target PLD1 (miR-182). Also depicted are the nucleotide sequences and positions on the mRNA of the binding pairs. It is of note that miR-3619 has two predicted binding sites on PLD2 mRNA.
Figure 12. Endogenous expression of mature miRNA in human breast cells. qRT-PCR was conducted of specific miRNA from whole cell lysates of three human breast cell lines. HMEC cells are non-cancerous human breast epithelial cells. MCF-7 and MDA-MB-231 cells are low and highly aggressive human breast cancer cell lines, respectively. Ct values were normalized to U6 housekeeping gene. All four miRNAs were downregulated in MDA-MB-231 cells when compared to MCF-7 cells and all but one were downregulated compared to non-cancerous HMEC cells. A probability (P) of <0.05 using the student t-test, indicates significance and a significant increase relative to HMEC is denoted by (*) while a significant decrease relative to HMEC is denoted by (#).
Figure 13. Exogenous miRNA reduces PLD protein expression. MicroRNA-encoding plasmids were transfected into MCF7 cells (A) or MDA-MB-231 cells (B-D) and PLD protein expression was measured by Western blot. Three microRNAs, miR-203, -887, and -3619 were predicted to regulate PLD2 whereas miR-182 was predicted to target the PLD1 isorm. Both PLD1 and PLD2 protein expression was decreased in the presence of isoform specific miRNA. Densitometry quantification for panel C is represented under the blot. Additionally, panel D shows a dose-dependent effect of exogenous miR-3619 on PLD2 protein expression. β-actin was used as a loading control.
Figure 14. Immunofluorescence of PLD protein after miRNA transfection. MDA-MB-231 cells were transfected with GFP-tagged miRNA-encoding plasmids. Subsequently, IF was performed to label PLD2 (A) or PLD1 (B) protein with TRITC. Thus, green signal represents cells expressing miRNA plasmid and red signal represents PLD protein.
Nuclei were stained with DAPI. In green-labeled cells, there was a decrease in PLD (red) signal when compared to empty vector and mock conditions. Empty Vector was used as a negative control and contains the same plasmid backbone with only the GFP gene. Images were taken at 100X magnification on a Nikon 50 Eclipse epifluorescence microscope under the same photomicrographic conditions. Images were then overlayed using Adobe Photoshop software.
Figure 15. Exogenous miRNA decreased PLD lipase activity. (A) PLD lipase activity was measured in the presence of individual exogenous miRNA in several cell lines. HMEC is a non-cancerous human breast cell line and MCF-7 and MDA-MB-231 cells are low and highly aggressive human breast cancer cell lines, respectively. Furthermore, MDA-MB-231 cells express the lowest endogenous amount of these miRNA (Figure 12) and all of the miRNA tested reduce PLD lipase activity. However, the miRNA have less effect in HMEC and MCF-7 cells. It should be noted these two cell lines also express less PLD protein. (B) PLD lipase activity was measured in the presence of combinations of miRNA in MCF-7 cells. Combinations of miRNA show a greater reduction in PLD lipase activity than individual miRNA. A probability (P) of <0.05 indicates significance by the student t-test and a significant decrease relative to Control is denoted by (#). In panel A, the Control for each cell line was used to compare experimental groups within that cell line.
Figure 16. Successful transfection of miRNA in human breast cancer cells. MicroRNA-encoding plasmids were transfected into MCF-7 and MDA-MB-231 cells. MicroRNA expression levels were measured by qRT-PCR and expressed relative to U6 as a housekeeping gene. Experiments in Figure 13-15 depend on successful transfection of miRNA and this figure demonstrates a significant increase in miRNA expression when transiently transfected in both cell lines.
Figure 17. Luciferase reporter assay supports miRNA binding to PLD mRNA. MDA-MB-231 cells were co-transfected with miRNA mimics and a luciferase reporter plasmid containing the luciferase gene and the PLD2 3’UTR sequence. (A) This reporter plasmid is transcribed into a fusion mRNA. If the miRNA mimic binds to the PLD2 3’UTR, it will suppress translation of the fusion mRNA and less luciferase will be measured by spectrometry. (B) Luciferase signal was decreased in the presence of PLD2-targeting miRNA when compared to the control (scramble) mimic and mock conditions. This suggests the miRNA can inhibit translation of PLD2 via binding to the 3’UTR. Statistical analysis by the student t-test was used with a probability (P) of <0.05 indicating significance and a significant decrease relative to Mimic Control is denoted by (#).
Subaim 1C: To assess the effect of microRNA regulation of PLD on breast cancer cell invasion.

Results

The overall objective of this study was to investigate the role of miRNAs as putative factors responsible for down-regulating PLD in healthy breast cells, and the lack thereof resulting in elevated PLD in highly aggressive cancer cells. Selective manipulation of gene expression of these miRNAs could be used to alter/control the severity of the invasive phenotype of these two human breast cancer cell lines. Overexpression of the miRNAs in both the highly aggressive MDA-MB-231 cells and the less aggressive MCF-7 cells and subsequent measurement of cell invasion was performed (Figure 18). Both the miRNA plasmids and the miRNA mimics were used in both cell lines and similar results were observed with both. In MDA-MB-231 cells, cell invasion was decreased in the presence of each miRNA individually. However, only miR-203 and miR-182 significantly decreased the cell invasion of MCF-7 cells. MCF-7 cells are much less invasive to begin with and further, this is consistent with data observed in Subaim 1B. MicroRNA had less effect on PLD expression and activity in MCF-7 cells compared to MDA-MB-231 cells.

Next, further investigation of miRNA-PLD regulation in invasion was pursued. Invasive properties of highly aggressive MDA-MB-231 cells were further increased by the overexpression of PLD1 and PLD2, which was then negated by overexpression of miRNA targeting each isoform (Figure 19A). MicroRNA-203 was chosen to represent PLD2 mRNA-targeting miRNAs due to its consistent inhibition of cell invasion shown in Figure 18.

As a companion experiment, specific siRNAs targeting miR-203 or miR-182 were transfected in MDA-MB-231 cells, and cell invasion was evaluated (Figure 19B). Next, the appropriate miRNA was co-transfected with the siRNA as a “rescue” experiment. Silencing miRNA was predicted to increase cell invasion while co-transfecting miRNA with siRNA was predicted to negate the effect of the siRNA. As shown in Figure 19B, MDA-MB-231 cells with silenced miR-203 or -182 showed increased invasion compared to cells co-transfected with siRNA against miRNA and miRNA mimics.
PLD-targeting miRNA expression was decreased in more metastatically invasive breast cancer cells (Figure 12), and further experiments were needed to demonstrate the molecular mechanism on how this is accomplished. Given that our laboratory has reported a role for PLD in metastasis (28), and that exogenous miRNA resulted in larger effects in MDA-MB-231 cells than MCF-7 cells, I hypothesized that this negative change in miRNA gene expression in highly invasive breast cancer cells was a function of the epithelial-mesenchymal transition. Cancer cells that have undergone EMT exhibit a more invasive, less differentiated phenotype. MDA-MB-231 cells have a spindle-like morphology, increased invasion capability, and limited cell-cell adhesions, whereas MCF-7 cells have rounder morphology, are less invasive, and grow in clusters. Thus, MDA-MB-231 cells are more representative of post-EMT cells and MCF-7 cells being more pre-EMT. Cells can be induced to undergo EMT or the reverse process MET. I used E-cadherin as a pre-EMT effector and vimentin as a post-EMT marker to investigate miRNA expression in response to EMT changes. Decreasing E-cadherin levels has been shown to induce EMT by activating several EMT transcription factors including Snail (127,128).

The relative levels of EMT marker expression in MCF-7 and MDA-MB-231 were listed in Figure 20A, supporting their use as pre- and post-EMT cells, respectively. Confirmation of the protein expression of E-cadherin (pre-EMT) and vimentin (post-EMT) in MCF-7 and MDA-MB-231 cells respectively is shown in Figure 20B. MicroRNA expression levels were evaluated when overexpressing EMT markers in MDA-MB-231 cells (Figure 21). Two pre-EMT markers (E-cadherin and β-catenin) and two post-EMT markers (vimentin and EGFR) were used. The most significant changes were detected with miR-3619 expression levels in the presence of E-cadherin and vimentin. MicroRNA expression increased in the presence of the pre-EMT marker, E-cadherin, but was decreased when overexpressing the post-EMT marker vimentin. This is consistent with the findings of decreased miRNA expression in highly invasive breast cancer cells. Furthermore, by silencing E-cadherin in MCF-7 cells, the decreased miR-3619 expression is more characteristic of MDA-MB-231 cells (Figure 22A). Conversely, by silencing vimentin in MDA-MB-231 cells, an increase miR-182 expression was
observed (Figure 22B). These data indicate PLD mRNA-targeting miRNA expression is influenced by EMT processes.

Finally, I am proposing a model (Figure 23) where PLD protein expression is down-regulated by a repertoire of microRNAs in healthy breast cells. In cancerous breast cells, expression of these miRNAs was suppressed as EMT progressed (particularly with the decrease of E-cadherin and appearance of vimentin), leading to increased PLD protein production. Furthermore, this surplus of PLD protein subsequently facilitated cell invasion, and thereby metastasis.

**Aim 1 Conclusions:** PLD1 and PLD2 are upregulated in invasive breast cancer cells compared to non-cancerous breast cells. Further, a set of miRNA were identified that were predicted to bind to PLD1 or PLD2 mRNA 3'UTR and the expression levels of these miRNA were inversely related to the protein expression of PLD1 or PLD2 in human breast cell lines. Exogenous addition of these miRNA reduced PLD1 and PLD2 protein expression and enzymatic activity and were able to decrease expression of a luciferase reporter plasmid. Physiologically, breast cancer cell invasion was reduced in the presence of these miRNA. Finally, the expression of these miRNA responds to EMT effectors. I propose that this repertoire of miRNA functions in healthy breast epithelial cells to maintain PLD protein at a normal expression level but upon EMT, these miRNA are downregulated allowing for an increase in PLD protein and thereby cell invasion.
Figure 18. Exogenous miRNA decrease breast cancer cell invasion. MCF-7 or MDA-MB-231 cells were transfected with miRNA-encoding plasmids (A) or miRNA mimics (B) and subjected to Matrigel cell invasion. Number of cells invading decreased in the presence of either miRNA plasmids or miRNA mimics, particularly in the more aggressive MDA-MB-231 cells. (C) Representative images of Matrigel inserts of MDA-MB-231 cell invasion with and without exogenous miRNA. A probability (P) of <0.05 via the student t-test indicates significance and a significant decrease relative to Mock + EGF is denoted by (#).
**Figure 19.** miRNA “rescue” cell invasive phenotype after PLD overexpression. (A) MDA-MB-231 cells were transfected with PLD1 (blue) or PLD2 (gray) plasmids and subjected to Matrigel cell invasion assay. A greater number of invaded cells were observed in PLD overexpressed cells compared to mock treated cells. Subsequently, MDA-MB-231 cells were co-overexpressed with either PLD1 or PLD2 and a respective miRNA (miR-182 for PLD1 and miR-203 for PLD2). The addition of the miRNA reduced the number of cells invading from PLD-overexpressing levels to approximately mock levels. (B) MDA-MB-231 cells with silenced miR-203 or -182 showed increased invasion compared to cells co-transfected with siRNA against miRNA and miRNA mimics. A probability (P) of <0.05 using the student t-test indicates significance and a significant increase is denoted by (*) while a significant decrease is denoted by (#).
Figure 20. Pre- and post-EMT markers in human breast cancer cell lines. (A) Commonly used markers for pre- and post-EMT and their relative expression in a low invasive breast cancer cell line (MCF-7) and highly invasive breast cancer cell line (MDA-MB-231). (B) Western blot showing protein expression in the cell lines of interest of a pre-EMT marker (E-Cadherin) and a post-EMT marker (Vimentin). These data suggest MCF-7 cells are representative of pre-EMT cells and MDA-MB-231 cells are representative of post-EMT cells.
Figure 21. miR-3619 expression in the presence of pre- and post-EMT markers. MDA-MB-231 cells were transfected with pre-EMT markers E-cadherin and β-catenin (green bars) or post-EMT markers vimentin and EGFR (red bars). In the presence of E-cadherin, miR-3629 levels increased. However, in the presence of vimentin, miR-3619 levels decreased. This expression profile of miR-3619 is consistent with levels in pre- and post-EMT cells.
**Figure 22.** miRNA expression after silencing EMT markers in human breast cancer cells. (A) Pre-EMT MCF-7 cells were silenced for E-cadherin and miR-3619 (PLD2) and miR-182 (PLD1) expression levels were measured by qRT-PCR. (B) Post-EMT MDA-MB-231 cells were silenced for vimentin and expression levels of miR-3619 (PLD2) and miR-182 (PLD1) were measured by qRT-PCR. (C-D) Western blot analysis shows silencing of intended protein, E-cadherin (C) and vimentin (D). Using the student t-test, a probability (P) of <0.05 indicates significance and a significant increase is denoted by (*) while a significant decrease is denoted by (#).
Figure 23. Proposed model of microRNA activity on PLD 3’UTR inhibiting cell invasion. In Pre-EMT cells, PLD-targeting miRNA are upregulated, thereby reducing PLD protein levels and reducing cell invasion. These miRNA are downregulated in response to EMT, allowing elevated protein expression of PLD leading to increased cell invasion. Exogenous miRNA, when added to MDA-MB-231 cells, can reduce the cell invasion capability via reduction of PLD protein expression and activity.
CHAPTER 4. AIM 2.

Specific Aim 2: To study the molecular regulation of classical PLD isoforms in breast cancer cells subjected to nutrient starvation.

Rationale

Phospholipase D (PLD) is a vital enzyme in many tissue types, including healthy breast tissue, and its enzymatic products exert influence over an abundance of cellular processes, including cytoskeletal rearrangement, membrane trafficking, and cell migration (26). Aim 1 demonstrated that PLD1 and PLD2 were upregulated in highly invasive breast cancer cells compared to low invasive and non-cancerous cells and the process of EMT allows cells to adopt a more invasive phenotype. Furthermore, expression of PLD-regulating miRNA responded to EMT effectors. Rapidly proliferating tumor cells are often subjected to stressors such as nutrient starvation, hypoxia, and overcrowding. Such stressors can promote EMT in tumor cells and promote invasion and metastasis. Thus, the miRNA regulation of PLD1 and PLD2 in breast cancer cells under stress is of interest to investigate.

Experimental Design

The goals of this aim were accomplished with two subaims. First, classical PLD protein and gene expression and enzymatic activity were measured under nutrient starvation. Nutrient starvation was the chosen stressor due to PLD’s known activation of mTOR in cell growth and proliferation. Second, miRNA expression under nutrient starvation and any cross-talk between PLD and miRNA expression were investigated.

In subaim 2A, PLD expression was evaluated by Western blot and qRT-PCR, as well as PLD enzymatic activity all under nutrient starvation. Culturing cells with serum-free media induced nutrient starvation. Three additional cell lines were included, an additional highly invasive breast cancer (BT-549), low invasive breast cancer (BT-474), and non-cancerous breast cell line (MCF-10A).

Then, in the second subaim, expression of the miRNA identified in Aim 1 was measured by qRT-PCR under conditions of increasing duration of nutrient starvation.
Next, the ability of miRNA to regulate PLD expression under nutrient starvation was evaluated via the luciferase reporter assay used in Aim 1. PLD expression was correlated with cell invasion ability under starvation conditions. Finally, it was investigated whether PLD, or its enzymatic product PA, can influence miRNA expression.

**Subaim 2A: To evaluate PLD1 and PLD2 expression in breast cancer cells subjected to stress.**

**Results**

To understand the reason for the high level of PLD observed in highly invasive cells, I hypothesized that cells in the rapidly growing tumor do not have full access to nutrients and begin to migrate out. PLD acts as a “stress-response signal” and its expression and activity change during stress conditions encountered in a nutrient deprived tumor. Dividing cells and cancer cells have highly anabolic metabolisms in order to generate the molecules needed to increase cell mass and successfully divide. It has been proposed that phosphatidic acid is an indicator of nutrient sufficiency due to its role in activating mTOR and the role of PLD as a stress response protein and further that cancer cell proliferation is PLD dependent (129,130).

Knowing that nutrient starvation of cancer cells is important to the expression of certain transcription factors and key signaling proteins that ultimately contribute to cancer cell survival, proliferation, and migration (40,41), PLD expression and enzymatic activity was tested in several human breast cancer cell lines and normal cells following serum starvation as a function of time. An increase in relative *pld1* and *pld2* gene expression was observed (Figure 24) that after approximately 6 hours remained relatively constant in most cell lines. This increase was most pronounced in the breast cancer cell lines, especially the highly aggressive MDA-MB-231 and BT-549 cells. A less robust yet still significant increase was observed in the less aggressive breast cancer cell lines, MCF-7 and BT-474.

Interestingly, PLD protein expression followed a different pattern in response to serum starvation. Starvation resulted in an initial steady increase in protein expression (Figure 25A-B) that peaked between 6 and 12 hours of starvation. After this point, PLD
protein levels returned to basal levels (or below) after prolonged (24 hour) starvation. This biphasic trend of PLD protein expression in response to starvation was observed in both MCF-7 and MDA-MB-231 cells. PLD lipase activity was also measured after increasing duration of starvation (Figure 25C), and the activity followed a similar biphasic pattern as protein expression. Furthermore, evaluation of MDA-MB-231 cell invasion after starvation correlated with PLD protein expression and lipase activity (Figure 26). Cell invasion increased initially with cell starvation but had returned to basal levels by 24 hours of starvation.

MicroRNA inhibit protein translation without necessarily reducing mRNA levels. Thus, I hypothesized that the molecular mechanism behind the biphasic PLD protein expression despite the steady increase in pld gene expression could be due to miRNA regulation. Therefore, evaluation of miRNA regulation of PLD during starvation was the focus of Subaim 2B.
Figure 24. *pld1* and *pld2* mRNA levels after starvation in multiple human breast cell lines, cancerous and non-cancerous. Cells were subjected to nutrient starvation for up to 24 hours, were collected at 0, 6, 18, and 24 hours post-starvation. *pld1* (A) and *pld2* (B) mRNA abundance were then measured by qRT-PCR. Both *pld1* and *pld2* gene expression increased with increasing duration of starvation, especially in the breast cancer cell lines (MDA-MB-231, BT-549, MCF-7, and BT-474). A probability (P) of <0.05 by student t-test indicates significance and a significant increase is denoted by (*).
Figure 25. PLD protein expression and lipase activity after starvation. (A-B) MCF-7 and MDA-MB-231 cells were subjected to nutrient starvation for 24 hours. Cells were collected at 0, 3, 6, 12, and 24 hours post starvation. Western blot analysis shows PLD1 (A) and PLD2 (B) protein expression in response to starvation. Both isoforms in both cell lines show a peak protein expression between 6-12 hours of starvation that subsequently returns to near basal levels by 24 hours of starvation. This is in contrast to the gene expression data shown in Figure 24. (C) PLD lipase activity shows a similar trend, peaking between 6 to 12 hours of starvation and returning to basal activity by 24 hours of starvation. A probability (P) of <0.05 by student t-test indicates significance and a significant increase is denoted by (*).
Figure 26. Cell invasion of MDA-MB-231 cells after increasing starvation. MDA-MB-231 cells were subjected to nutrient starvation for 24 hours. Cells were collected after 0, 3, 6, 12, and 24 hours of starvation and subsequently used in a Matrigel invasion assay. Cell invasion increases initially and peaks at 6 hours of starvation, returning to basal levels by 24 hours of starvation. These data correlate with PLD protein and lipase activity in response to starvation (Figure 25). A probability (P) of <0.05 by student t-test indicates significance and a significant increase is denoted by (*).
Subaim 2B: To study microRNA regulation of PLD1 and PLD2 in breast cancer cells under nutrient starvation.

Results

First, the expression levels of the miRNA in breast cancer cells as a function of increasing starvation duration was evaluated. As shown in Figure 27, the expression of all of the PLD mRNA-targeting miRNAs tested here varied upon starvation. Interestingly, initially (up to 6 hours) several miRNAs were baseline, whereas beginning at approximately 6 hours miRNA expression increased up to 24 hours of starvation. The time of the miRNA increase (6-12 hours) corresponds to the peak of PLD protein expression. Thus, during the time points when miRNA levels were highest, PLD protein was lowest, suggesting the increase in miRNA could be responsible for the observed decrease in PLD protein. MicroRNA expression was measured in both MCF-7 (gray graphs) and MDA-MB-231 cells (blue graphs). The largest increases in miRNA expression for the MDA-MB-231 cells were the PLD2 mRNA-targeting miR-887 and the PLD1 mRNA-targeting miR-182. However, the changes for each individual miRNA were typically larger fold increases in MCF-7 cells than MDA-MB-231 cells. Further, miRNA levels in non-cancerous MCF-10A cells were measured using the same time points measured for MCF-7 and MDA-MB-231 cells (Figure 28). Only minor changes in miRNA expression were observed in MCF-10A cells suggesting this process is specific to the cancer cell lines. The data indicate that one of the fundamental and significant differences between cancer and non-cancer cells is that the former are able to modulate their response to environmental cues (in this case starvation) by increasing or decreasing microRNAs.

To gain more direct evidence that miRNA regulate PLD protein translation during prolonged starvation, a luciferase reporter assay was used to study PLD2 translation in the presence of miRNA and under starvation conditions. In cells containing only the PLD2 reporter plasmid, an initial increase in luciferase signal was observed, followed by a decreased signal with prolonged nutrient starvation (Figure 29B). Further, when miRNA mimics were added prior to beginning starvation, the initial spike in luciferase signal was diminished and there was an even greater decrease in luciferase signal by 24
hours of starvation. This suggests that the presence of miRNA can inhibit the increase of PLD protein production in response to starvation and further that miRNA could be eliciting the downregulation of PLD protein observed with prolonged starvation.

This initial increase in PLD activity caught my attention, which I hypothesized could be related to other well-known biological phenomena in which PLD is implicated, namely, regulation of mTOR, as it is linked to nutrient response. An activated mTOR pathway (including ribosomal S6K) results in increased cell growth and proliferation. I predict that as a highly sensitive stress response protein, PLD protein levels initially increases during nutrient starvation, facilitating cell migration. One could reason at this point that the initial increase in PLD serves as a stress response in the cell that allows functions other than nutrient-related to occur, such as cell migration that could be encountered initially in tumor cells lacking nutrients. I also hypothesize a second phase exists in the starved cell when mTOR and S6K were no longer activated by PA (as PLD is low). Under these conditions, cell migration ceases, at which point nutrient conservation and autophagy begin.

Aim 1 showed that miRNA reduced PLD protein expression and based on the results of this study so far and the biphasic nature of PLD protein expression during starvation, I hypothesized that a mechanism of negative feedback exists in which the enzymatic product of PLD, PA, increases expression of miRNA with subsequent post-translational regulation of PLD. To reproduce the effects of prolonged nutrient starvation on miRNA, cancer cells were incubated with DOPA (1,2-dioleoyl-sn-glycero-3-phosphate), the commercially available PA most similar to the PA produced by PLD. Prolonged incubation (4 hour) resulted in an increase in miRNA gene expression (Figure 30B and D) compared to a slight decrease in gene expression after only 20 min of incubation (Figure 30A and C).

In rapidly growing tumors, cells often lack full access to nutrients. Starvation induces a stress response in these cells, leading to many cellular changes, including elevated protein expression of PLD. For its established roles in signaling pathways and cell migration, the observed increase in PLD protein aids cell invasion. With prolonged starvation (24 hours), PLD protein levels decreased to below basal level. Additionally, an increase in gene expression of the miRNAs targeting PLD was found. The increase in
miRNA gene expression correlated with decreased PLD protein expression. Therefore, I propose a model in which the enzymatic product of PLD, PA, mediates an increase in miRNA gene expression (Figure 31).

**Aim 2 Conclusions:**

In response to nutrient starvation, PLD protein exhibited biphasic expression. This may be explained by the induction of PLD-regulatory miRNA gene expression with prolonged starvation. I propose a model whereby the PLD enzymatic product phosphatidic acid (PA) induced a miRNA-mediated negative feedback on PLD protein expression in prolonged nutrient starvation of breast cancer cells.
Figure 27. miRNA expression after starvation in breast cancer cells. PLD-targeting microRNA expression is increased in breast cancer cells in response to long-term starvation. (A-H) Cells were deprived of serum for the indicated lengths of time. qRT-PCR was performed to measure endogenous levels of miRNAs, i.e., miR-182 (A, B), miR-203 (C, D), miR-887 (E, F), and miR-3619-5p (G, H) in MCF-7 cells and MDA-MB-231 cells, respectively. A probability (P) of <0.05 by the student t-test, indicates significance and a significant increase is denoted by (*) while a significant decrease is denoted by (#).
Figure 28. miRNA expression after starvation in non-cancerous breast cells. Endogenous gene expression of PLD-targeting miRNAs under nonstarved and starved (24 h) conditions was measured by qRT-PCR in noncancerous human breast cells, MCF-10A. A probability (P) of <0.05 by the student t-test, indicates significance and a significant increase is denoted by (*) while a significant decrease is denoted by (#).
Figure 29. Luciferase assay shows miRNA inhibit increase in protein after starvation. (A) Luciferase reporter and scheme of cotransfections with miRNAs. (B) Luciferase reporter assay shows decreased PLD2 protein production in the presence of miRNA with increasing duration of starvation in MCF-7 cells. A probability (P) of $<0.05$ indicates significance, by the student t-test, and a significant increase is denoted by (*) while a significant decrease is denoted by (#).
Figure 30. miRNA expression in breast cancer cells after stimulation with PA. Gene expression of miR-203, miR-887, miR-3619-5p, and miR-182 after incubation with increasing amounts of DOPA (1,2-dioleoyl-sn-glycero-3-phosphate) for 20 min or 4 h in MCF-7 or MDA-MB-231 cells. Note that the y-axis scales are different in panels B and D since miRNA expression varies in cell line and with time stimulated with PA.
Figure 31. Model depicting the proposed effect of starvation on microRNA in cancer cells. Initially, when placed under starvation conditions, cancer cells increase protein expression of the stress response protein PLD. The enzymatic product of PLD, PA, subsequently serves to signal miRNA gene expression, thereby regulating PLD protein translation in a negative-feedback pathway. The model compiles the repertoire of miRNAs that suppress PLD translation in response to an elevated PA level. The elevated PLD level results in a rise of the cellular PA level, therefore inducing expression of miRNAs that suppress PLD translation. PLD gene, protein, and activity are enhanced specifically in cancer cells under conditions of cellular stress frequently occurring at the tumor core, such as hypoxia, starvation, or overconfluence. This elevation of PLD activity results in an increased expression of multiple miRNAs in low-invasive tumor cells leading to feedback inhibition of PLD expression. However, high-invasive tumor cells lose the ability to induce these miRNAs, resulting in an uncontrolled PA level and the subsequent inhibition of apoptosis through the mTOR pathway.
CHAPTER 5. AIM 3.

Specific Aim 3: To determine the effect of upregulated PLD6 on breast cancer cell invasion.

Rationale

PLD6, a “non-classical” PLD isoform that is localized in the outer mitochondrial membrane, has been found to be upregulated in mammary tumors by von Eyss et al (131). Von Eyss et al. demonstrated that the oncogenic transcription factor c-myc directly binds the PLD6 promoter inducing transcription (131). Myc expression is often dysregulated in triple negative breast cancer, which is the subtype MDA-MB-231 cells represent (131). Myc promotes cell growth and proliferation, contributing to the aggressive nature of triple negative breast cancer cells (131). As a downstream effector of myc, PLD6 was investigated in the context of breast cancer cell invasion.

While still understudied, it is known that PLD6 hydrolyzes cardiolipin to generate PA and thereby promote mitochondrial fusion (132). In turn, mitochondrial fusion inhibits apoptosis and enables the cell to cope with stress and perform more oxidative phosphorylation. In tumor cells that are rapidly proliferating, increased mitochondrial fusion and ATP generation as well as decreased apoptosis would be highly beneficial. Thus, it would seem logical to assume that overexpression of PLD6 would promote cell growth and energy production necessary for tumor cells. Preliminary results indicated the localization of PLD6 in the leading edge, lamellipodia, of breast cancer cells, suggesting PLD6 has a role in breast cancer cell invasion. Herein, the hypothesis that upregulation of PLD6 in breast cancer cells promotes cell invasion was investigated.

Experimental Design

In the first subaim, the expression PLD6 in three breast cancer cell lines was determined via Western blot and qRT-PCR under basal condition. As additional evidence of PLD6 expression in breast cancer patient tumors, data mined from the oncomine database was presented. Immunofluorescence microscopy was used to determine the subcellular localization of PLD6 in breast cancer cells compared to non-cancerous breast cells. Finally, to investigate PLD6 in a tumor environment, immunohistochemistry to
detect PLD6 protein was performed in *ex vivo* tumors of xenotransplanted MDA-MB-231 cells into mice.

In the second subaim, the effect of starvation and EGF stimulation on PLD6 expression and subcellular localization was evaluated by Western blot and IF microscopy. Nuclear fractionation coupled with Western blot analysis as well as confocal microscopy was used to study possible nuclear localization of PLD6. In parallel, PLD6 protein at the membrane ruffling of lamellipodia was observed using immunofluorescence microscopy. Finally, cell invasion was evaluated with the overexpression or silencing of PLD6.

**Subaim 3A: To establish the expression of PLD6 in breast cancer cells.**

**Results**

As shown in Figure 32A, data mined from the Oncomine database (oncomine.org) supports an increase in PLD6 in human invasive breast cancer tumors when compared to adjacent normal tissue. The Finak breast study (133) performed microarray analysis on 53 human tumor samples and adjacent normal tissue. However, the study included several subtypes of invasive breast cancer, (mined by Karen Henkels). The microarray analyses show an increase in PLD mRNA in invasive breast tumors with a p value of 0.0273, however, a much smaller p value is expected to support significance in studies of this kind. Therefore, this data is presented as further supporting the notion that PLD6 may be elevated in certain types of invasive breast tumors.

Pld6 mRNA (Figure 32B) and protein (Figure 32C) levels were evaluated in HMEC cells (non-cancerous breast cells), MCF-7 cells (low aggressive breast cancer), and MDA-MB-231 cells (highly aggressive breast cells). Protein and mRNA levels were measured by Western blot and qRT-PCR respectively from untreated whole cell lysates. Both PLD6 protein and mRNA were elevated in MDA-MB-231 cells when compared to HMEC and MCF-7 cells, indicating that PLD6 may be important in invasive breast cancer cells.

Previous research in the field has shown that PLD6 protein localizes to the outer mitochondrial membrane in normal cells, however it is unknown if a similar localization
pattern exists in breast cancer cells. Therefore, immunofluorescence microscopy of HMEC and MDA-MB-231 cells was completed using antibody specific for PLD6 protein in combination with a mitochondrial marker, called MitoTracker Red Fluorescent Protein (RFP) (Figure 33). PLD6 protein was not detectable in IF images of MCF-7 cells. However, PLD6 protein co-localized with mitochondria in both HMEC and MDA-MB-231 cells (Figure 33). Further, there was more PLD6 staining in MDA-MB-231 cells than HMEC cells, which is consistent with PLD6 levels from the Western blot and qRT-PCR data.

Our lab recently xenotransplanted MDA-MB-231 cells into immunocompromised mice and investigated the role of PLD1 and PLD2 in tumorigenesis (28). Here, immunohistochemistry was performed on mouse mammary tumor sections from a similar study to evaluate PLD6 protein expression in these tumors. IF microscopy revealed positive PLD6 staining at the periphery of the wild type MDA-MB-231 primary mammary tumor (Figure 34). In mice that were xenotransplanted with MDA-MB-231 cells stably transfected with shPLD2, diminished PLD6 staining was observed in the mammary tumors. The authors of a previous study (28) demonstrated that the shPLD2 MDA-MB-231 tumors were less aggressive, producing smaller primary tumors and less distant metastases. Additionally, Henkels et al. (28) found that inhibiting PLD2 resulted in a better outcome than inhibiting PLD1, meaning smaller primary tumors and less lung metastases. Furthermore, the shPLD2 was MISSION shRNA from Sigma-Aldrich (cat no. NM_002663) and no corresponding sequence was found in the 3′UTR of PLD6, suggesting there is no off-target effect of the shPLD2 on PLD6 mRNA. This lends further support for PLD6 having a role in breast cancer cell invasion.

Having identified PLD6 in xenotransplanted mammary tumors, further investigation into the localization of PLD6 within these tumor was conducted. Serial sections of the same tumors were stained with hematoxylin and eosin (Figure 35). Skeletal muscle and adipose cells are easily identifiable by their unique appearance and are denoted in the figure. The tumor peripheries show stronger purple hematoxylin staining of nuclei indicating higher levels of living cells. In contrast, areas of predominately pink eosin staining show a decrease in nuclei and represent regions where cell death has occurred. This is consistent with necrosis of a hypoxic and starved core of
a rapidly proliferating tumor. Comparing the H&E slides with the IF slides, PLD6 staining appears to co-localize with regions of strong hematoxylin staining. This represents the highest density of living cells that are most likely to invade. In the shPLD2 tumor, there was a larger region of strong hematoxylin staining and less cell death observed. This observation suggests a tumor where growth rate does not exceed the available nutrients and oxygen, thereby reducing cell death. In the original mouse study, the authors found the shPLD2 cells generated a less aggressive tumor resulting in a smaller primary tumor with less distant metastases formed, and as such, their need for elevated PLD6 activity is diminished.

Tumors are a heterogenous collection of cells including cancerous cells as well as other resident stromal cells such as fibroblasts, immune cells, adipose cells, and epithelial cells (134). In the xenotransplanted tumor model, human MDA-MB-231 cells were injected into SCID mice, meaning in the tumors were comprised of human cancer cells and mouse stromal cells. Because the PLD6 antibody used detects both human and mouse PLD6, the cells showing positive PLD6 staining could not be definitively identified as either human or mouse. Therefore, PLD6 protein levels were evaluated in the MDA-MB-231 cell lines that had been stably silenced with shRNA, either shControl or shPLD2. A decrease in PLD6 protein was observed in shPLD2 cells when compared to shControl (Figure 36). Thus, I propose that the shPLD2 cells in the tumor contain less PLD6 than their shControl or wild type counterparts. Further, it is well known that cancer cells communicate with cells in the tumor microenvironment, (134), therefore posing an intriguing question of whether PLD6 levels in cancer cells affects PLD6 levels in tumor stromal cells. However, PLD6 levels in mouse tumor-associated cells was not evaluated at this time. A more definitive answer could be achieved using microdissection to isolate the stromal cells from the tumor and analyzing PLD6 protein or gene levels and compared to adjacent normal tissue.

In this subaim, elevated transcript and protein expression of PLD6 was shown in triple negative, invasive MDA-MB-231 cells when compared to non-cancerous cells. Further, PLD6 localized to the mitochondria under basal conditions in HMEC and MDA-MB-231 cells. Finally, PLD6 protein was detected in breast cancer tumors from xenotransplanted mice.
**Figure 32.** PLD6 expression in breast cancer cells. (A) Data mined from the Finak Breast Study on the Oncomine Database shows an increase in pld6 gene expression in invasive breast cancer tissue when compared to adjacent non-cancerous tissue. (B-C) pld6 gene (B) and protein (C) expression were measured by qRT-PCR and Western blot respectively in a non-cancerous human breast cell line (HMEC), a low aggressive breast cancer cell line (MCF-7), and a highly aggressive breast cancer cell line (MDA-MB-231). Both PLD6 mRNA and protein are elevated in the highly aggressive MDA-MB-231 cells. Error bars represent SEM and a probability (P) of <0.05 indicates significance as calculated with the student t-test. A significant increase is denoted by (*) while a significant decrease is denoted by (#).
**Figure 33.** Fluorescence microscopy of PLD6 and MitoTracker in breast cells. Endogenous PLD6 localizes to the mitochondria in HMEC and MDA-MB-231 cells. PLD6 protein is labeled green with FITC and mitochondria were labeled red with MitoTracker-RFP. Images were taken at 100x magnification on a Nikon 50 Eclipse epifluorescence microscope and pictures were overlayed using Adobe Photoshop software.
Figure 34. PLD6 expression in xenotransplanted mammary tumors. MDA-MB-231 cells were xenotransplanted into SCID mice and tumors were allowed to grow (28). Three conditions are shown here: Intact MDA-MB-231 cells and MDA-MB-231 cells that have been stably transfected with either Control or PLD2 short hairpin (sh) RNA. This study demonstrated that shPLD2 MDA-MB-231 cells, but not the shControl cells, formed smaller primary tumors and less lung metastases, indicative of a less aggressive cancer. Here, the shPLD2 tumors are being used as representative of a less invasive breast cancer phenotype. Serial sections of primary tumors were fixed onto microscope slides and stained with anti-PLD6-FITC antibody and DAPI stain. IF images were taken at 10X magnification and images were overlayed using Adobe Photoshop software. As indicated by the white arrows, a band of higher intensity PLD6 staining was observed near the tumor periphery. However, this staining is absent in the less aggressive shPLD2 samples.
Figure 35. H&E staining of xenotransplanted mammary tumors. Hematoxylin and eosin staining was performed on serial sections of the same tumors prepared in Figure 34. Black arrows point to the tumor periphery where abundant purple hematoxylin staining indicates a high cell density of living cells. Areas of pink eosin staining, denoted by **, represent regions of higher cell death more similar to a necrotic tumor core. White arrowheads indicate non-stained adipose cells. Skeletal muscle adjacent to the wild type tumor is denoted by SM. Larger regions of cell death are observed in the wild type and shControl tumors consistent with necrotic cores of rapidly growing tumors. Images were taken at 10x magnification.
**Figure 36.** PLD6 levels in MDA-MB-231 cell lines stably transfected with control and PLD2 shRNAs. PLD6 protein level was determined by Western blot in MDA-MB-231 cells stably transfected with either shControl or shPLD2. GAPDH was used as a loading control.
Subaim 3B: To determine the effect of starvation and EGF signaling on PLD6 expression and subcellular localization on breast cancer cell invasion.

Results

Starvation and EGF signaling promote cell invasion and increase PLD1 and PLD2 signaling as demonstrated in Aims 1 and 2. However, the effect of starvation and EGF signaling on PLD6 remains unknown. PLD1 and PLD2 protein expression change in response to starvation (Aim 2), indicating that these PLD isoforms can be considered as “stress-response proteins”. Here it was investigated if this phenomenon was applicable to the PLD6 isoform, particularly since PLD6 localizes to the mitochondria and starved cells could have functional changes in oxidative metabolism. First, MDA-MB-231 cells and MCF-7 cells were serum starved for up to 24 hours, and then, PLD6 protein was measured by Western blot and by immunofluorescence microscopy. Using Western blot analyses, there was no obvious change in the levels of PLD6 protein expression in MDA-MB-231 cells as a function of increasing starvation time (Figure 37A). PLD6 protein was not detectable in MCF-7 cells at any time pre- or post-starvation (Figure 37). MDA-MB-231 cells cultured for IF microscopy were starved and treated with EGF for 15 minutes. Two methods of EGF treatment were used. One was global application of 10 nM EGF in starvation media incubated with cells, denoted global EGF. Alternatively, a concentrated amount of EGF was added in one corner of the well, denoted localized EGF. IF staining of these cells indicate PLD6 moving away from mitochondria (MitoTracker-RFP) towards a more diffuse localization throughout the cytoplasm and also possibly within the nucleus (Figure 37B). This would be the first documentation of PLD6 localizing in the nucleus, even though PLD6 is not predicted to contain a nuclear localization signal (NLS). PLD6 does contain a MLS (132), and there can be overlap between MLS and NLS sequences such as an abundance of lysine and arginine residues (135). Figure 38 shows the amino acid sequence of PLD6 protein, denoting the MLS and potential NLS. Amino acid sequences rich in lysines or arginines often comprise an NLS. The MLS of PLD6 ends in a string of arginines that could serve as a NLS. Alternatively, PLD6 could associate with other proteins and be shuttled into the nucleus.
Next, nuclear fractionation was used to investigate the potential nuclear localization of PLD6 (Figure 39). MDA-MB-231 cells were starved for up to 24 hours and were then collected at 0, 3, 6, 12, and 24 hours after the beginning of starvation. The cell pellets were next subjected to nuclear fractionation and PLD6 protein was measured by Western blot. GAPDH detection in the cytoplasmic fraction and Histone H3 detection in the nuclear fraction suggested successful nuclear fractionation. Using this approach, PLD6 was only detected in the cytoplasmic fraction (Figure 39A). Additionally, MDA-MB-231 cells were starved overnight and subsequently treated with 10 nM EGF for 15 minutes. These cells were subjected to nuclear fractionation and PLD6 protein analyzed by Western blot. Again, PLD6 was only detected in the cytoplasmic fractions (Figure 39B). Nuclear fraction assays are susceptible to protein leakage from the nuclear fraction and therefore the fractionation Western blot was probed for a soluble nuclear protein, p53 transcription factor. p53 protein was detected in both the cytoplasmic and nuclear fractions (Figure 39A). While it is expected to detect some p53 in the cytoplasm due to protein synthesis and p53 signaling, higher levels should be observed in the nuclear fraction since p53 is a transcription factor. This suggests the nuclear fractionation experiments may have encountered protein leakage and thus these experiments cannot definitively exclude PLD6 from the nucleus.

To further investigate nuclear localization of PLD6, immunofluorescent confocal microscopy of MDA-MB-231 cells was used (Figure 40). MDA-MB-231 cells were serum starved overnight and treated with 10 nM EGF for 15 minutes. As shown in Figure 37B, these treatments produced a population of cells with apparent nuclear PLD6 staining. EGF-treated MDA-MB-231 cells were stained with FITC-labeled anti-PLD6 antibody, RFP-MitoTracker, and DAPI. An Olympus FV1000 confocal microscope was used to image 0.3 µm slices at 60 x magnification with 1.5 digital zoom. Serial images are shown in Figure 40 with the first image being closest to the glass slide and progressing upward in the z-axis. The red MitoTracker stain began perinuclear and below the nucleus. Going upward, a nuclear outline was obvious with perinuclear MitoTracker staining. However, a degree of green PLD6 staining was observed in the nuclear region all slices in some cells. Those cells are denoted with white arrows in Figure 40. In other cells, a clearly defined nuclear region devoid of red and green signal
was present. This suggests some cells may exhibit nuclear PLD6 localization and further quantification is necessary to determine the number of cells with PLD6 nuclear staining in each condition. This effect may not directly be due to EGF stimulation as not all cells on the same slide or field demonstrate the same nuclear PLD6 staining. Silencing PLD6 in MDA-MB-231 cells and the subsequent devoid of PLD6 antibody staining in the nucleus by confocal microscopy would lend further support to PLD6 nuclear localization. Additional controls are needed to further support PLD6 staining within the nucleus. These include secondary antibody alone or isotype control to illustrate any non-specific binding of secondary antibody. Furthermore, another nuclear stain that does not directly target DNA or DNA-associated proteins would

Next, PLD6 localization to sites of membrane ruffling and cell adhesion prior to the formation of lamellipodia in invasive cells was investigated with immunofluorescence microscopy. As shown in Figure 37, a more diffuse staining of PLD6 protein was observed after starvation and EGF stimulation, including staining at cell margins. MDA-MB-231 cells were co-transfected with PLD6 and either Cdc42 or PAK1. Cdc42 is a Rho family GTPase involved in cytoskeletal rearrangement, cell-to-cell adhesion, and cell migration. PAK1 is a serine/threonine kinase and a target for GTP binding proteins Cdc42 and Rac. In this manner, both Cdc42 and PAK1 regulate cytoskeletal rearrangement necessary for cell motility. These transfection conditions are being used here to induce an obvious invasive cell phenotype. Forty-eight hours post transfection these cells were stained for IF microscopy to evaluate PLD6 expression in lamellipodia. Cells were additionally stained with MitoTracker-RFP or with TRITC-labeled Phalloidin stain. As controls, cells that were transfected with PLD6 alone and also PLD6-overexpressing cells that were additionally treated with 10 nM EGF were included. As shown in Figure 41, transfection of PLD6 alone did not show PLD6 protein near the plasma membrane of cells. However treatment with EGF produced cells with an invasive morphology and PLD6 staining at membrane ruffling. Similar staining was observed for PLD6 in cells co-transfected with Cdc42 or PAK1. PLD6 staining was interspersed with phalloidin at the membrane ruffling of lamellipodia in these cells.

Finally, the effect of PLD6 overexpression or silencing on breast cancer cell invasion was investigated (Figure 42). PLD6 was overexpressed in MCF-7 cells, as well
as MDA-MB-231 cells. Additionally, PLD6 was silenced with siRNA in MDA-MB-231 cells. Forty-eight hours post-transfection, these cells were collected and were used for Matrigel invasion assays. Cells were starved for 2 hours prior to invasion and 10 nM EGF was used as a chemoattractant. Silencing and overexpression were confirmed via Western blot and qRT-PCR (Figure 43). Overexpressing PLD6 in MDA-MB-231 cells increased breast cancer cell invasion and conversely, silencing PLD6 in MDA-MB-231 cells significantly reduced in the number of invading cells. However, overexpression of PLD6 in MCF-7 cells had no significant effect on the number of cells that invaded the Matrigel inserts. Successful transfection of PLD6 is shown in Figure 43C with an increase in PLD6 protein levels 48 hours after transfection. Data from Aim 1 demonstrated that overexpression of PLD1 and PLD2 increased cell invasion of both MCF-7 and MDA-MB-231 cells. Likewise, silencing PLD1 and PLD2 in MDA-MB-231 cells decreased cell invasion. Thus, Western blot analysis was done of the protein levels of PLD1 and PLD2 in MDA-MB-231 cells in the presence of 50 nM siPLD6 (Figure 43). Compared to the scramble control (siNEG), PLD1 and PLD2 protein levels do not decrease when silencing PLD6. This further supports that the cell invasion effect observed by silencing PLD6 is in fact from diminished PLD6 levels and not concomitant silencing of PLD1 or PLD2. Noteworthy, is the decreased PLD1 and PLD2 protein expression in the siNEG compared to the Mock transfection (Figure 43). This suggests an offtarget effect of siRNA on PLD1 and PLD2 protein expression. While siPLD6 does not further decrease PLD1 and PLD2 protein below siNEG levels, there was an observed decrease below Mock transfection conditions. This decrease in PLD1 and PLD2 protein from mock levels could impact the cells. Therefore, the decreased PLD1 and PLD2 protein could have contributed to the decreased cell invasion observed in MDA-MB-231 cells with siRNA against PLD6 (Figure 42C).

**Aim 3 Conclusions:**

PLD6 is upregulated in invasive breast cancer cells and is also found in PLD2-overexpressing MDA-MB-231 xenotransplanted tumors. Under basal conditions, PLD6 localizes to the mitochondria but upon starvation or EGF treatment, PLD6 adopts a more diffuse localization in the cytoplasm with possible nuclear localization. Total PLD6
protein does not change under these conditions but PLD6 is recruited to lamellipodia. Finally, the presence of PLD6 promotes breast cancer cell invasion in MDA-MB-231 cells.
Figure 37. Endogenous PLD6 expression and subcellular localization after starvation and EGF stimulation. (A) MDA-MB-231 and MCF-7 cells were starved for up to 24 hours and collected at the indicated times of starvation duration. Whole cell lysates were used for Western blot analysis to detect PLD6 protein levels. Levels of PLD6 protein appear unchanged in MDA-MB-231 cells during the starvation time course and further, PLD6 protein was not detected in MCF-7 cells. (B) MDA-MB-231 cells were treated either with starvation overnight, starvation + global (10 nM) EGF for 15 minutes, or starvation + localized deposit of EGF, incubated for 15 minutes. Placement of EGF in localized
treatment is indicated by white *. White arrows indicate PLD6 staining near the cell periphery. Cells were stained red with MitoTracker-RFP and green with FITC-labeled anti-PLD6 antibody. Nuclei are stained blue with DAPI. Images were taken at 100x magnification and images were merged using Adobe Photoshop software.
Human PLD6:

**MGRLSWQVAAAAAVGLALTLEALPWVL**RWR**LRSRRRRPRRE**APFFPSQVTCT
EALLRAPGAEALPEGCPGLPHGESALSRLRALLAARASLDLCLFAFSSPQ
LGHA**VQLLHQRGVRVRV**VTDCDYMALNGSQIQLLR**KAGIQR**VRHDQDPGYMHR**
KFAIVDKRVLITGSLNWTTQAIQNNRENVLITEDDEYVRLFLEEGERIWEQFNPT
KYTF**PPKKSHGSCAPPVSRAGGRLLSWHRTCGSTSESQT**

**Figure 38.** Human PLD6 amino acid sequence. The mitochondrial localization signal (MLS) as validated by (132) is highlighted in blue and consists of the first 39 amino acids. Within the MLS there is a string of arginines (in red), which could potentially serve as a nuclear localization signal (NLS). Additionally, the catalytic HKD motif is highlighted in green.
Figure 39. Nuclear fractionation and measurement of PLD6 protein expression. (A) MDA-MB-231 cells were subjected to a starvation time course, collected, and subcellular fractionation was performed. (B) Alternatively, MDA-MB-231 cells were either starved overnight alone or starved and subsequently treated with 30 nM EGF for 15 minutes. Cells were collected and subcellular fractionation was performed. PLD6 protein was only detected in the cytoplasmic fractions. In both experiments, GAPDH was used as a cytosolic marker while Histone H3 was used as a nuclear marker. p53 protein was used as a soluble nuclear protein and the level of p53 in the cytoplasmic fraction may indicate a degree of leakage from the nuclear fractions.
Figure 40. Confocal microscopy images of endogenous PLD6 in EGF-treated MDA-MB-231 cells. MDA-MB-231 cells were serum starved overnight and subsequently treated with 10 nM EGF for 15 minutes. Endogenous PLD6 protein was stained with FITC-labeled anti-PLD6 antibody. MitoTracker-RFP stained mitochondria red. DAPI-stained nuclei appear blue. Images were captured at 60x magnification with 1.5 Digital Zoom on an Olympus FV1000 confocal microscope. Serial images were taken at 0.3 µm apart on the z-axis. Image 1 in the upper left corner is the section closest to the glass cover slip and subsequent images progress upward to the top of the cell in the z-axis.
Only the final image contains all three filters. DAPI was removed from the other images to illustrate FITC staining in the nuclear region. White arrows indicate cells with nuclear PLD6 staining.
Figure 41. PLD6 expression in MDA-MB-231. MDA-MB-231 cells were transfected with PLD6 alone or co-transfected with PLD6 and Cdc42 or PAK1 to induce morphology changes. The second row of images are cells that were additionally treated with 10 nM EGF to stimulate morphology changes. All other cells were not treated with EGF. Cells were stained with FITC-labeled anti-PLD6 antibody and either MitoTracker-RFP or TRITC-labeled phalloidin stain. Images were taken at 100x magnification and photographs were merged using Adobe Photoshop software.
Figure 42. Cell invasion of cells overexpressing or silencing PLD6. Matrigel-based invasion assays were performed using either MCF-7 (A) or MDA-MB-231 (B) cells. Both cell lines were transfected with PLD6 plasmid, however, only the PLD6-overexpressing MDA-MB-231 cells showed an increase in cell invasion. (C) PLD6 was silenced in MDA-MB-231 cells using siRNA and these cells exhibited a drastic reduction in cell invasion. Error bars represent SEM and a probability (P) of <0.05 indicates significance using the student t-test. A significant increase is denoted by (*) while a significant decrease is denoted by (#).
Figure 43. Successful overexpression and silencing of PLD6. (A) qRT-PCR of pld6 mRNA levels in MCF-7 and MDA-MB-231 cells. Cells transfected with pld6-encoding plasmid show a significant increase in PLD6 mRNA. (B) Western blot of PLD6 protein levels in MDA-MB-231 cells treated with siRNA against PLD6 or a scramble siRNA (siNEG). PLD6 protein is decreased in the presence of 50 nM siPLD6. Further, PLD1 and PLD2 protein levels remain unchanged in the presence of 50 nM siPLD6 relative to scramble siNEG. (C) Western blot of PLD6 protein in MCF-7 and MDA-MB-231 cells when transfected with PLD6 plasmid. β-Actin was used as a loading control.
CHAPTER 6. DISCUSSION

In this dissertation, the signaling of three members of the PLD family and how their dysregulation promotes breast cancer cell invasion and survival were investigated. In the first Aim, the post-transcriptional regulation of the classical PLD isoforms, PLD1 and PLD2, were explored. Specific miRNAs present in non-cancerous cells downregulate PLD protein and this regulation is lost in invasive breast cancer cells. Furthermore, the addition of exogenous miRNAs (miR-203, -887, -3619, and -182) to MDA-MB-231 cells downregulates PLD protein, and this regulation is lost in invasive breast cancer cells. The addition of exogenous miRNA to MDA-MB-231 cells downregulates PLD protein production and subsequently inhibits cell invasion. In Aim 2, PLD expression and regulation under nutrient starvation was investigated, a condition that favors the epithelial-to-mesenchymal transition that may lead to cell invasion. Finally, in Aim 3, the role of PLD6 in breast cancer cell invasion was investigated, including the effect of starvation and EGF signaling on PLD6 expression and subcellular localization. Overall, this study contributes to increasing the understanding of the molecular regulation and signaling of the PLD family in breast cancer invasion.

A connection between EMT markers and the expression of four miRNAs mediated by the signaling enzyme PLD was shown. Low aggressive MCF-7 and BT-474 breast cancer cells have low endogenous PLD enzymatic activity and cell invasion concomitant with high expression of miR-203, -887, and -3619 (that decrease endogenous PLD2 levels and translation of a luciferase reporter) and miR-182 (targeting PLD1). The combination of miRNAs abolished ~90% PLD enzymatic activity. Conversely, post-EMT MDA-MB-231 and BT-549 cells have low levels of expression of the same miRNAs and high levels of PLD1/2 expression and high aggressiveness. The latter was reversed by ectopically transfecting the miRNAs, which was negated by silencing miRNAs with specific siRNAs. In this study, a molecular mechanism was determined where E-cadherin triggers expression of the miRNAs in pre-EMT cells, whereas vimentin dampens expression of the miRNAs in post-EMT, invasive cells. This novel work identifies for the first time that a set of miRNAs that is activated by a major pre-EMT marker and deactivated by a post-EMT marker, boosts the transition from low
invasion to high invasion, as mediated by the key phospholipid metabolism enzyme PLD.

The present data identify the mechanism of action of pre- and post-EMT drivers on breast cancer cell invasion through a discrete set of micro-RNAs that directly regulate the lipid-signaling enzyme PLD. Here, I propose a model by which regulation of PLD protein expression was disrupted in highly aggressive MDA-MB-231 breast cancer cells. Four microRNAs were identified and shown to regulate either PLD1 or PLD2 mammalian isoforms with evidence to suggest direct binding to the respective PLD-3’UTR. The tumor suppressor-like miRNAs had endogenously decreased levels of gene expression in highly aggressive MDA-MB-231 breast cancer cells compared with HMEC non-cancerous breast cells. This decrease in miRNA gene expression correlated with increased PLD protein expression. By overexpressing the tumor suppressor-like miRNAs, miR-203, -887, and -3619, targeting PLD2, and miR-182 targeting PLD1, the respective PLD protein expression was decreased as well as PLD enzymatic activity in MCF-7 and MDA-MB-231 breast cancer cells. These experiments suggest a post-transcriptional regulation of PLD by these tumor suppressor-like miRNAs. Based on these data, I propose a mechanism of direct binding of these miRNAs to their respective PLD mRNA and subsequent inhibition of protein expression. Additionally, I propose this mechanism occurs naturally in non-cancerous HMEC breast cells, which is dysregulated in highly invasive MDA-MB-231 breast cancer cells.

MicroRNA-loaded RISC can inhibit protein translation by either inhibiting ribosomal function or inducing the degradation of mRNA (136). Zhang et al. demonstrated that the miRNA-loaded RISC can associate with the deadenylase PARN and carry PARN to target mRNA where PARN cleaves the poly(A) tail thereby destabilizing the mRNA (136). This mechanism of miRNA-mediated deadenylation has also been shown with other deadenylases including the Ccr4-Not and Pan2/Pan3 complexes (137,138). Further investigations into the mechanism of action of miRNA-mediated downregulation of PLD in breast cancer are necessary.

Phospholipase D is a critical membrane protein at the center of complex signaling networks increasing both cell movement and cell proliferation. PLD acts through protein-protein interactions with signaling molecules including Grb2, Rac2, WASP, S6K, and JAK3 and also through the enzymatic production of a vital lipid secondary messenger,
phosphatidic acid (28). PLD has been implicated in perpetuating cancer progression by serving as a cell survival signal and by facilitating invasion, the first step in metastasis (28,139,140). Elevated PLD protein expression and activity have been associated with more advanced cancer cell lines (140-143).

Furthermore, overexpressing these miRNAs confirmed the hypothesis that the tumor suppressor-like miR-203, miR- 887, miR-3619, and miR-182 were able to diminish the invasively aggressive properties of MDA-MB-231 cells. Suppressing the PLD-mediated effect on cell invasion was presumably accomplished through inhibition of PLD2 translation. As a consequence the well-known effects of PLD on phosphatidic acid-mediated cell migration would be inhibited, as well as the formation of protein-protein interactions of PLD and proteins of the cell motility machinery (namely, Grb2, Wasp, Arp3, and actin). Silencing these miRNAs (miR-203 and -182) could reverse their inhibitory effect on cell invasion. Thus, I report for the first time specific miRNAs that play a major role in breast cell cancer biological activity to explain the role of PLD.

As EMT progresses and breast cancer cells became more mesenchymal, a set of miRNAs specific for PLD is down-regulated as is the PLD gene, while protein expression is increased. By silencing a pre-EMT marker protein (E-cadherin), the expression of the tumor suppressor-like miR-3619 and miR-182 were diminished. Conversely, expression of these miRNAs was increased by silencing the post-EMT marker vimentin. Therefore, these tumor suppressor-like miRNAs are under regulatory control of the EMT status of the cell, and thus cells undergoing EMT prepare for invasion by accumulating PLD protein via downregulation of these miRNAs. Additionally, one miRNA can have numerous mRNA targets within a cell and these miRNA could target other EMT effectors. TargetScan was used to predict miRNA binding of well known EMT effectors including vimentin, fibronectin, E-cadherin, EGFR, β-catenin, Snail, Slug, Twist1, Zeb1, and Zeb2. Notably, several miRNA studied herein were also predicted to target some of these mRNA (TargetScan). MicroRNA-203 is predicted to target the mRNA of fibronectin, E-cadherin, EGFR, Slug, Twist, and Zeb1. With the exception of E-cadherin, the other predicted targets all promote EMT and cell invasion. Additionally, miR-182 is predicted to target fibronectin, Slug, Twist, and Zeb2, all of which promote EMT. This suggests miR-203 and -182 could exert more influence in suppressing EMT through
targeting multiple targets and furthermore, could be beneficial therapeutic agents. In this way, miRNA can be useful therapeutic agents to target multiple pathways simultaneously. In contrast, silencing RNA (siRNA) are also being explored as therapeutics in cancer treatment (144) and posses greater specificity in their target. Therefore, siRNA can be used to target specific oncogenes or can be designed for patient-specific mutations (144). However, siRNA are less stable that miRNA in the vascular circulation and thus delivery systems are important considerations. The long-term application of this study could be realized as a potential clinical application in assessing the efficacy of tumor suppressor-like miRNA-mediated down-regulation of PLD in combination with inhibitors of PLD activity directed toward inactivating the aggressive cell invasion phenotype.

In the second Aim, the effect of starvation on PLD and miRNA regulation were investigated. It is well-known that mTOR is a strong inhibitor of apoptosis. One of the intracellular activators of mTOR is PA. The activity of the enzyme that synthesizes PA, PLD, is increased in cancer cells subjected to serum deprivation, making PLD a “survival” or “stress response” protein (27). However, the mechanism for this increase during starvation has remained unsolved. PLD rises and decreases with nutrient starvation while miRNA expression steadily rises with time of starvation, and miRNAs are responsible for the decrease of PLD expression. The interaction between PLD/PA and the microRNAs is biphasic and depends on a feedback loop that is absent in noncancer cells.

A new mechanism of regulating the response to nutrient starvation by microRNAs targeting PLD is reported here. PLD plays an important role in the metastasis of breast cancer cells (27), and several protein-protein interactions relevant to cell migration have been discovered. The aim of the current study was to identify and further characterize microRNAs, which bind with imperfect complementarity to the 3’UTR of PLD mRNA, leading to repression of its translation. Additionally, the effect, or biological function, of nutrient starvation on miRNA-mediated post-transcriptional regulation of the signaling enzyme PLD was determined.

It is known that several miRNAs can regulate the expression of a particular protein, presented here are a specific set of miRNAs that affect PLD translation. In
combination with current software, it was determined that miR-182 was predicted to bind to PLD1 and that miR-203, miR-887, and miR-3619-5p were predicted to bind to PLD2. Determining the regulatory role of these miRNAs on PLD protein expression in breast cells, both noncancerous and cancerous, was the goal.

Multiple studies describe the dysregulation of miRNAs in cancer tissue and cell culture compared to noncancerous samples (145-148). Most often, miRNAs are repressed in cancer, as is described here with the PLD-regulating miRNAs. However, there have been examples of oncogenic miRNAs that are overexpressed in cancer, such as miR-155 and miR-21, where overexpression was sufficient to induce lymphomagenesis in mice (105,149). MicroRNAs have many roles in cell function, are pivotal intersections of communication, and thus have been implicated in many areas of cancer disease, including initiation (105,149), proliferation, metastasis, drug resistance (150), and biomarkers (147,148). This highlights the crucial role of miRNAs in cancer development and progression. I proposed in this study that miRNA regulation of PLD protein expression is a vital component of the nutrient depletion stress response in breast tumor cells.

As shown by the Foster and Frohman groups, starvation and lack of nutrients in cancer cells initiate a survival program (34,151-154). Cells under hypoxic conditions and that also lack nutrients have elevated PLD activity. The endogenous levels of PLD expression and phospholipase activity in luminal MCF-7 and claudin-low MBA-MB-231 breast cancer cells followed a biphasic pattern with a maximal expression at 6 to 12 h after starvation and a decrease below control levels at 24 h. After 12 to 24 h of starvation, microRNAs were elevated in MDA-MB-231 cells and MCF-7 cells 20- to 40-fold, respectively, and after 24 h of starvation, PLD2 protein mass was minimal. The miRNA expression levels changed prior to significant decreases in PLD2 protein mass. The four PLD-specific miRNAs (miR-203, miR-182, miR-3619-5p, and miR-887) played a key role in decreasing PLD protein levels. Still, all four of these miRNAs that had elevated expression (miR-203, miR-887, miR-3619, and miR-182) could in combination be responsible for influencing PLD2 protein levels.

These data are consistent with the current model for phosphatidic acid (PA) activation of mTOR. PA, the enzymatic product of PLD, binds and activates mTOR (155,156). The activated mTOR pathway induces increased cell growth and proliferation
As a highly sensitive stress response protein, the PLD protein mass initially increased during nutrient starvation, which would facilitate cell migration. Additionally, an increase in PA results in mTOR-induced cell growth. After prolonged starvation, it is thought that cells can no longer afford the cost of increased mTOR activity and cell growth associated with increased PLD and PLD-mediated cell migration. Therefore, I propose a negative-feedback mechanism wherein cells undergoing prolonged starvation increase gene expression of PLD-regulating microRNAs, resulting in the downregulation of PLD protein mass.

Starvation induces autophagy, which is associated with protein degradation (157,158). It is still possible that PLD2 protein degradation could be mediated by an additional mechanism in parallel with miRNAs. For example, autophagy is coupled with a reduction in histone H4 lysine 16 acetylation (H4K16ac) via downregulation of hMOF, a histone acetyltransferase (159,160). If hMOF is degraded (leading to a decrease in H4K16ac at the PLD2 promoter), then this could influence the levels of PLD2 protein levels.

The p53 mutation status is one of the molecular differences between MCF-7 (wild-type p53) and MDA-MB-231 cells. The p53 replacement mutation at codon 280 is located in the DNA binding domain of p53, which causes altered transcriptional activity. MicroRNA-203 (161) and miR-182 expression is regulated by p53. In cardiac fibroblasts, serum starvation resultes in an elevated expression of p53 (162). Upon starvation, levels of p53 might be altered differentially in MCF-7 cells and MB-231 cells. These altered p53 levels might influence miRNAs and also PLD2 levels.

Loss or gain of function of specific miRNAs probably contributes to breast epithelial cellular transformation and tumorigenesis (163). A model of starvation effects on PLD in cancer cells by the David Foster group indicates that cells in the core of a tumor mass become hypoxic and increase the environmental pressure to leave the tumor, seeking a new environment; these cells resemble mesenchymal cells, and the expression of PLD is augmented (164). Future work should investigate whether the miRNAs that are documented here are downregulated under starvation and hypoxic conditions.

Evidence is provided here for a negative feedback loop, where PLD induction upon starvation would lead to increased PA. PA signaling could induce expression of
miRNAs, which in turn inhibit PLD2 translation. The physiological relevance for breast cancer cells is that PA can activate cell invasion and then, due to the negative feedback, can deprive mTOR and S6K of their natural activator, prevent inhibition of apoptosis, and survive nutrient deprivation, which normal cells cannot do. It seems logical there may be a common mechanism of altering the expression of these four miRNA in starved cells or cells treated with PA. All four miRNA are located on different chromosomes and little is currently known about the promoter regions of each miRNA. The exception is miR-203, which is dysregulated in numerous cancer types. For example, the miR-203 was found to be downregulated in metastatic breast cancer cell lines due to hypermethylation of the CpG island in its promoter (165). CpG islands are frequently seen in promoters of tumor suppressor genes and therefore epigenetic changes of the four miRNA genes could be a possible regulatory mechanism in breast cancer cells. Furthermore, Ding et al. (166) showed that the EMT-promoting transcription factor Slug (SNAI2) can directly bind and repress the miR-203 promoter. Additionally, Zhan et al. demonstrated that Snail (SNAI1) transcription factor can directly bind and repress the miR-182 promoter in breast cancer cells (167). Snail is a well-known driver of EMT and one or several of the EMT transcription factors may act upon the promoters of these four miRNA in breast cancer cells. The Cambronero lab previously showed that PA can bind to the transcription factor PPARα (168) and it would be interesting to investigate binding of PA to other transcription factors including EMT drivers. A better understanding of PLD regulation in these cells could also lead to the design of better treatment options.

The third specific Aim, was designed to understand an understudied isoform of the PLD family, PLD6. Most well-characterized for its role in spermatogenesis, PLD6 was identified through microarray analysis to be upregulated in some breast cancer tissue samples. Located in the outer mitochondrial membrane, PLD6’s catalytic activity promotes mitochondrial fusion and discourages apoptosis, which would be advantageous for cancer cells. Furthermore, von Eyss et al. (131) found that PLD6 is under direct regulation of the transcription factor c-myc, which is mutated and/or upregulated in many cancers. Myc is well known for its role in promoting cell growth and moreover, mitogen signals such as EGF activate myc. In previous two aims, it was demonstrated that PLD1 and PLD2 are influenced by EGF as well as starvation conditions. PLD1 and PLD2 are
stress response proteins and their increased expression and activity result in increased cancer cell invasion. Thus I hypothesized that PLD6 has a similar role in breast cancer. Namely, does PLD6 catalytic activity respond to the same signaling pathways and is its activity related to cell invasion.

For the study of PLD6, the same breast cell lines as in the previous two aims were used, MDA-MB-231, MCF-7 and HMEC cells. Elevated PLD6 mRNA and protein expression in MDA-MB-231 cells were observed compared to the non-cancerous HMEC cells. Further, PLD6 detection in MCF-7 cells was none to very minimal. This data is consistent with elevated expression of myc in MDA-MB-231 cells compared to MCF-7 cells. It is interesting that MCF-7 cells express less PLD6 than even non-cancerous HMEC cells. While MCF-7 cells are a less invasive cell line, they are more rapidly proliferating than non-cancerous cells. Because PLD6 promotes increased ATP production, it is interesting that PLD6 expression would be lower in MCF-7 cells than HMEC cells. MCF-7 cells likely have alternative methods of coping with increased energy demands such as increased glycolysis.

In examining xenotransplanted MDA-MB-231 tumors from mice, PLD6 staining was evident near the tumor periphery. Wild type tumors showed greater PLD6 staining than the less aggressive shPLD2 tumors, consistent with the notion of PLD6 promoting breast cancer cell invasion. Furthermore, this finding suggests a role for PLD6 in a tumor environment, which is more complex than cancer cells in cell culture. The tumor microenvironment is composed of cancer cells as well as non-cancerous cells including fibroblasts, immune cells, adipocytes, and epithelial cells, as well as the supporting collagen and tissue (134). Cross talk between cancer cells and the tumor microenvironment plays a significant role in tumor growth (134). A pertinent example is how oxidative stress in tumor stromal cells promotes breast cancer development and progression. Studies have shown that ROS produced by tumor stromal cells acts via paracrine signaling promoting cancer cell tumorigenic transformation, resistance to apoptosis, and cell migration (169,170). Additionally, oxidative stress promotes the transformation of tumor fibroblast to myofibroblast, which are highly mobile and contractile cells expressing mesenchymal markers (134,171). Myofibroblasts promote tumor initiation and progression by generating high levels of ROS and secretion of
growth factors (134,172,173). A major producer of ROS is mitochondrial oxidative phosphorylation and in a normal cell, excessive ROS leads to mitophagy and autophagy. Autophagy of tumor stromal cells provides amino acids, nucleic acids, and fatty lipids to cancer cells, promoting their growth (134,174-176). Furthermore, breast cancer cells exhibit increased mitochondrial oxidative activity compared to tumor-associated fibroblasts (177). PLD6 promotes mitochondrial fusion thereby inhibiting mitophagy and therefore, I expect PLD6 protein to be elevated in cancer cells and less expressed in tumor stromal cells. PLD6 expression likely varies throughout disease progression. For instance, PLD6 would likely favor ROS generation seen in tumor-associated myofibroblasts. With time, cellular mechanisms regulating mitophagy and autophagy in the myofibroblasts would likely alter PLD6 expression. Conversely, in cancer cells, increased mitochondrial activity and avoidance of mitophagy perseveres despite accumulating ROS.

Under basal conditions, PLD6 co-localized with mitochondria in both HMEC and MDA-MB-231 cells, consistent with published data. When MDA-MB-231 cells were treated with starvation alone or starvation + EGF, PLD6 adopted a more diffuse localization throughout the cytoplasm. Unlike PLD1 and PLD2, PLD6 protein levels did not change after starvation or EGF treatment. Both starvation and EGF promote cell migration and thus the re-distribution of PLD6 could be beneficial to cell migration. It is possible that the more diffuse localization of PLD6 indicates a relocalization of mitochondria in response to starvation or EGF. Mitochondria are found at the leading edge of migrating cells and distribution of mitochondria throughout a migrating cell would provide ATP diffusely for use in migration. By IF microscopy, it was unclear if the PLD6 at the cell periphery was localized to mitochondria. The mitochondrial signal was so strong in the perinuclear region, that any mitochondrial staining in the thinner lamellipodia could be masked. Fluorescent confocal microscopy would be better suited to answer such a question.

MDA-MB-231 cells starved and treated with EGF also appeared to have a nuclear PLD6 staining upon IF microscopy. To my knowledge, PLD6 has not been reported to be in the nucleus and a PLD6 NLS has not been validated. However, reported overlap of typical MLS and NLS sequences could indicate that a portion of the MLS on PLD6 could
function as a NLS. Furthermore, PLD6 functions as both a lipase and an endonuclease in the processing of piRNA. Thus, nuclear PLD6 is an intriguing notion and its nuclease activity may be involved in RNA processing. Nuclear fractionation of MDA-MB-231 cells treated with starvation and EGF and fraction analysis by Western blot did not detect the presence of PLD6 in the nuclear fraction. The original nuclear marker used was Histone H3 and because it is bound to chromatin, it is difficult to detect the occurrence of any protein leakage from the nuclear fraction during the fractionation process. A soluble nuclear protein p53, was used to further investigate this possibility. p53 protein was observed in the cytoplasmic fractions, which may indicate protein leakage from the nuclear fractions. An additional problem could be the polyclonal PLD6 antibody used. The antibody could have non-specific binding to a different nuclear protein. However, a monoclonal PLD6 antibody against a specific epitope is not currently commercially available. Using confocal microscopy, PLD6 antibody signal was found within the nucleus in a population of cells. An additional experiment would be overexpressing myc-tagged PLD6 plasmid and probing with a myc-tag specific antibody with strong signal specificity. However, this approach would not detect endogenous protein and it is possible endogenous protein behaves differently than overexpressed protein.

Using confocal microscopy, I did observe PLD6 staining in the nuclear regions of cells. However, this staining was only present in a small population of cells treated with EGF, suggesting EGF stimulation may not be sufficient to induce PLD6 nuclear localization. Alternatively, PLD6 nuclear localization could be cell cycle dependent. PLD6 functions as both a lipase and an endonuclease and both of these activities could be critical in a growing cell preparing to divide. PLD6’s mitochondrial lipase activity and production of PA is known to promote mitochondrial fusion generating larger mitochondria able to meet the energy demands of a growing cell. Additionally, PLD6 functions as an endonuclease on precursor piRNA. piRNA are important in repressing transposons in spermatocytes and maintaining DNA integrity. This function of PLD6 has been reported in the nuage of spermatocytes, the mitochondrial-rich perinuclear region, during meiosis (69). An interesting study would be to synchronize cells and observe PLD6 subcellular localization in different phases of the cell cycle.

Overexpression of PLD6 increased cell invasion in MDA-MB-231 cells but failed
to elicit any change in cell invasion in MCF-7 cells. This suggests PLD6 alone is not sufficient to promote cell invasion or MCF-7 cells have a mechanism of counteracting the effects of PLD6. A specific assay to determine total PA levels within the cell as well as PA co-localization with PLD6 would be beneficial. This could demonstrate if overexpression of PLD6 results in increased cellular PA. Additionally, it could help correlate increased cell invasion with an increase in PLD6 lipase activity. First, as discussed above, MDA-MB-231 cells have higher expression levels of the transcription factor myc. Myc protein directly binds to the PLD6 promoter and increases PLD6 transcription, contributing to the elevated levels of PLD6 in MDA-MB-231 cells. In addition, myc promotes the transcription of many genes involved in cell proliferation and cell growth including cyclins, ribosomal RNA and proteins. To date, there is no literature suggesting myc regulates PLD1 or PLD2, however, PLD1 and PLD2 promote that stabilization of myc protein (178). PLD6 activity promotes mitochondrial fusion and presumably increases oxidative phosphorylation and ATP production within the cell. Thus, it could be that the cell uses this energy to power cytoskeletal reorganization and other processes necessary for cell invasion, which the machinery is already present in MDA-MB-231 cells but not MCF-7 cells. In addition, EGF was as a chemoattractant in the invasion assays. MDA-MB-231 cells express higher levels of EGFR than MCF-7 cells. Further, EGF signaling stimulates myc, but also PLD1 and PLD2 activity. Data in Aim 1 showed that PLD1 and PLD2 both promote cell invasion and they are both expressed at higher levels in MDA-MB-231 cells. Thus, it is possible that PLD6 promotes cell invasion in a cell that is capable of invading, such as a cell that has already undergone EMT.

Overall, this study demonstrated that three members of the PLD family are upregulated in invasive breast cancer cells. The classical PLD isoforms, PLD1 and PLD2, are regulated by at least four microRNA in health breast cells. However, in cancer cells that have undergone EMT, these miRNAs are downregulated, promoting the increased protein translation of PLD1 and PLD2. Further, PLD1 and PLD2 are stress response proteins, and are increased initially upon serum starvation. During prolonged starvation, the same miRNA are upregulated and serve to downregulate PLD1 and PLD2 protein. Additionally, PLD6 is a mitochondrial protein whose catalytic activity promotes
mitochondrial fusion. Under certain conditions, PLD6 may localize to the nucleus and/or the leading of invading cells. The overexpression of PLD6 in MDA-MB-231 cells increased the cell invasiveness while silencing PLD6 decreased cell invasion. Interestingly, this effect was cell line dependent, as no effect was observed with overexpressing PLD6 in MCF-7 cells. In conclusion, the dysregulation of the three PLD isoforms studied herein contribute to breast cancer cell invasion.
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