# Phospholipase D couples survival and invasion in cancers

## ----possible answer to the cancer progression puzzle

by

Yang Zheng

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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# Abstract

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Metastasis is a late stage of malignant cancer. However, recent evidence suggests that mutations contributing to metastasis may be acquired by cancer cells at much earlier stage. This has been referred to as the cancer progression puzzle. Phospholipase D (PLD) is a ubiquitous enzyme that hydrolyzes phosphatidylcholine to produce phosphatidic acid (PA) and choline where PA is considered to be the critical lipid second messenger. Both PLD expression level and enzymatic activity are elevated in variety of human cancers. We have shown that, under the stress condition of serum withdrawal, PLD activity is elevated and provides a survival signal in the human breast cancer cell line MDA-MB-231, likely to be critical early in the tumor progression, to suppress default apoptotic programs. Of the significance, we also found that the elevated PLD activity enhances the invasive behavior of MDA-MB-231 cells, thus linking the early survival advantage with later metastatic potential. Serum reduction in culture is similar to the conditions endured by early stage cancer cells in an unvascularized solid tumor, where there is a lack of growth factors and nutrients. Under such conditions, we hypothesize that some cancer cells respond by elevating their PLD activity. These cells would have a better chance at

not only surviving, but also migrating to new sites and forming new colonies where blood supply is available. Further mutations among the population of these colonies would further advance progression to malignant cancer. This hypothesis provides a possible answer to the cancer progression puzzle. We propose that some malignant cancers acquire at least part of the metastatic capability at an early stage of tumorigenesis, by selection for the survival of the emerging cancer cell, against the pressure of a poor blood supply in poorly vascularized tumors.

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# Chapter 1: Introduction

## 1.1 Introduction to cancer

### 1.1.1 Cancer and evolution

Cancer is a disease arising from multiple mutations accumulated through years or even decades. These mutations include activation of oncogenes, loss of function of tumor suppressor genes, and mutations of genes involved in DNA repair and genomic stability. These mutations lead to uncontrollable growth of somatic cells into tumors, and in the worst case scenario of metastasis, cancer cells spread to other sites in the body.

One has to study cancer from an evolutionary stand point in order to understand its development. To maintain the integrity of the genome, all organisms are equipped with genetic measurements to counter DNA replication errors. With both increased genomic complexity and longevity, there exists a greater probability for mutations to occur in more advanced organisms. Thus a more sophisticated defense mechanism is necessary. As a result of natural selection, all species, including humans, have evolved very efficient anticancer mechanisms at multiple physiological levels. That is the reason why cancer is often a disease of old age (61).

In spite of all the defense mechanisms, cancers still occur. Over a long lifespan, many somatic cells will die and be replaced. By contacts with mutagens or merely by chance, very few of these newly divided somatic cells will acquire the critical mutations. These mutations can help the cell to multiply faster and avoid being eliminated. It is an

evolutionary process at the cellular level, whereby cell clones are selected for against the pressure applied by anticancer mechanisms, so that only the fittest cell clones can form colonies and potentially develop into cancer (61, 68, 123).

### 1.1.2 Six common physiological changes necessary for cancer

Many types of cancers are diagnosed in the human population with an age-dependent incidence implicating four to seven rate-limiting, stochastic events (135), which indicate that there are multiple barriers that cancers need to overcome. Hanahan and Weinberg (61) suggested that a normal cell needs to acquire six physiological changes to overcome these barriers in order to become a malignant cancer. These physiological changes include: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and metastasis. Each of these physiological changes represents a successful breach of an anticancer mechanism possessed by cells and tissues. Hanahan and Weinberg proposed that most, if not all, human cancers have these six abilities which are acquired step by step over decades (61).

*Uncontrolled Proliferation (including self-sufficiency in growth signals, and insensitivity to antigrowth signals)*

Proliferation potential, including self-sufficiency in growth signals, and insensitivity to growth-inhibitory signals, are the first changes cancer cells acquire by mutation. With these initial requisite traits, a single somatic cell can quickly multiply without the normal constraints on cell proliferation. From this population of cells,

additional mutations can lead to the acquisition of traits that give the cell a further selective advantage.

Normal cell proliferation is dependent on appropriate mitogenic signals. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules such as: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. Oncogenes can deregulate the growth signaling pathway at different levels by **(a)** producing their own growth factors ─ examples include the production of PDGF (platelet-derived growth factor) and TGFα (transforming growth factor α) by glioblastomas and sarcomas, respectively (3); **(b)** overexpressing growth factor receptors such as epidermal growth factor receptor (EGF-R/*erbB*) in stomach, brain, and breast tumors, and the HER2/*neu* receptor in stomach and mammary carcinomas (153, 184); **(c)** switching the types of extracellular matrix receptors (integrins) they express, favoring ones that transmit progrowth signals (52, 104); **(d)** interfering with the downstream circuitry that receives and processes the growth signals; these factors include the oncogenes Ras and Src (17).

To proliferate, not only do cancer cells need to be self-sufficient in growth signals, they also need to breach the anti-growth signaling. Anti-growth signaling forces normal cells into the quiescent ($G_0$) or postmitotic state where the cells temporarily or permanently lose their proliferation ability. At the molecular level, many, and perhaps all, antiproliferative signals are funneled through the retinoblastoma protein (pRb) and its two relatives, p107 and p130. When in a hypophosphorylated state, pRb blocks proliferation by sequestering and altering the function of E2F transcription factors that

control the expression of genes essential for progression from G1 into S phase (177). TGFβ is one of the antigrowth factors which signal through pRb pathway to prevent the phosphorylation that inactivates pRb, thus blocking advancement through G1 phase. In some cell types, TGFβ suppresses expression of the *c-myc* gene which regulates the G1 cell cycle machinery in unknown ways (116).

The pRb signaling circuit, as governed by TGFβ and other extrinsic factors, can be disrupted in a variety of ways in majority of human tumors, such as downregulation or dysfunctional mutant of TGFβ receptors (50, 110), mutations on the downstream components including Smad4, (142, 192), loss of pRb function by mutation, or sequestration by oncoproteins such as the E7 oncoprotein of human papillomavirus (35).

*Evading apoptosis*

After gaining the proliferation capability, somatic cells can multiply to form a small colony. All the cells in this colony now face an immediate selective pressure of apoptosis induced by tumor suppressor genes such as p53. Programmed cell death (apoptosis) is probably hardwired in all cells. Once triggered by internal or external signals, the apoptosis molecular machineries will execute precisely a series of pre-programmed steps which result in a series of cell physiology changes in 30-120 minutes, and finally result in cell death typically within 24 hours (179). External apoptotic signals can be received by various death factors/receptors, including Fas/CD95, TNFR1, DR3, DR4 and DR5 (5). Intracellular sensors monitor the cell's well-being and activate the death pathway in response to detecting abnormalities, including DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia (37).

Many of these signals then function through the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis. Bcl-2 family members that are either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function in part by governing mitochondrial death signaling through cytochrome C release. The p53 tumor suppressor protein can elicit apoptosis by upregulating expression of proapoptotic Bax in response to sensing DNA damage; Bax in turn stimulates mitochondria to release cytochrome C (61). Cytochrome C and death receptors such as FAS then activate a group of intracellular proteases, termed caspases, which execute the death program through selective destruction of subcellular structures (164).

There is evidence suggesting that overexpression of oncogenes such as myc and Src can induce apoptosis in fibroblasts upon serum withdrawal (78, 191). It is reasonable to assume that elimination of mutant cells with activated oncogenes by apoptosis would be the primary defense mechanism against tumorigenesis. On the other hand, successful cancer cells must avoid apoptosis. This can only be achieved by acquiring further mutations, including the inactivated mutants of p53 gene which are found in over 50% of human cancers (64). Antiapoptotic mechanisms also include the PI3 kinase–AKT/PKB survival pathway which can be activated by extracellular factors such as IGF-1/2 or IL-3 (37), intracellular signals emanating from Ras (33), or loss of the PTEN tumor suppressor, a phospholipid phosphatase that normally attenuates the AKT survival signal (19). We have shown that phospholipase D provides an alternative survival signal other than PI3-Kinase/AKT pathway in some human cancer cell lines (22).

*Unlimited proliferation potential*

Human cells in culture can only divide about 60-70 times. Considering the multitude of barriers cancer cells face and the low mutation rates, 60-70 times of doubling does not allow cancer to develop into macroscopic tumors (11, 161). The counting device for cell generations is the ends of chromosomes, known as telomeres, which get shortened by each dividing in normal cells. Telomere maintenance is evident in stem cells and in virtually all types of malignant cells (148) to give the cancer cells unlimited proliferation ability. The concept of cancer stem cells suggests that only a small portion of the cells in a tumor retain the proliferation ability (126). It is not clear whether the cancer stem cells originate from normal tissue stem cells or from differentiated tissue cells (126), but in any case, these cancer stem cells have acquired unlimited proliferation ability.

*Sustained Angiogenesis*

All cells have to reside within 100μm of a capillary blood vessel because of the need for oxygen and nutrients. To ensure that, the growth of new blood vessels, angiogenesis, is conducted under precise control during lots of normal physiologic activities such as organogenesis and wound healing. After a tissue is formed, angiogenesis is dormant and under careful regulation. Cancer cells also need oxygen, nutrients, and probably some growth factors in the blood. So for cancer cells to develop from the incipient micro colonies into a sizable tumor, angiogenesis must be induced. Evidence has shown that the cells within aberrant proliferative lesions initially lack

angiogenic ability, thus limiting their capability for expansion. In order to progress to a larger size, incipient neoplasias must develop angiogenic ability (16, 43, 60).

There are currently more than two dozen known angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF1/2), and a similar number of endogenous inhibitor proteins including thrombospondin-1 (44). Integrin signaling also plays important role in angiogenesis (61). Tumors can achieve angiogenesis by changing the balance of angiogenesis inducers and inhibitors. Overexpression of VEGF and/or FGFs are found in many cancers, while in some other cancers, expression of endogenous inhibitors such as thrombospondin-1 is downregulated (172). p53 upregulates thrombospondin-1 level in some cells, so the loss of p53 functions, which is commonly observed in most human cancers, can contribute to the angiogenesis in these cancers (28). Activation of the Ras oncogene or loss of the VHL tumor suppressor gene in certain cell types causes upregulation of VEGF expression (111, 133).

*Invasion and metastasis*

The last and lethal stage of cancer is metastasis which is responsible for 90% of human cancer deaths (157). At some point during cancer development, the initial primary tumor cells invade the local tissue by breaching the adjacent tissues and then traveling to distant sites via blood vessels and/or the lymphatic system to form new colonies. This process is called metastasis. Invasion of the local tissues and metastasis to distant sites are closely related processes which have been intensively studied for decades but still poorly understood at the genetic and histological level. Both processes require similar

functions for breaching the tissues to settle at new sites, either at local tissues or distant parts of the body. This suggests that there may be one general property acquired during cancer development that contributes to both invasion and metastasis. Both utilize similar operational strategies involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases (61).

For invasion to happen, the cancer cells need to “squeeze” through the surrounding extracellular tissues which requires the cancer cells to be able to 1) survive with little or no cell-cell contact with fellow cancer cells, 2) secrete proteases such as matrix metalloproteinase-9 (MMP-9) which helps to breakdown the tissues, and 3) move mechanically by morphological changes such as pseudopodia formation and relocation of focal adhesion points. For metastasis to happen, invading cancer cells need to breach blood vessel and/or lymphatic tube epithelial cells, enter the circulation and/or lymphatic system. Although most cancer cells are less anchorage-dependent than normal tissue cells, most cancer cells would still die in circulation. Only very few cancer cells can survive and then stick somewhere along the circulation path, usually at capillaries. There, even fewer cancer cells can again breach the capillary epithelial wall into the local tissues and form secondary tumors. The regulatory circuits and molecular mechanisms that govern metastasis remain elusive and, at present, seem to differ from one tissue environment to another (61).

### 1.1.3 Evolutionary puzzle of cancer progression

As discussed above, cancer development is a multistep evolutionary process. At each step, cancer cells encounter a new proliferation-limited factor, thus only cell clones

with right mutation can be selected for. This selection process continues until tumor cells finally become malignant cancers. Weinberg's cancer model of six physiological changes is a good summary of this process (61).

However, as pointed out by Weinberg himself, this prevailing model carries with it a striking conceptual inconsistency: the genes that give rise to the final step of cancer progression— metastasis — would not seem to confer increased proliferative benefit at the primary site. That is, there is no reason to think that a metastatic phenotype enables cells to proliferate more effectively within the primary tumor mass, thereby increasing their representation in the overall tumor-cell population. Hence, rare cells in the primary tumor mass that happen to acquire metastatic capability will remain rare. As the success rate of individual cells undertaking metastasis is extraordinarily low, this makes it difficult to imagine how metastasis can ever proceed (12).

Weinberg suggested that the mutations required for metastasis happened in earlier stages of tumorigenesis (12). It is apparent that tumorigenesis can take different paths. Each physiological change necessary for tumorigenesis can be acquired through one of many gene mutations. On the other hand, some gene mutations such as the loss of functional p53 gene can contribute to more than one of six physiological changes. So, it is possible that certain mutations at the earlier stage of tumorigenesis not only give proliferative advantages to the mutant cells at the time, but are also critical for the metastasis, which happens much later. This idea has three implications. First, metastasis ability may be determined at a much earlier stage of cancer. Second, the genes responsible for metastasis may be among the genes which have been studied for decades.

Third, even relatively small primary tumors may already have the ability of metastasis (12).

This argument is supported by several lines of evidence. In some cases of small, well-localized human breast cancers, individual carcinoma cells were found in bone marrow. And, revealed by DNA microarray analysis, metastasis tumor cells are often strikingly similar to the primary tumor cells, suggesting that the dominant population of primary tumor cells is identical to the metastasis cells (12). Practically, this means that the early detection in at least some cancers may not save life, given the current technology and practices. The effectiveness of breast cancer early detection has been broadly studied by many groups. For example, in a 10 year study of more than 266,000 women in China, breast self exam (BSE) provided no survival benefit, but more tumors that were smaller and benign were detected (163). And similar conclusion was drawn by Canadian researchers who analyzed all major studies on breast self-examination conducted between 1966 and October 2000 (9). Other than breast self exams, the benefit of mammograms is also questionable (54, 114, 115, 124, 125). Olsen and Gotzsche reviewed the seven randomized mammogram trials and concluded that mammograms do not give a substantial survival benefit. Their opinion has been broadly criticized and debated (30, 34, 48, 95), but the supporting evidence is also there (13, 114, 115).

### 1.1.4 Ask the right questions

At the early stage of cancer development, the scheme could be like this:

Most of the originally mutated cells with proliferation advantage could develop into microscopic colonies. Because of the defensive apoptotic pressure, most of these colonies would remain small in size or eventually be eliminated. Only few cells in the colony may acquire antiapoptotic mutation which give it the proliferative advantage over other cells in the colony. These cells soon become the dominant genotype of the colony. With these colonies being established, what comes next?

Facing no immediate urgency, it is feasible to speculate that the other three physiology changes necessary for a malignant cancer including unlimited proliferation potential, sustained angiogenesis, invasion and metastasis, may be acquired at any time point later by further mutations, without a specific order. For example, some cancers may develop angiogenesis first, while others may acquire the unlimited proliferation ability, or local tissue invasion ability first. Depending upon the genotype of particular cancer and the local tissue environment, different approaches to achieve these physiology changes may result in differences in cancer progression.

With the six step cancer model and the progression puzzle in mind, we can ask more questions. Is it simply a coincidence that the mutations which give proliferative advantages at early stage of tumorigenesis also confer the metastasis ability much later? Or were these mutations somehow selected for? Since invasion of local tissue is closely related to metastasis, could the invasion trait be the one that is selected for? If so, how and when does this happen? Work generated in this thesis indicates that PLD not only provides a survival signal but also confers enhanced migration/invasion capability of

cancer cells. Thus the elevated PLD activity may provide a mechanism for the metastasis trait to be selected for in an early stage of tumorigenesis.

## 1.2 Introduction to phospholipase D

### 1.2.1 Phospholipase D functions

In higher eukaryotic organisms, the generation of "lipid second messengers" at the site of ligand-receptor interactions has been indicated to provide varied, rapid and complex responses to extracellular signals (159). The phospholipids-metabolizing enzymes producing such messengers have been under investigations for decades. Among these enzymes, PI kinases and phospholipase C (PLC) are the most widely studied (166). They both modify phosphatidylinositol (PI). For the past 15 years or so, another such enzyme, phospholipase D (PLD) has also emerged as a critical component of intracellular signal transduction. Phospholipase D (EC 3.1.4.4; PLD) was first discovered in plants. However, widespread interest in this enzyme began once experiments in cultured animal cells revealed its rapid and dramatic activation to extracellular stimuli (24, 40).

Phospholipase D is a ubiquitous enzyme that hydrolyzes phosphatidylcholine to produce phosphatidic acid (PA) and free choline headgroup. While choline was indicated to be involved in the regulation of cell growth (69), it is the PA signaling pathway that has been better studied and considered to be most critical for the PLD's functions. PA itself can function as second messenger to activate various kinases including the mammalian target of rapamycin (mTOR) (41). PA can also be further converted to diacylglycerol (DAG) and lyso-PA, both of which have second messenger functions that could contribute to the effects of PLD (38, 39).

PLD has been implicated in a lot of cellular activities including membrane trafficking, cytoskeletal reorganization, receptor endocytosis, exocytosis, and cell migration (38, 39). Recent studies also suggest that PLD is involved in mitogenic activities (45). First, PLD activity has been reported to be elevated in response to various growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor(FGF), epidermal growth factor (EGF), insulin, insulin –like growth factor 1 (IGF1), growth hormone, and sphingosine 1-phosphate. Second, PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src, v-Ras, v-Fps, and v-Raf (45).

### 1.2.2 PLD enzymatic reactions

PLD hydrolyzes the distal phosphodiester bond in phospholipids such as phosphatidylcholine (PC), transiently forming a phosphatidyl-enzyme intermediate which is normally hydrolyzed by water, generating phosphatidic acid (PA) (Fig. 1.1A) (62). Primary short-chain alcohols (e.g. 1-butanol, ethanol) are much more preferred by PLD in this reaction as a substitute of water. So, in the presence of 1-butanol, for example, PLD will utilize 1-butanol instead of water in a transphosphatidylation reaction to produce phosphatidylbutanol instead of PA (Fig. 1.1B). While PA can be generated not only by PLD, but also by diacylglycerol kinase and by acylation of glycerol 3-phosphate, phosphatidylalcohols are uniquely formed by PLD. On the other hand, PA can be further metabolized to diacylglycerol (DAG) and lyso-PA (LPA), while as phosphatidylalcohols are metabolically stable and will accumulate in cells upon PLD activation. Because cellular phosphatidylalcohol levels are normally extremely low, their accumulation upon

PLD activation is readily detectable. This means two things. First, we can use primary short-chain alcohols to block the PA production by PLD, essentially inhibiting the PLD physiological functions. Second, with the presence of primary short-chain alcohols, the production and accumulation of phosphatidylalcohol can be a good indicator of PLD activity *in vitro* and *in vivo* (89, 107, 112, 174).

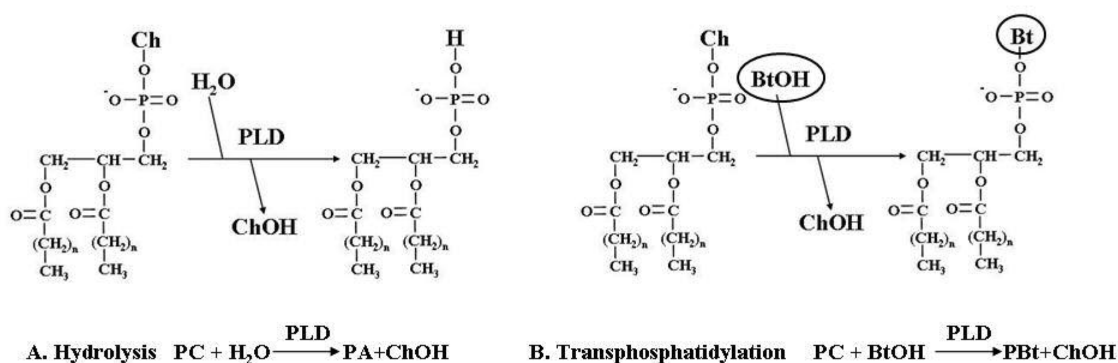


Figure 1.1 Phospholipase D (PLD) Catalyzed Reactions

### 1.2.3 PLD isoforms

Two mammalian PLD genes (PLD1 and PLD2) with about 50% homology have been reported. PLD1 has two splice variants, hPLD1a and hPLD1b, but very little is know about differences between these variants (25). Structures of PLD1 and PLD2 are shown in (Figure 1.2). Both PLD1 and PLD2 have the conservative regions I-IV. N-

terminal of PLD1 and PLD2 contains pleckstrin homology (PH) and phox homology (PX) domains (160). Phosphatidylinositol 4,5-bisophosphate ($PIP_2$) is required for PLD activity and PIP2 binds at the PH domain and at a conserved sequence located between conserved sequences II and III (73, 144). C-terminal of PLD1 and PLD2 has four conserved amino acids which are critical for catalytic activity (101, 180). The amino acid sequence of PLD1 contains regions that are conserved with PLD2 as well as other non-mammalian species. In addition it contains a "loop region" that is unique to PLD1. Possible functions that have been proposed or demonstrated for these regions are shown in Figure 1.3.

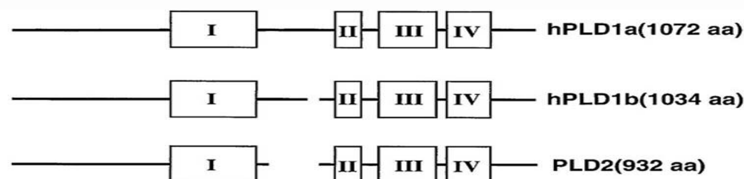


**Figure 1.2 Conserved structures of mammalian PLD1 and PLD2 (39)**

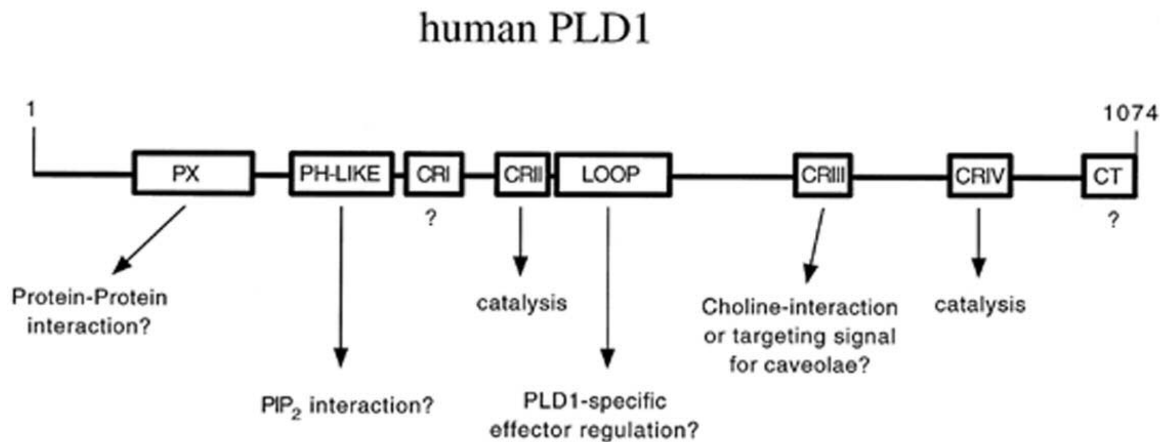


**Figure 1.3 Conserved and unique features for human PLD1 (160).** The PLD1 amino acid sequence encodes regions of sequence that either is unique to PLD1 (loop region) or is conserved with mammalian PLD2 and some or all PLD's from non-mammalian species (other boxed regions). Possible functions that have been proposed or demonstrated for these regions are listed underneath each box. (CR, conserved region; CT, carboxyl terminus; LOOP, loop region).

PLD1 and PLD2 also have different subcellular localizations. Although the exact location of either isoform has been inconclusive, it is clear that PLD1 and PLD2 are both membrane-associated through palmitoylation on conserved Cys residues. PLD1 is found throughout the cell but particularly in perinuclear, Golgi, and heavy membrane fractions. In contrast, PLD2 is localized almost exclusively on the plasma membrane in light membrane "lipid raft" fractions that co-fractionate with caveolin. PLD1 can also be found in the lipid rafts (45).

Both PLD1 and PLD2 activity require PI-4,5-bisphosphate (PIP2). PLD1 is activated by the ADP ribosylation factor (ARF)-, Ral-, and Rho- family GTPases, as well as by protein kinase C alpha (PKC-α). PLD2 is activated by fatty acids. PLD2 is constitutively active in *vitro* and this activity is unaffected by GTPases or PKC-α. Characters of PLD isoforms are summarized in Table 1.

| CHARACTERISTICS | PLD1 | PLD2 |
|---|---|---|
| PKC/ARF/Rho Responsive | Yes | No |
| PIP2 Dependence | Yes | Yes |
| Molecular Weight kDa | ~120 | ~106 |
| Basal activity | Low | High |
| Substrate Specificity | PC | PC |
| Transphosphatidylation | Yes | Yes |
| Subcellular localization | PM,CEM,ES | PM,CEM |

**Table 1 Characteristics of PLD1 and PLD2**

### 1.2.4 PLD signaling pathways

*Upstream factors of PLD*

Several small GTPases have been reported to activate PLD1 in vivo and in vitro, including ARF-, Ral-, and Rho-family members (39). These GTPases are involved in cell membrane trafficking, cytoskeleton regulation, and survival signal pathways, coincidently but not surprisingly, PLD also plays a role in these functions.

ADP-ribosylation factor (ARF) family of small GTP-binding proteins plays a central role in membrane trafficking and cytoskeletal remodeling (102). ARF proteins were reported to be required for PLD activities elevated by various mitogenic factors including PDGF, EGF, insulin, phorbol esters, and H-Ras (45), although it is not clear how ARF gets activated by mitogenic signals. Among the members of ARF family, ARF6 co-localizes with PLD1 (131) and ARF6 is localized in the lipid raft fractions, while ARF4 was recently implicated in the activation of PLD2 (93).

Rho-family GTPases regulate cell membrane traffic and actin dynamics. Rho-family members were implicated in mitogenic signaling through PLD (46, 70), and more commonly Rho-family members were shown to mediate PLD responses through agonists that stimulate secretion (131).

RalA mediates Ras signaling, and directly interacts with PLD1(85, 105). Although RalA alone can not activate PLD1 either in vitro or in vivo (85), it is required for the activation of PLD activity by EGF, PDGF, insulin, Src, Ras, Raf, and phorbol esters (45). These evidence suggest that RalA is critical in PLD response stimulated by mitogenic or oncogenic signals, possibly by working together with Arf6 (45).

Other than small GTPases, Phosphatidylinositol 4,5-bisphosphate (PIP2) is also required for both PLD1 and PLD2 activation. PIP2 can be generated by various PI kinases including PI-4-P 5-kinase, while PI3-kinase, on the other hand, converts PIP2 to PIP3. PI-4-P 5-kinase is a downstream target of PLD signaling, so by generating PIP2, it may provide a positive feedback loop. Since both Arf6 and RalA stimulate PLD activity through interaction with PLD1, but evidences suggest that PLD2 is the major responsible

isoform for the mitogenic signal stimulated PLD activity (181), and that PLD2 activation was suggested to be dependent on the activation of PLD1 (117), we proposed a model (Figure 1.4) in which mitogenic signals stimulate PLD1 activation through the interaction of Arf6 and RalA, which then lead to the activation of PLD2 which mainly reside on the lipid rafts of the plasma membrane (45).

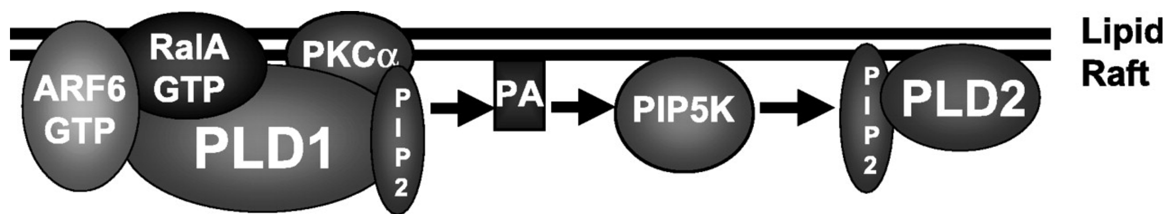


**FIGURE 1.4 Mitogenic signaling through PLD1-dependent activation of PLD2.** In this model, it is proposed that PLD1 is activated through the synergistic actions of ARF6 and RalA in lipid rafts. PIP2 is required for both PLD1 and PLD2 activity. On activation of PLD1, all available PIP2 is used by PLD1, which is present in low levels in the lipid rafts. PLD2, which is present at high levels in lipid rafts, is inactive in the absence of sufficient PIP2. The generation of PA by PLD1 would activate PI-4-P 5-kinase (PIP5K), which would then generate sufficient PIP2 to activate the high levels of PLD2.

*Downstream targets of PLD*

While lots of evidence indicates PLD involvement in various cell activities, the relevant downstream targets of PLD are still mostly elusive. Raf has a PA binding site and its association with PA was implicated to facilitate the recruitment of Raf to the plasma membrane, where it can participate in the activation of the mitogen-activated protein (MAP) kinase pathway (45, 136).

The mammalian target of rapamycin (mTOR) has recently emerged as an important downstream target of PLD. mTOR is a protein kinase directly involved in both cell cycle progression and cell growth by regulating translation, transcription, membrane traffic, and protein degradation (96, 141), and mTOR is also a downstream target of PI-3-kinase/Akt survival pathway (121, 145). It's recently reported that PA binds to mTOR competitively with rapamycin and is required for activation of mTOR (20, 41). Consistently, elevated PLD activity seems to confer resistance to rapamycin in some human cancer cells (23). All these evidence suggest the role for mTOR as a particularly important downstream target of PLD signaling pathway.

### 1.2.5 PLD and cancer: survival advantages provided by PLD

As discussed above, in the PLD signaling pathway, a lot of components including upstream RhoA, Arf6, RalA, and downstream target mTOR are involved in critical cell functions including cytoskeleton modulation, growth control and survival signaling. These functions are vital for the cell and are also common targets of tumorigenesis process. Among the multiple paths of cancer development, could an altered PLD

signaling pathway be one of the components? The answer seems to be “yes”. Elevated expression of PLD1 and elevated PLD activity was reported in breast cancer tissues (122, 168). PLD activity has been shown to be elevated in other human cancers including renal cancers (189), gastric cancers (167). And a polymorphism of the PLD2 gene was recently reported to be associated with the prevalence of colorectal cancer(182).

PLD can cooperate with tyrosine kinase to transform rat fibroblasts. While overexpression of tyrosine kinase c-Src alone in 3y1 fibroblast cells result in apoptosis, inducing PLD1 or pLD2 into these cells can rescue the cells from apoptosis (88). Unlike c-Src, v-Src can upregulate PLD activity, and overexpression of v-Src alone in 3y1 fibroblast is enough for cell transformation without causing apoptosis (88, 191). Overexpression of tyrosine kinases such as Her-1 Her-2/neu, EGFR, ErbB, Src is common in various types of cancers (67, 129, 134). This strongly suggests that the PLD is involved in cancer development, possibly by providing survival signals which help cancer cells to avoid the apoptosis induced by other cancerous mutations (45).

On the other hand, since Arf-, Rho- family GTPases are involved in cytoskeleton modulation and evidence also suggests a direct PLD and actin stress fiber interaction (90, 97, 100, 130), it is reasonable to speculate a role for PLD in the cancer invasion and metastasis, because these process require deregulated and enhanced cell motility and cytoskeleton dynamics.

The work of this thesis focuses on the role of PLD as an alternative survival signal provider in various human cancers and the PLD’s involvement in human cancer

migration/invasion. A discussion of how these two functions facilitate the tumorigenesis and possibly result in metastasis is also included.

# Chapter 2: methods and materials

*Materials*. 1-Butanol (1-BtOH), *t*-BtOH, cytochalisins B and D, colchicine and Rapamycin were obtained from Sigma. [$^3$H]Myristic acid was obtained from PerkinElmer Life Sciences. Honokiol were kindly provided by Dr. Jack Arbiser (Emory University). The secondary antibodies to rabbit or mouse immunoglobulin conjugated with horseradish peroxidase were from Bio-Rad. The anti-mouse immunoglobin conjugated with Rhodamine Red-X and the anti-rabbit immunoglobin conjugated with cyanine were from Jackson ImmunoResearch. Antibodies for AKT, p-AKT, hypoxia-inducible factor-1 $\alpha$ (HIF1 $\alpha$ ), actin, HA and hemagglutinin were purchased from Santa Cruz Biotechnology. Precoated silica 60A thin layer chromatography plates were from Whatman. Plasmid expression vectors for PLD2 (pCGN-mPLD2) and mPLD2-K758R (pCGN-mPLD2-K758R) (25) were the generous gift of Dr. Michael Frohman (SUNY, Stony Brook). Migration and Invasion Boyden chambers were from BD-bioscience. Diff-Quik stain set were from Dade Behring Inc.

*Cell culture conditions*. Parental and 3Y1 rat fibroblasts transformed by v-Src ($3Y1^{v\text{-}Src}$ cells) have been described previously (188). These cells and the stable or transient transfectants derived from them were maintained at 37 °C in a humidified 5% $CO_2$ atmosphere, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) bovine calf serum. Cells were grown to 50% confluency and then made quiescent by replacing fresh DMEM containing 0.5% serum one day before the experiment. All human cancer cell lines used in this study

were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium with 10% bovine calf serum.

*Transfection and cell line establishment.* 3Y1 and 3Y1$^{v\text{-}Src}$ cells that overexpress recombinant PLD proteins were obtained by transfection of plasmids encoding human PLD1, mouse PLD2 or the catalytically inactive mutants human PLD1-K898R or mouse PLD2-K758R, using LipofectAMINE Plus reagent (Life Technologies) according to vendor's instructions. Transient transfectants were made quiescent 24 h after transfection and used for experiments 48 h after transfection. Stable transfectants were obtained by cotransfection with the hygromycin B selection vector pCEP4 (Invitrogen). Transfected cultures were selected with hygromycin B (200 mg/ml) 24 h after transfection for 10–14 days. At that time antibiotic-resistant colonies were picked, amplified under selective conditions, and used for experiments. All of the PLD proteins expressed were Flu-tagged and could be detected using anti-HA antibody raised against the Flu epitope. Transfections on human cancer cell lines were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was determined by transfection of pEGFP-C1 (Clontech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 70%. The generation of MCF-7 cells expressing PLD2 (MCF-7-P2 cells) has been described previously (79). These cells represent a pool of clones selected for G418 resistance as described (79).

*Western Blot Analysis*—Extraction of proteins from cultured cells and Western blot analysis of extracted proteins was performed using the ECL system (Amersham Biosciences) as described (103).

*PLD activity assays*. Cells were placed in 60 mm culture dishes in DMEM with 10% Bovine calf serum for 1 or 2 days to reach 70-80% confluency, then the medium were changed to fresh DMEM with desired serum% according to each experiment for 24 hours. Then cells were labeled for 4 h with [$^3$H]myristate (40 Ci/mmol) at a final concentration of 1 µCi/ml, followed by 15 min of incubation with 0.8% (v/v) 1-BtOH. Afterwards, cells were put on ice, washed twice with cold phosphate buffered saline, and collected in 0.5 ml of methanol/6 M HCl (50:1, v/v). Lipids were extracted by adding 0.5 ml of chloroform. Phase separation was achieved by the addition of 155 µl of 1 M NaCl and the organic phase was recovered after centrifugation. This was followed by reextraction through the addition of 350 µl $H_2O$, 115 µl 1 M NaCl, and 115 µl methanol. An aliquot of thus obtained organic phase was then counted in a liquid scintillation counter for normalization. Lipids of equal amount of radioactivity were dried under a stream of nitrogen and redissolved in 50 µl of chloroform/methanol (9:1, v/v). Samples were then spotted on precoated silica (60 A) plates and separated by thin layer chromatography with a solvent system of ethylacetate/trimethylpentane/acetic acid/$H_2O$ (9:5:2:10). The product of metabolically labeled PBt was visualized by autoradiography of the thin layer chromatography plates and the films were scanned on a densitometer for quantification.

*Migration and Invasion Assays (Boyden chamber assay)* —The assays were carried out using BIOCOAT™ cell culture inserts that had polyethylene terephthalate filters (8-µm

pore size) on the bottom. For migration assays, inserts were used directly without coating; and for invasion assays, the inserts were coated with Matrigel™ purified from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins, which closely mimics the basement membrane *in vivo*. Single cell suspensions in varying serum concentrations were added into the inserts. The inserts were set into 24-well plates that held 0.75 ml/well growth medium with the indicated serum concentration and incubated under normal growth condition for 24 h. Cells that had not penetrated the filters were wiped out with cotton swabs, and cells that had migrated or invaded to the lower surface of the filters were fixed and stained by Diff-Quik Stain set. The number of migrated or invaded cells was counted under microscope. The mean of three individual fields was obtained for each well.

# Chapter 3: PLD generates survival signals in human cancers

Our lab has been working on the hypothesis that PLD provides proliferation and survival signals in some human cancers. I participated in some of these studies, the results of which are directly related to the topic of this thesis. This chapter will summarize these results and discuss possible mechanisms by which PLD provides survival signals in cancers.

## 3.1 PLD confers rapamycin resistance in human breast cancer cells (23)

mTOR (mammalian target of rapamycin) is a protein kinase regulating cell cycle progression and cell growth (63). Rapamycin binds to the immunophilin FK506-binding protein 12 (FKBP12) which then binds mTOR and inhibits its function in a poorly understood fashion (20).As a highly specific inhibitor of mTOR, rapamycin is in clinical trial for treatment of breast and other cancers. It has been reported that mTOR activation requires phosphatidic acid (PA) binding which was proposed to be competitive to rapamycin-FKBP12 binding on the mTOR (20, 41). Since PA is a product of PLD and PLD was elevated in most of the tested breast cancers (122), we investigated the effect of rapamycin in breast cancer cell lines with different levels of PLD activity (23).

We examined three breast cancer cell lines. MCF-7 is a less aggressive cancer cell line. MDA-MB-231 cell line, on the other hand, grows fast and is highly invasive. MCF-7R cell line is a fast-growing variant of MCF-7 cells. As shown in Figure3.1 (23), we found a correlation between PLD activity and rapamycin resistance on the cell proliferation rate of these cells. MCF-7 cells have low PLD activity and are highly

sensitive to rapamycin with an IC50 of 20nM; MDA-MB-231 cells have high PLD activity and are resistant to rapamycin with an IC50 of 10uM. MCF-7R cells are intermediate in both respects.

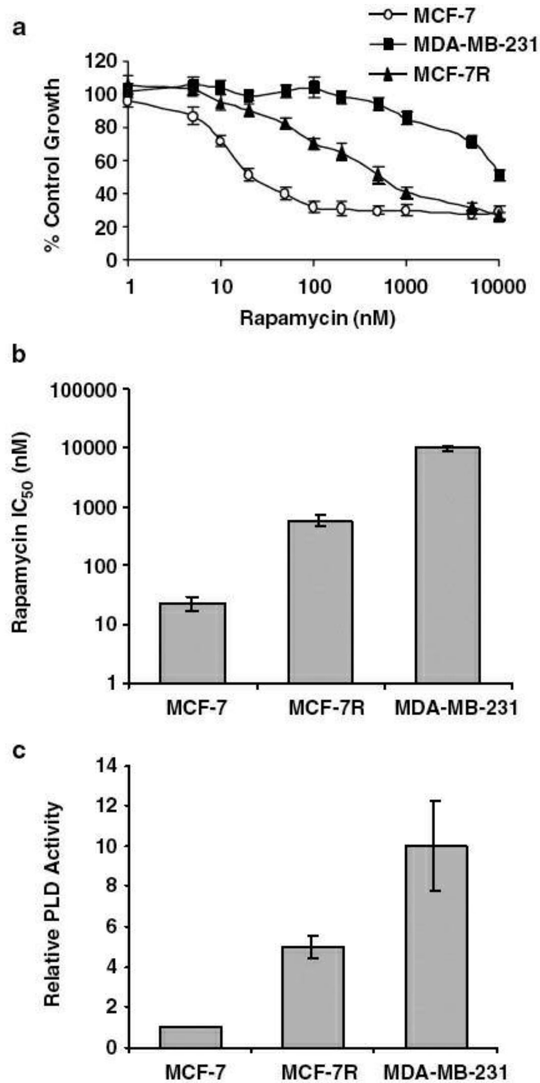


**Figure3.1 Increasing PLD activity in breast cancer cells correlates with resistance to rapamycin.** (borrowed from (23)) (**a**) MCF-7, MCF-7R, and MDA-MB 231 cells were plated in 96-well culture plates in DMEM with 10% bovine calf serum. The MCF-7 cells were plated at 6000 cells per well; the MCF-7R and MDA-MB 231 cells were plated at 3000 cells per well. After 24 h, rapamycin was added with increasing concentration as shown. At 2 days later, metabolically active cells were determined using Promega's CellTiter 96 AQueous cell proliferation assay according to the manufacturer's protocol (Promega) and described by (187). The effect of rapamycin on cell proliferation was calculated as the percentage of metabolically active cells relative to the untreated controls, which were given a value of 100%. The data shown are the average of three independent experiments with error bars representing the standard deviation. (**b**) $IC_{50}$ values, the concentration of rapamycin required to inhibit proliferation by 50%, were averaged over three independent experiments. Error bars represent the standard deviation. (**c**) PLD activity in the MCF-7, MCF-7R, and MDA-MB 231 cells was determined as described previously (149). The PLD activity in the cell lines were normalized to that in MCF-7 cells, which was given a value of 1. Error bars represent the standard deviation for three independent experiments

Transient expression of PLD2 in MCF-7 cells results in a three fold increase of PLD activity and also an increased resistance (IC50 from 20nM to about 200nM) to rapamycin for the cell proliferation inhibition. On the other hand, introduction of catalytically inactive PLD2 mutant into MDA-MB-231 cells reduced PLD activity in these cells by 60% and reduced resistance (IC50 from 10uM to 100nM) to rapamycin for the cell proliferation inhibition (23)(Data not shown). This data suggests the involvement of PLD in the mTOR-dependent proliferation signaling pathway in these cancer cells.

## **3.2 PLD provides survival signals in MDA-MB-231 cells.** (191)

Upon serum starvation, normal cells and some cancer cells will go into apoptosis after a short period of time. However, MDA-MB-231 cells can survive well under the same condition. We investigated the PLD involvement in the survival of MDA-MB-231 upon serum starvation.

As mentioned in the introduction, 1-butanol and iso-butanol can block the production of phosphatidic acid (PA) by PLD while 2-butanol and t-butanol can not. MDA-MB-231 cells will not go into apoptosis upon serum starvation, but 1-butanol and iso-butanol can induce apoptosis in these cells upon serum starvation, as indicated in Figure 3.2A by cleaved Parp protein and in Figure 3.2B by percentage of non-viable cells. 2-butanol and t-butanol here serve as controls showing the apoptotic effect is not merely induced by any alcohol. Expression of HA-tagged catalytically inactive PLD1 or PLD2 in MDA-MB-231 cells also lead to apoptosis upon serum starvation, as shown in Figure 3.2C. This data suggests that PLD signaling is required for the survival of MDA-MB-231 cells under serum starvation- induced stressful conditions.

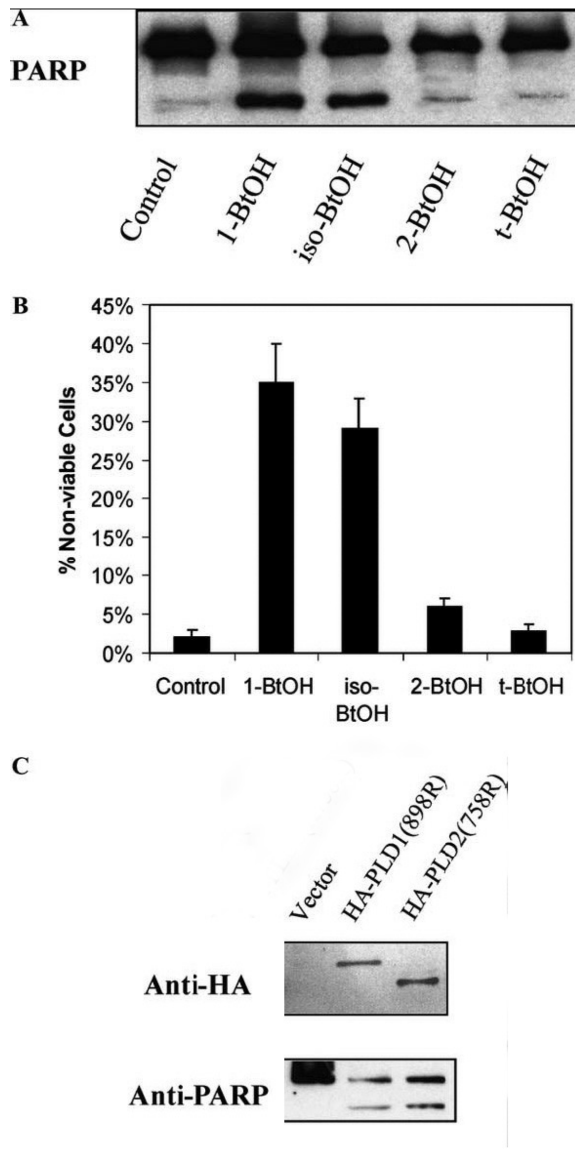


**Figure 3.2 Inhibiting PLD activity induces apoptosis in serum starved MDA-MB-231 cells** (borrowed from (191))**.** MDA-MB-231 cells were placed in DMEM without serum for 14 h, at which time the cells were treated with the indicated alcohols (1%) for an additional 6 h. PARP cleavage (**A**) and the percentage of non-viable cells (**B**) were determined then. (**C**) Vectors expressing HA-tagged catalytically inactive mutants of PLD1 (K898R) and PLD2 (K758R) were transiently transfected into the MDA-MB-231. Twenty hours after transfection, PARP cleavage was determined in the absence of serum. PLD expression was verified by Western blot analysis using an antibody that recognizes the HA tag as shown.

## 3.3 mTOR dependent PLD survival pathways

### 3.3.1 Alternative PLD/mTOR survival signal in human breast cancer cells

Cancer cells need to generate survival signals to overcome the apoptosis induced by the organism's anti-cancer mechanism. The best studied and frequently dysregulated survival pathway in human cancers is the Phosphatidylinositol-3-kinase (PI3K)/AKT pathway. PI3K phosphorylates PI-4,5-bi-phosphate (PIP2) to generate PI-3,4,5-tris-phosphate (PIP3) which leads to the activation of Akt protein kinase. Akt then phosphorylates and subsequently, inactivates many substrate proteins that negatively regulate cell cycle progression and/or facilitate apoptosis (171). PTEN, a phosphatase that dephosphorylates PIP3 to PIP2, is frequently lost or inactivated in many human cancers, thus activating the PI3K/AKT survival pathway (171).

One downstream effector of PI3K/AKT pathway is mTOR, a protein kinase that regulates cell cycle progression and proliferation by phosphorylating several substrate proteins that regulate protein synthesis. It has been proposed that regulators of protein synthesis provide survival signals (138). mTOR was also proposed to be a downstream target of PLD through PA interaction, and mTOR inhibitor rapamycin interferes with mTOR in a competing fashion with PA (20, 41). As aforementioned, we have found that PLD confers rapamycin resistance for the cell proliferation in some human cancers (23), and can provide survival signals in MDA-MB-231 human breast cancer cells (191). Thereby, we further investigated the role of PLD in human cancer survival pathways.

Our data indicates that elevated PLD activity in the MDA-MB-231 human breast cancer cell line generates an mTOR-dependent survival signal that is independent of PI3K. In contrast, MDA-MB-435S breast cancer cells, which have very low levels of PLD activity, are dependent on PI3K for survival signals. These results suggest that an alternative survival signal that is dependent on PLD and mTOR could be active in breast cancers where the PI3K/AKT survival pathway is not active, as illustrated in Figure 3.3 (22).

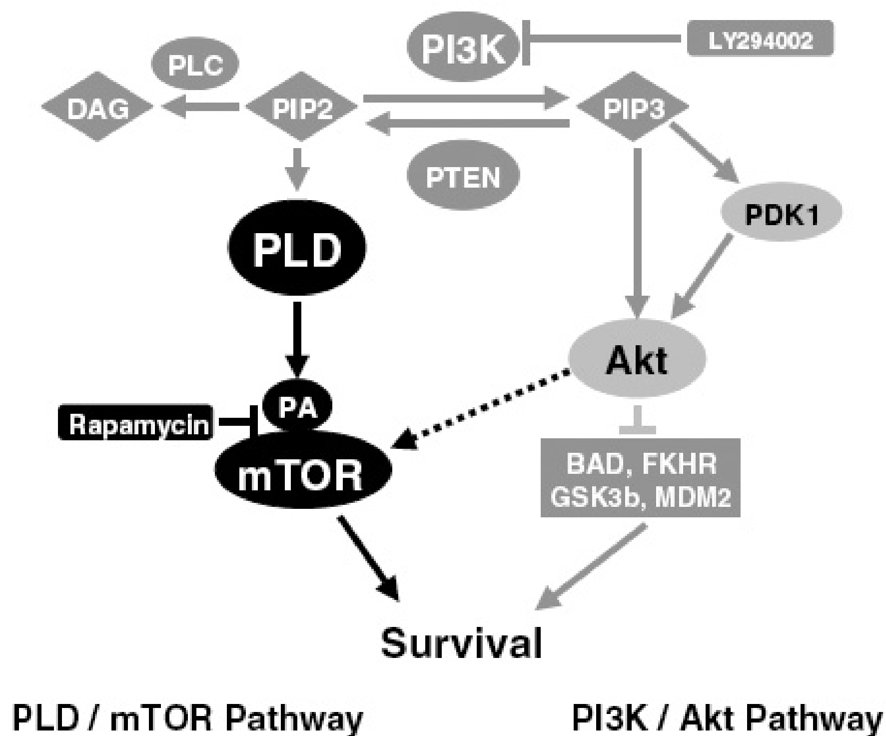


**Figure 3.3 Alternative survival signaling through the PLD/mTOR and PI3K/Akt pathways (22).** Alternative survival signals are generated by the activation of the lipid modifying enzymes PLD and PI3K. PI3K generates PIP3, which recruits PDK1 and Akt. PDK1 phosphorylates and activated Akt. Akt then phosphorylates and inactivates several substrate proteins that negatively regulate cell proliferation or stimulate apoptosis. These include

GSK3β, BAD, forkhead family transcription factors (FKHR) and MDM2 (171). Akt also activates mTOR indirectly which indicates that there is overlap in the two pathways. PLD generates PA which leads to the activation of mTOR. The two pathways are also connected by their different lipid requirements. Akt requires PIP3 and PLD requires PIP2, thus the generation of PIP3 from PIP2 by PI3K would deplete PIP2, whereas PTEN would generate PIP2 from PIP3. It is also possible that PLC could be a critical negative regulator of both survival pathways by removing PIP2, thus shutting down both pathways

### 3.3.2 PLD/mTOR survival signal is mediated by the suppression of PP2A and activation of Myc

Phosphorylation of S6-kinase and 4E-BP1 by mTOR (65) leads to the translation of select mRNA transcripts important for cell survival (141). Protein phosphatase 2A (PP2A) dephosphorylates the S6-kinase and 4E-BP1 (128). SV40 small t-antigen (21, 57), which interacts with and suppresses PP2A, is required for transformation of human cells by H-Ras (56).Thus, it has been proposed that PP2A is a tumor suppressor gene (169). The ability of PLD to cooperate with a signaling oncogene to transform cells (88, 103) is similar to what SV40 early region genes be able to accomplish in cell transformation and tumorigenesis. We therefore asked whether elevated PLD activity, similar to SV40 small t-antigen, suppresses PP2A. Our data suggest that the elevated PLD activity in the human breast cancer cell line MDA-MB-231 causes an mTOR-dependent suppression of PP2A that is critical for the survival signals generated by PLD, as illustrated in Figure 3.4 (80).

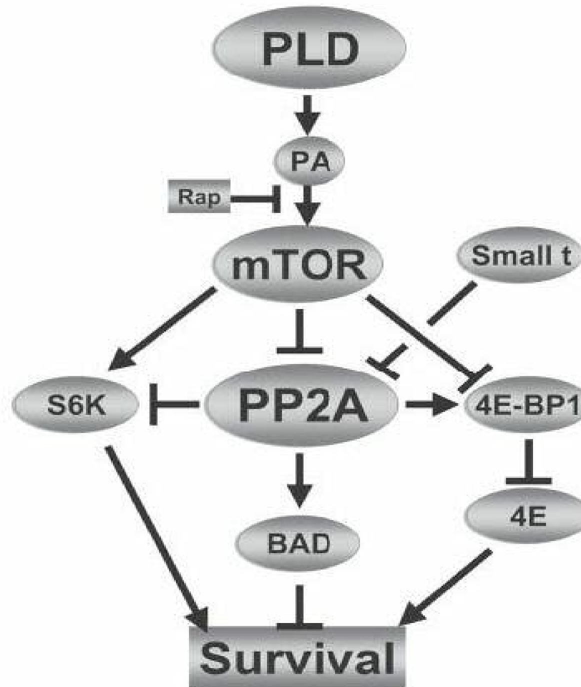


Figure 3.4 (80) Model for targeting of PP2A by PLD-generated survival signals. PLD generates phosphatidic acid (PA) from phosphatidylcholine, which can then stimulate mTOR. Activated mTOR could then phosphorylate PP2A and suppress its activity. This leads to dissociation of PP2A from the mTOR substrates S6-kinase and 4E-BP1. The suppression of PP2A also suppressed dephosphorylation of BAD at the PP2A site Ser-112. PP2A is also suppressed by SV40 small t-antigen, which is critical for the transformation of human cells.

Estrogen has long been implicated in the development of human breast cancers. Anti-estrogen drugs such as tamoxifen and aromatase inhibitors are effective in preventing breast cancer (86, 87). Estrogens, predominantly 17-β-estradiol (E2), mainly function as tumor promoters (66). One aspect of tumor promotion is the suppression of default apoptotic programs that are activated in cells with inappropriate growth signals or

damaged genomes (61). Not surprisingly, E2 has been reported to suppress apoptosis in the estrogen receptor (ER)-positive breast cancer cell line MCF-7 (2, 42, 77, 162).

About 15-20% of normal breast epithelial cells are ER positive, while 70% of breast cancers are ER positive. This suggests that ER expression was selected in the breast tumorigenesis, at least partially because ER positive cancer cells have anti-apoptotic advantages. However, most cultured breast cancer cell lines are ER negative. Also, the loss of estrogen responsiveness is a hallmark of poor prognosis in breast cancers (156). These findings seem contradictory, but they might actually mean two things. First, there is alternative pathway in ER negative breast cancer cells, compensating for the loss of estrogen induced anti-apoptotic effect. Second, estrogen-dependent breast cancers are less aggressive, and the loss of estrogen dependency could somehow make the tumors more aggressive, possibly also by the alternative pathway which substitutes the estrogen survival pathway.

Based on what's been discussed before, PLD seems to be a good candidate for this alternative pathway. We investigated the role of E2 and PLD on the apoptosis of ER+ MCF7 breast cancer cell. Our data indicates that an mTOR and PLD dependent activation of Myc is necessary for the suppression of apoptosis by E2 and that elevated PLD activity in these cells provides an estrogen-independent survival signal, as shown in the Figure 3.5 (137).

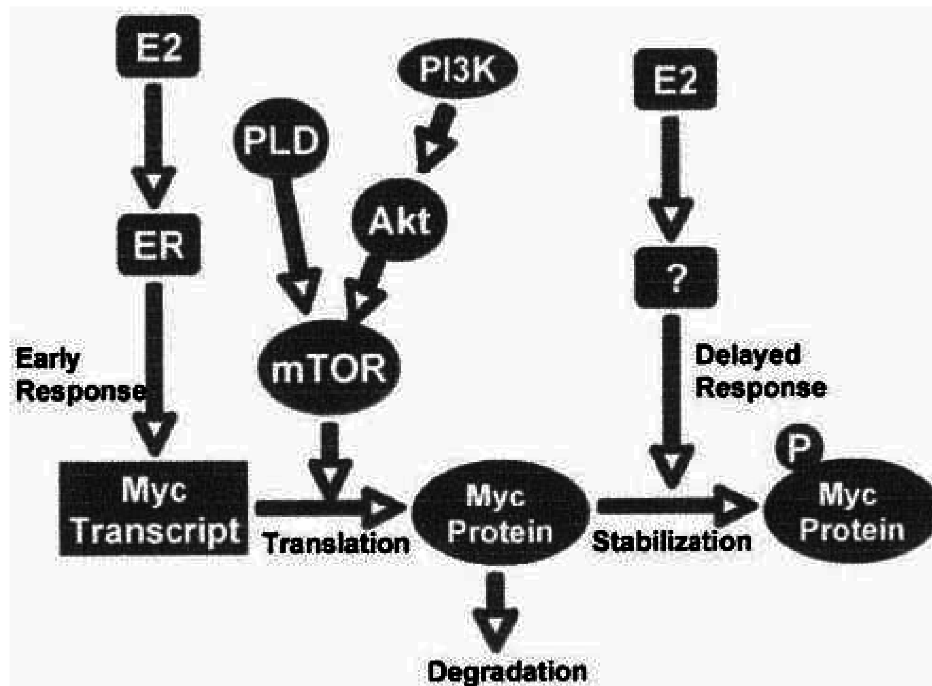


Figure 3.5 (137) Model for E2- and PLD-driven Myc-dependent survival signals in MCF-7 cells. It is proposed that E2 induces an early transient increase in Myc expression at the level of transcription. Beginning on day 2 and peaking at day 5, there is a delayed increase in Myc expression that is likely due to stabilization of Myc protein through phosphorylation (P). Although there is no increase in mTOR activity induced by E2, the delayed increase in Myc expression is dependent upon basal levels of mTOR activity and the activity of the upstream regulators PLD and PI3K. In the absence of E2, it is proposed that elevated PLD activity can increase mTOR activity to translate basal levels of Myc transcript to increase Myc expression. It is not clear whether PLD would lead to increased Myc stabilization. Elevated PLD activity provides an E2-independent survival signal that could be critical for progression to an ER-negative status in breast cancer progression.

## 3.4 mTOR independent PLD survival pathways

p53, the tumor suppressor gene, is the most commonly mutated gene found in over 50% of human cancer cells (58). While other tumor suppressor genes such as Rb and APC are commonly deleted in cancers, 85% of the p53 mutants have missense mutation (58, 72, 74). Moreover, mutant p53 is usually expressed at high level, and is more stable than wild-type p53 in human cancers (4, 113, 127). These observations suggest a positive selection for mutants, especially the missense type of mutants of p53 over cancer development.

As a tumor suppressor gene, the loss of function properties of p53 mutations has been well studied. It's rare if not impossible for both alleles of the p53 gene to get the loss of function mutations at the same time. One explanation is that the mutant p53 allele could function as "dominant negative" to interact with and inactivate the wild-type p53 gene (15). However, mutant/wild-type heterozygous genotypes are rare in human cancers (15). p53 mutants have been shown to confer tumorigenic properties in cells lacking wild-type p53 (32, 76, 98, 143), indicating the effect of p53 mutant are not limited to blocking the functions of wild-type p53 gene. Also, tumor-derived p53 mutants have been shown to activate promoters not activated by wild-type p53 including those for the epidermal growth factor (EGF) receptor (31), c-Myc (47) and c-Fos (132), and others (18, 170). These evidences suggest that mutant p53 may gain some functions to facilitate cancer development.

MDA-MB-231 cells overexpress mutant p53, and also have elevated PLD activity that provide survival signal which suppress apoptosis induced by the stress of serum

withdrawal. So we investigated the impact of PLD on mutant p53 in MDA-MB-231 cells. Our results indicate that the elevated p53 level in MDA-MB-231 cells is dependent on elevated PLD activity, in a MAP kinase dependent but mTOR independent way, and that mutant p53 is required for the survival signals generated by PLD to prevent apoptosis induced by serum withdraw (81).

The PLD related survival pathways discussed above are summarized in Figure 3.6

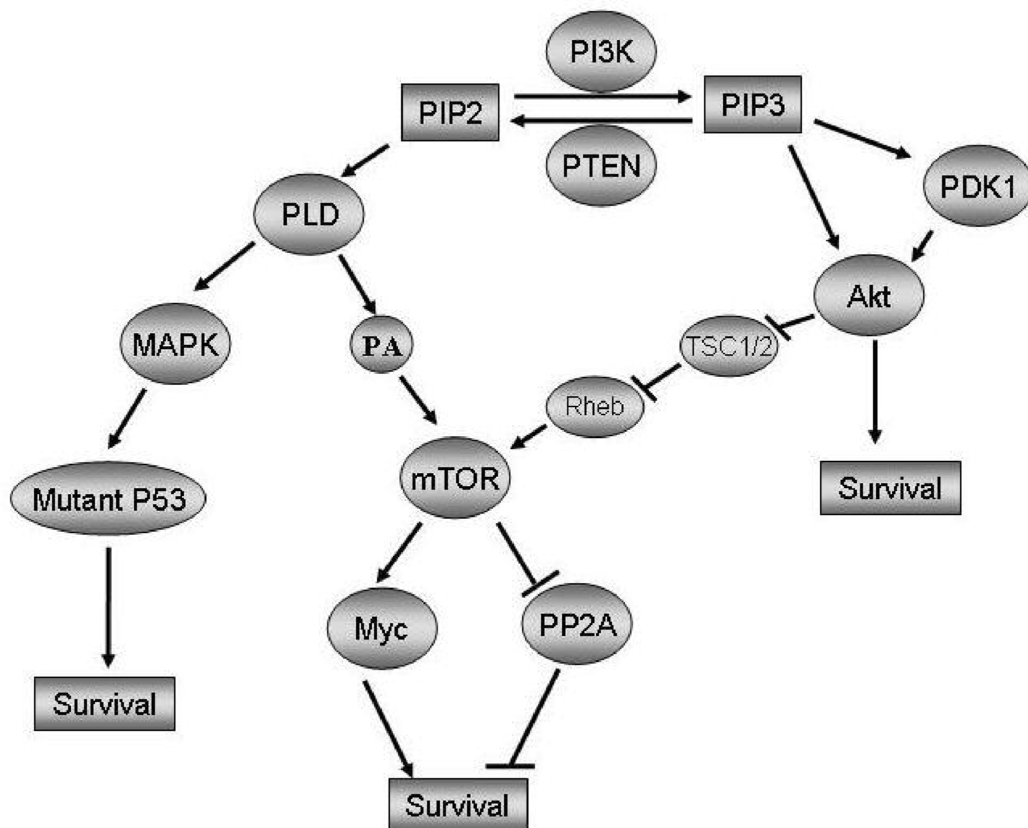


**Figure 3.6 Alternative survival pathways through PLD.** PLD provides survival signals through mTOR dependent and MAP kinase dependent pathways. The mTOR dependent pathway involves the upregulation and possibly stabilization of Myc protein level (observed in MCF7 cells), and

the suppression of PP2A activity (observed in MDA-MB-231 cells). In MDA-MB-231 cells the MAP kinase dependent pathway leads to the elevated mutant P53 level which is required for the survival of MDA-MB-231 cells, possibly as a gain-of-function P53 mutation.

# Chapter 4: PLD involvement of cancer invasion

While collaborating with others on the projects of PLD survival pathways in human cancers, my work focuses on PLD's role in another important aspect of cancer: invasion and metastasis. This chapter will present data regarding this subject.

## 4.1 PLD is required for cell protrusions and enhanced cell migration in v-Src transfected 3y1 fibroblast

### 4.1.1 Introduction

Cancer metastasis is a very complicated process which requires cancer cells to gain multiple survival capabilities, relocate and form new colonies at distant parts of the body other than the primary tumor site. Among these abilities, invasion of tumor cells to the local tissues is necessary at the beginning and the end of metastasis process when tumor cells need to migrate in and out of blood vessels and tissues. For invasion to occur, cancer cells usually have morphological changes and dysregulated cell motility.

Cytoskeleton networks, including actin microfilaments, microtubules, and intermediate filaments, govern cell motility. Regulation of the actin network has been the subject of intense studies on tumor invasion (91, 99, 118). Microtubules have also been found critical for cell polarity and the direction of cell migration (49, 155). It is thought that the actin cytoskeleton provides the driving force for the motile cells, while microtubules steer the cells and guide their movement in a directed manner (36, 49, 53, 75, 155, 176). There have been many reports of microtubule disrupting drugs arresting

tumor invasion both in tissue culture and in tumor-transplanted animal studies (51, 108, 186).

Non-receptor tyrosine kinase Src can mediate the regulation of cytoskeleton networks by extracellular signals such as growth factors, cytokines, cell–cell, and cell–matrix interactions (82). Src was found to localize along actin filaments and microtubules as well as at focal adhesions and adherens junctions (14). At these sites, Src interacts with a lot of downstream effectors controlling the processes of adhesion, migration, and invasion (82, 119). Src kinase activity and sometimes Src protein levels were found to be elevated in many types of cancer, with a correlation often observed between the increase in Src kinase activity and the degree of malignancy/invasiveness (14).

### 4.1.2 PLD activity is required for v-Src induced cell protrusions in 3Y1 fibroblasts

While studying the PLD's role as a survival signal provider in the rat 3Y1 fibroblasts transformed by Src, we observed different morphology of 3Y1 cells transfected by c-Src and v-Src. As shown in Figure 4.1, while $3Y1^{c\text{-}Src}$ cells remain morphologically much similar to the parental 3Y1 cells, the $3Y1^{v\text{-}Src}$ cells looks distinctively different with multiple protrusions stretching out from the cell body. It has been shown before that v-Src activates PLD activity in 3Y1 cells while c-Src does not (88, 191). Thus, we queried whether or not PLD is involved in this morphological difference.

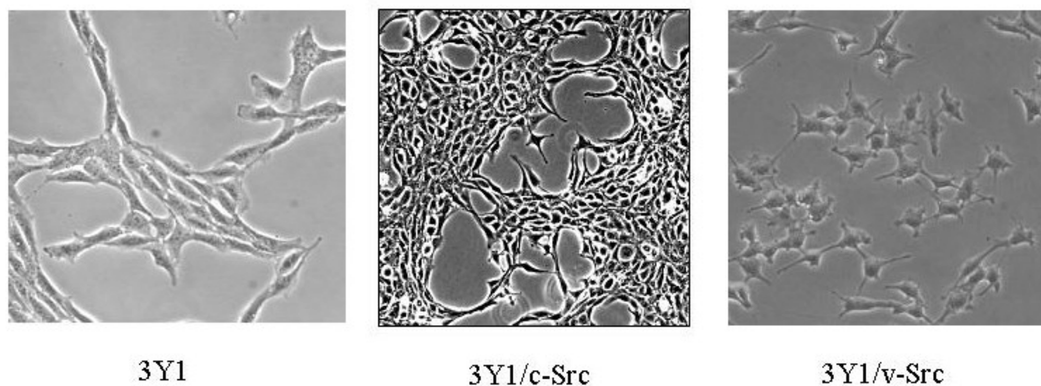


**Figure 4.1 (150) v-Src but not c-Src induces protrusions in 3Y1 rat fibroblasts.** 3Y1 cells were stably transfected by v-Src or c-Src. While 3Y1$^{c\text{-}Src}$ cells remain morphologically much similar to the parental 3Y1 cells, the 3Y1$^{v\text{-}Src}$ cells looks distinctively different with multiple protrusions stretching out from the cell body. Pictures represent morphologies of these cells observed repeatedly.

We introduced wild-type PLD2 and catalytically inactive mutant PLD2-K758R, which function as a dominant negative mutant (149, 181), into 3Y1 and 3Y1$^{v\text{-}Src}$ cells. As shown in Figure 4.2A, overexpression of PLD2 caused longer protrusions in the v-Src-transformed cells. In contrast, catalytically inactive PLD2 abolished the protrusions in 3Y1$^{v\text{-}Src}$ cells (Figure 4.2A). Neither wild-type nor catalytically inactive PLD2 could change the morphology of parental 3Y1 cells. Expression levels of the HA-tagged PLD2 proteins are shown in Figure 4.2B. The same differential expression level of PLD proteins was obtained in two additional sets of independently derived clones. Although

there were differential levels of PLD2 expression in the 3Y1 and $3Y1^{v\text{-}Src}$ cells, the highest levels of PLD2 expression were observed in the parental 3Y1 cells where no cell protrusion was observed. Thus, the effect of PLD2 expression upon cell protrusion was dependent upon the presence of v-Src. Figure 4.2C shows PLD activity of $3Y1^{v\text{-}Src}$, $3Y1^{v\text{-}Src}$ transfected by wild-type PLD2 or catalytically inactive mutant PLD2. The PLD activities of these clones are positively correlated with their protrusion potentials. This further suggested that PLD enzyme activity is involved in the formation of these protrusions.

We then performed the "alcohol trap assay" (149) where primary butanol is used to generate phosphatidylbutanol and consequently, blocks the production of phosphatidic acid by PLD. Tertiary butanol serves as the negative control because its bulky side groups, which do not allow it to properly insert into the membrane, make it a poor substrate. As shown in Figure 4.3, 1-butanol abolished the protrusions in both $3Y1^{v\text{-}Src}$ and $3Y1^{v\text{-}Src}$/PLD2 cells, while t-butanol had no effect on the protrusions of both cells. However, the effect of 1-butanol on these cells is temporary. After a few hours the cells treated with 1-butanol will return to their normal shape with re-established protrusions, possibly due to the depletion of the primary butanol and PLD started to function normally again. These data further confirm that the effect of PLD2 and catalytically inactive PLD2 observed in Figure 4.2 is due to PLD activity and that PLD activity is required for the protrusion induced by v-Src in 3Y1 cells. But, PLD2 alone is not sufficient to cause any morphological changes in parental 3Y1 cells as shown in Figure 4.2 A.

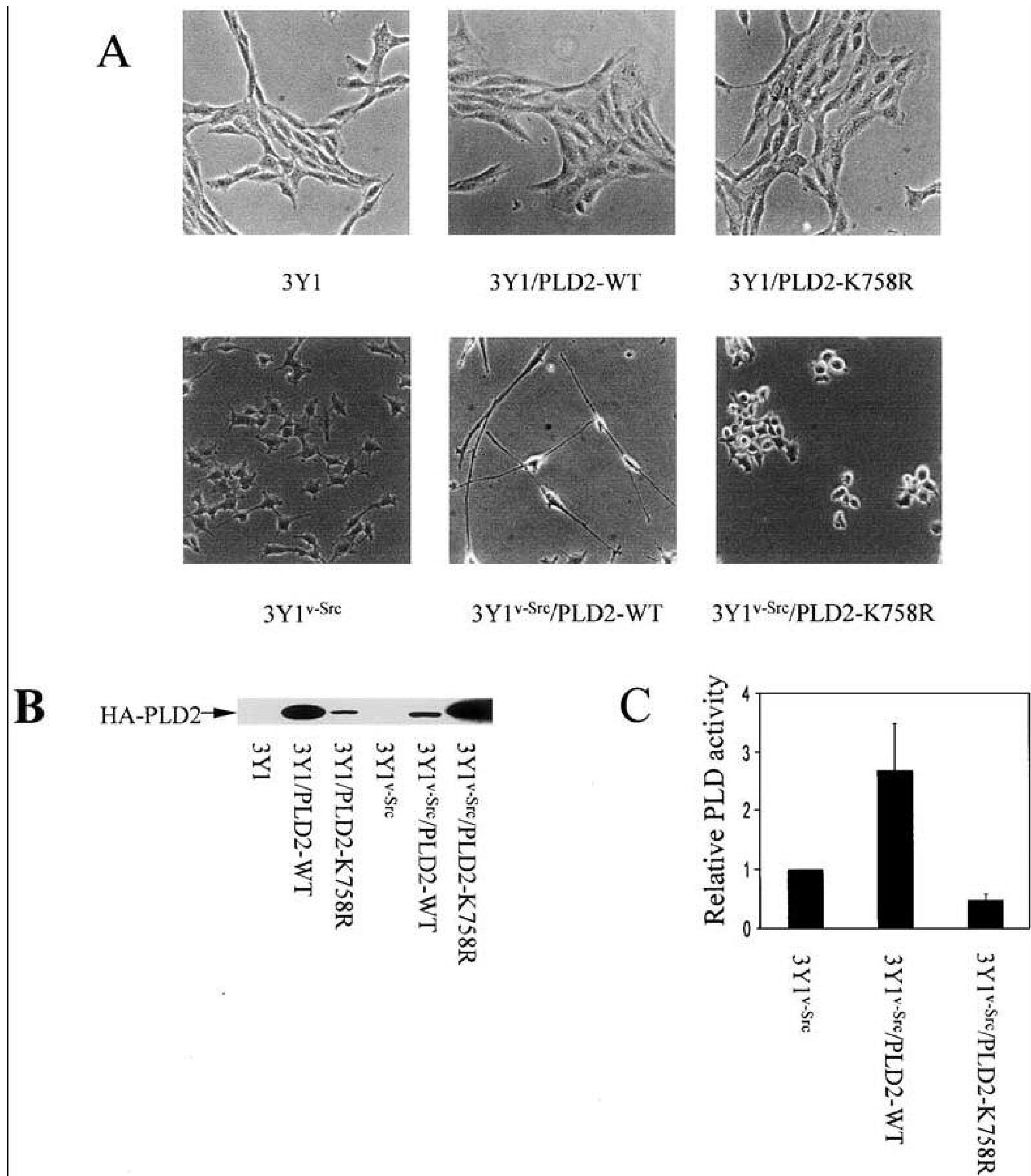


**Figure 4.2 (150) PLD2 regulates v-Src-induced cell protrusion in 3Y1 cells.** (A) Parental and v-Src-transformed 3Y1 cells, stably transfected with a HA-tagged PLD2 and catalytically inactive PLD2 have different morphologies, suggesting a PLD requirement for the v-Src induced cell protrusions. The data shown are representative of the morphology observed for two other independently derived clones. (B) PLD expressions in these cells were analyzed by Western blot analysis using HA-antibody. (C) PLD

activities in the 3Y1$^{v\text{-}Src}$, 3Y1$^{v\text{-}Src}$/PLD2WT and 3Y1$^{v\text{-}Src}$/PLD2KR cells used in (A) and (B) were determined using the transphosphatidylation reaction and normalized to the 3Y1$^{v\text{-}Src}$ cells. Cells were pre-labeled with [$^3$H]myristate for 4 h and then incubated with 1-BtOH (1% v/v) for 15 min. Generated PBt was resolved by thin layer chromatography and visualized by autoradiography. The PBt was quantified by densitometry as described in Materials and methods. Error bars represent the standard deviation of three independent experiments performed in duplicate.

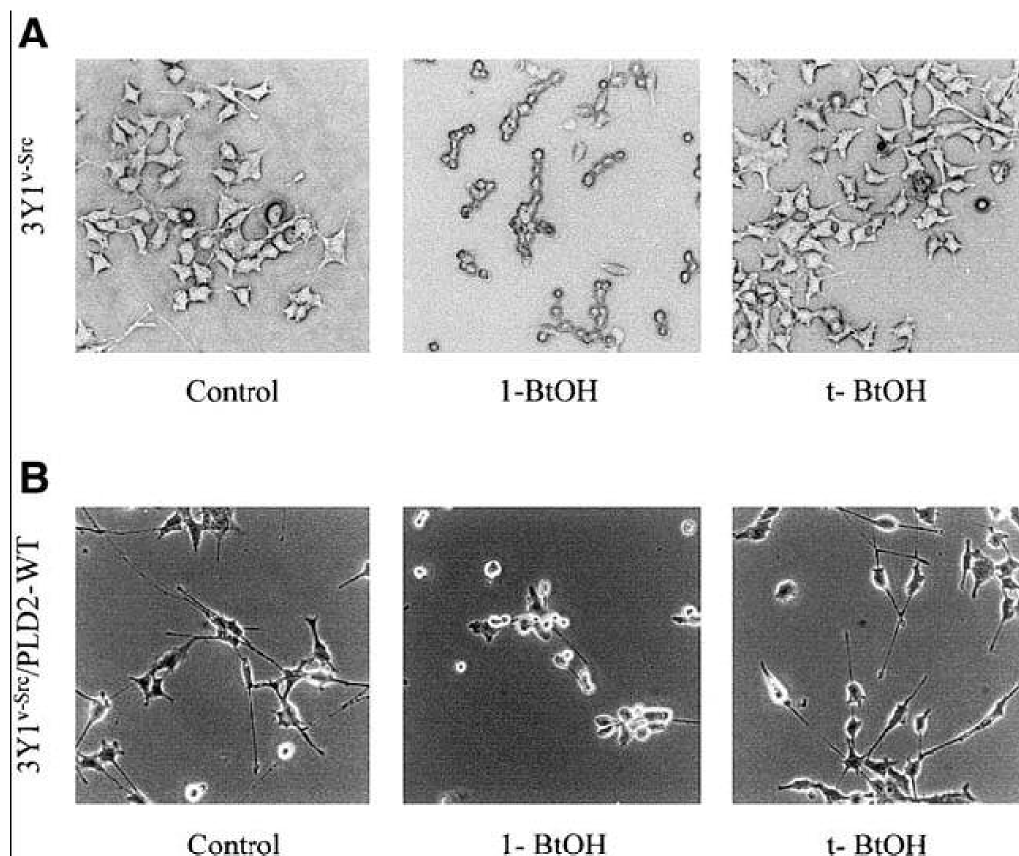


**Figure 4.3 (150) v-Src-induced cell protrusions are dependent upon PLD activity.** 3Y1$^{v\text{-}Src}$(A), and 3Y1$^{v\text{-}Src}$/PLD2WT (B) cells were either untreated or treated with 1% (v/v) primary butanol or tertiary butanol respectively for 15 minutes under the regular incubation condition (37C and 5% $CO_2$), then pictures of the cells were taken under a microscope. The data presented are representative results of several experiments done separately.

### 4.1.3 v-Src-induced cell protrusions are dependent upon microtubule assembly

To investigate cytoskeletal components responsible for the cell protrusions, we tested compounds that interfere with the polymerization of actin filaments or microtubules. Cytochalasin B, which blocks actin polymerization, rounded up the cell bodies but did not retract the cell protrusions in the 3Y1$^{v\text{-}Src}$ and 3Y1$^{v\text{-}Src}$/PLD2 cells (Figure 4.4A). In contrast, colchicine, an inhibitor of microtubule polymerization, caused complete retraction of the protrusions without rounding up cell bodies (Figure 4.4B). This data indicates that microtubule formation is required for maintaining the protrusions. The data does not rule out a role for actin in the formation of the protrusions, but does suggest that actin polymerization is less important or unnecessary for maintenance of the structure.

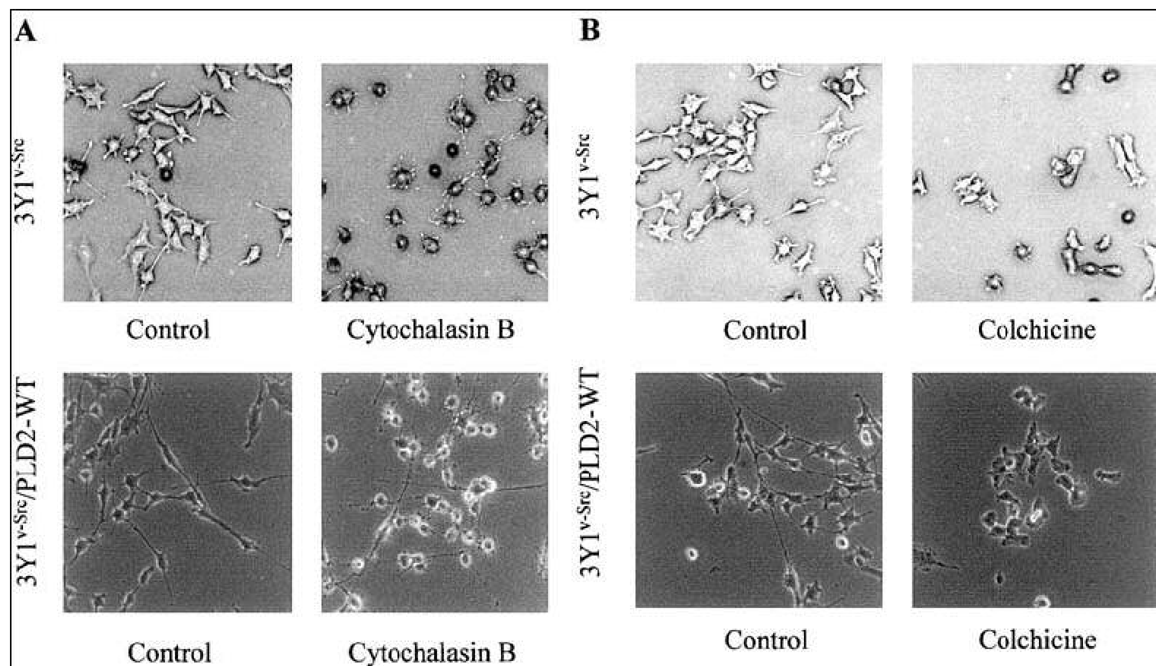


**Figure 4.4 (150) Inhibition of microtubule formation retracts the v-Src-induced cell protrusions.** (A) 3Y1$^{v\text{-}Src}$ and 3Y1$^{v\text{-}Src}$/PLD2WT cells

were treated either with DMSO (Control) or with cytochalasin B (15 μM in DMSO). After 30 min, the cells were visualized microscopically. Fields revealing the morphology of the majority of the cells are shown. (B) $3Y1^{v-Src}$ and $3Y1^{v-Src}$/PLD2WT cells were treated with either DMSO (Control) or colchicine (50 μM) for 30 min. Fields revealing the morphology of the majority of cells are shown. The data presented are representative results of experiments repeated three times.

### 4.1.4 v-Src induced migration of 3Y1 rat fibroblasts is mediated by PLD

We then tested the migration ability of these cells by Boyden chamber migration assay. As shown in Figure 4.5, 3Y1v-Src cells have much higher migration rate than 3Y1 parental cells and introduction of catalytically inactive PLD2 into 3Y1v-Src cells greatly reduced its migration ability, suggesting that PLD activity is required for v-Src induced migration in 3Y1 cells. Unexpectedly, introduction of wild-type PLD2 into 3Y1v-Src cells did not further enhance migration, although the migration rate was still higher than those in parental 3Y1 and 3Y1v-Src transfected by catalytically inactive PLD2. This may be due to the extremely elongated cell shape caused by overexpression of wild-type PLD2 in 3Y1v-Src cells which is not favored by the cell for migration. On the other hand, introduction of wild-type or catalytically inactive PLD2 into parental 3Y1 cells have no effect on cell migration (data not shown).

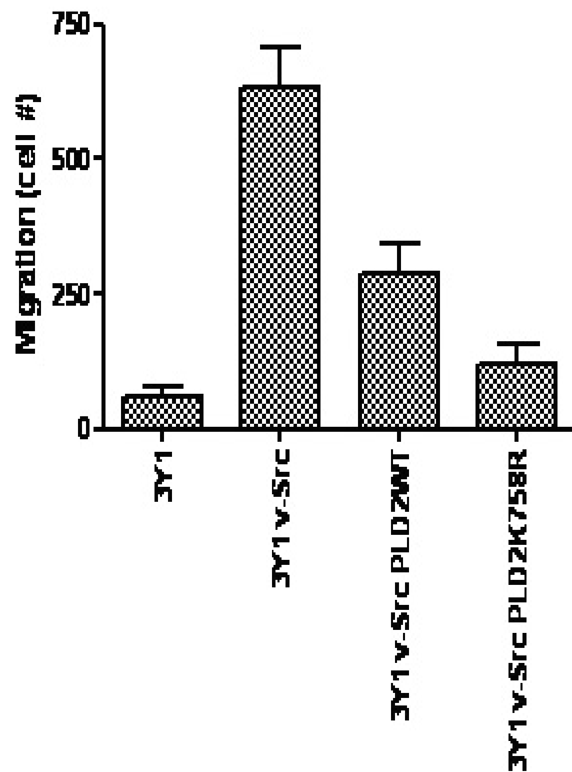


**Figure 4.5 PLD activity is required for the v-Src induced cell migration of 3Y1 rat fibroblasts.** Boyden chamber assay as described in the Chapter 2 Methods and materials was performed. 20,000 cells of each indicated cell line were placed with DMEM containing 0.5% serum in the upper chamber, while DMEM with 10% serum were added to the bottom chamber as chemoattractant. Error bars represent the variation among duplicates.

### 4.1.5 Discussion

Our data indicate that the protrusions observed in v-Src transformed 3Y1 cells are microtubule-based structures and are dependent upon PLD2. While PLD2 is necessary for the protrusions in transformed cells, expressing PLD2 in non-transformed 3Y1 cells did not result in protrusions. This suggests that both v-Src and PLD are required for the protrusion structures, and v-Src in 3Y1 cells is doing more than stimulating PLD activity for the protrusion formation.

It is not clear how v-Src and PLD are cooperating to generate cell protrusion. However, Src has been shown to phosphorylate tubulin on tyrosine residues (26), and Src

deficiency can cause impaired motility similar to those caused by microtubule depolymerization (75). These observations indicate Src involvement in the formation and maintenance of microtubules.

Overexpression of PLD1 is not tolerated well by cells. Most of the v-Src-transformed cells that expressed high levels of PLD1 became heavily vesiculated and soon stopped dividing (103). Thus, the accumulation of vesicles in cells overexpressing PLD1 could have masked any effect on cell protrusion. Therefore, this data does not exclude a role for PLD1 in the generation of the cell protrusions reported here.

Cell protrusion has been implicated in cell motility and metastasis (91, 99, 118). Our data correlate the enhanced cell migration and the protrusion structures in v-Src transformed 3Y1 fibroblasts, both with a dependency of PLD. Although our data suggests that continued microtubule assembly is required for maintaining the cell protrusions in v-Src transformed cells, it is widely believed that the driving force for cell migration is provided by actin filament formation (36, 49, 53, 75, 118, 155, 176), and our data does not exclude a role for actin on the formation of the protrusions. Consistently, PLD activity has been implicated in the reorganization of actin cytoskeleton (1, 25, 27, 55, 90, 139, 140). Thus, it is possible that in v-Src-transformed cells, PLD2 may regulate both actin and microtubule cytoskeletal networks to manipulate morphological and migratory behaviors.

## 4.2 PLD couples cell survival and migration/invasion in human cancers under the stress of serum reduction

Elevated expression of PLD1 and elevated PLD activity was reported in breast cancer tissues (122, 168). PLD activity has also been shown to be elevated in other human cancers including renal cancers (189) and gastric cancers (167). Furthermore, a polymorphism of the PLD2 gene was recently reported to be associated with the prevalence of colorectal cancer(182). Our lab has been investigating the role of PLD as a survival signal provider in human cancers. With the observation of PLD requirement on the protrusions and cell migration of v-Src transformed rat 3Y1 fibroblasts, we hypothesized that PLD may also be involved in the invasive properties of human cancers.

### 4.2.1 PLD activity is correlated with cell migration and invasion ability of human breast cancer cell lines.

We selected two human breast cancer cell lines for this study: MDA-MB-231 which is fibroblast-like and highly invasive, and MCF7 which is luminal epithelial-like and weakly invasive in contrast. MCF7 cells have been used in many studies as a benign cancer cell line comparing to the malignant MDA-MB-231 (84, 154). We observed a positive correlation between PLD activity and the migration/invasion ability in these cells. MDA-MB-231 cells have high PLD activity and a high migration/invasion rate. MCF7 cells, on the other hand, have about 1/20 PLD activity compared to MDA-MB-231 cells and barely migrate/invade at all (Figure 4.6). We have checked the PLD protein level in these two cell lines before and found that MDA-MB-231 cells express more PLD1 while MCF7 cells express more PLD2, but the expression of these PLD isoforms were still at

comparable level (191). This suggest that the much higher PLD activity observed in MDA-MB-231 cells is more likely due to the regulatory mechanisms instead of overexpression of PLD protein.

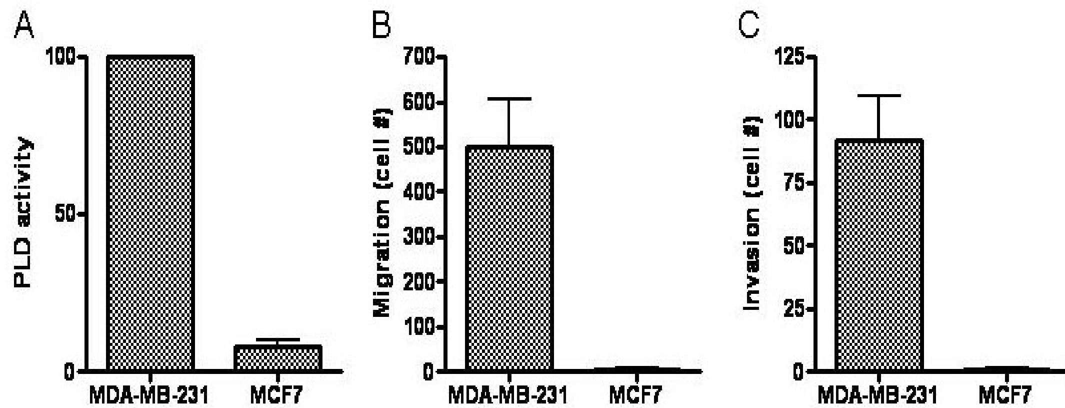


**Figure 4.6 (190) PLD activity correlates with cell migration and invasion ability of human breast cancer cell lines.** (**A**) PLD activity assay was performed as described in Chapter 2 Methods and Materials. Before the assay, cells were quiescented in DMEM containing 0.5% serum for 24 hours. Results were normalized to the PLD activity of MDA-MB-231 cells. (**B**)&(**C**) Cell migration and invasion abilities were examined by Boyden chamber assay as described in Chapter 2. 20,000 cells of each cell line were placed into the upper chamber with DMEM containing 0.5% serum, while the bottom chambers were filled with DMEM containing 10% serum as a chemoattractant. Data represents results from many experiments done separately.

### 4.2.2 Effects of serum/serum withdrawal on PLD activity of MDA-MB-231 cells

*Low serum condition elevates PLD activity of MDA-MB-231 cells*

It has been shown before that PLD activity can be stimulated by mitogenic signals, including those in serum (165), so to reduce the background of PLD activity, we usually quiescent cells in DMEM medium containing 0.5% serum overnight before performing PLD activity assay. Therefore it was a surprise when we found that MDA-MB-231 cells in 0.5% serum have much higher PLD activity as compared to those in 10% serum. However, it should be noted that the serum's effect on the PLD activity of MCF7 cells is minor (Figure 4.7). The different PLD response to serum in these two disparate cancer cells is very interesting, especially considering that PLD has been reported to provide survival signals in transformed 3Y1 rat fibroblasts upon apoptotic stress induced by serum withdrawal (191). These observations furthered our investigation for the PLD-mediated survival signals in human cancer cells, as discussed in chapter 3.

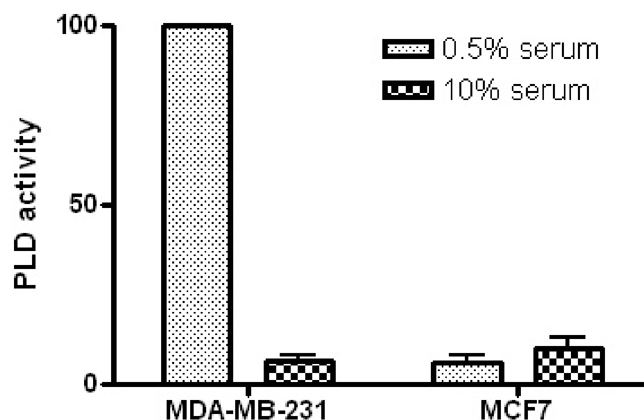


**Figure 4.7 Serum withdrawal elevates PLD activity in MDA-MB-231 human breast cancer cells.** PLD activity assay was done as described in

Chapter 2 Methods and Materials. Cells were placed in DMEM with 10% serum for 2 days, then before the PLD assay, cells were treated either by DMEM containing 0.5% serum or by DMEM containing 10% serum respectively, for 24 hours. Results were normalized to the PLD activity of MDA-MB-231 cells treated by 0.5% serum. Data represents at least 3 individual experiments.

*Adding serum/serum withdrawal instantly inhibit/elevate PLD activity of MDA-MB-231 cells*

Higher PLD activity of MDA-MB-231 cells, as compared to that of MCF7 cells, is more likely due to regulation instead of expression level of PLD. We wondered whether the elevated PLD activity by serum withdrawal in MDA-MB-231 cells is also due to regulation of PLD, so we performed a PLD activity assay with a time course, as shown in Figure 4.8. Significant increases of PLD activity could be observed as early as 10 minutes after serum withdrawal, although at 4 hours the PLD activity were further elevated (Figure 4.8A). Adding serum back to MDA-MB-231 cells which were in medium containing 0.5% serum would immediately (observed as early as 10 minutes) suppress the PLD activity to a comparable level of PLD activity in 10% serum for 24 hours (Figure 4.8B). The swiftness of the PLD response to serum indicates that this response is due to regulation instead of expression of PLD. It also suggests that the elevated PLD activity in MDA-MB-231 cells is a response to the lack of growth factors in serum.

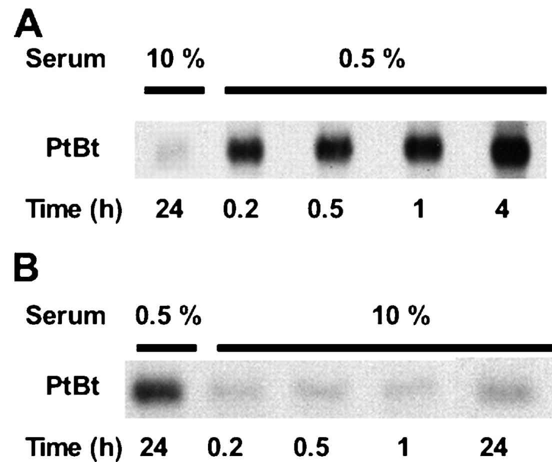


**Figure 4.8 (190) Serum withdrawal/addition instantly affects PLD activity of MDA-MB-231 cells.** (**A**) MDA-MB-231 cells were placed in DMEM containing 10% serum for 2 days and then changed to medium containing 0.5% serum at the indicated time points before PLD activity assay. (**B**) MDA-MB-231 cells were placed in DMEM containing 10% serum for 1 day, and then changed to 0.5% serum for another day, then 10% serum was added back at the indicated time points before PLD activity assay. PLD activity was represented by the PtBt band which indicates the production and accumulation of phosphatidylbutanol. Data represents results from two separate experiments.

*Prolonged serum withdrawal resulted in weaker elevation of PLD, but serum can still block the elevated PLD activity*

We also examined the effect of serum on PLD activity of MDA-MB-231 cells under prolonged periods of different serum treatments including 0% serum condition. The results are represented in Figure 4.9. In this set of experiments, MDA-MB-231 cells were placed in DMEM with 10% serum for 24 hours, then the medium was switched to

fresh DMEM with 10% (lane 1,2), 0.5% (land 3,4,5), or 0% (lane 6,7,8) serum for 48 hours. Then at 4 hours or 20 minutes before the PLD activity assay, the medium was switched back to 10% serum (lane 4,5 and lane 7,8), or to 0.5% serum (lane 2). A few deductions can be made from the data. First, although the PLD activity of the cells left in 0.5% serum for 2 days (lane 3) is greater than the PLD activity of the cells which were always in 10% serum (lane 1), it is not as high as the PLD activity of the cells with only 4 hours of 0.5% serum treatment (lane 2). This suggests that the upregulated PLD activity by low serum condition will eventually decrease, given a longer exposure to low serum condition. Second, the complete depletion (0%) of serum will not further upregulate PLD activity; instead, the PLD activity in the 0% serum treatment (lane 6) was closer to the PLD activity in the 10% serum (lane 1), suggesting that although 10% serum can block PLD activity, a minimal amount of serum (0.5% in this case) is still required for the upregulation of PLD activity. Third, although the prolonged exposure to 0.5% or 0% serum leaves the cells with relatively low PLD activity, adding serum back still significantly blocked the PLD activity under these conditions (lane 4,5; lane 7,8). This further confirms serum’s inhibitory effect on PLD activity. MDA-MBA-231 cells in 0.5% or 0% serum for 2 days do not undergo apoptosis, instead, they still divide and increase in numbers (data not shown), indicating the relatively lower PLD activity under these condition are not due to cell death.

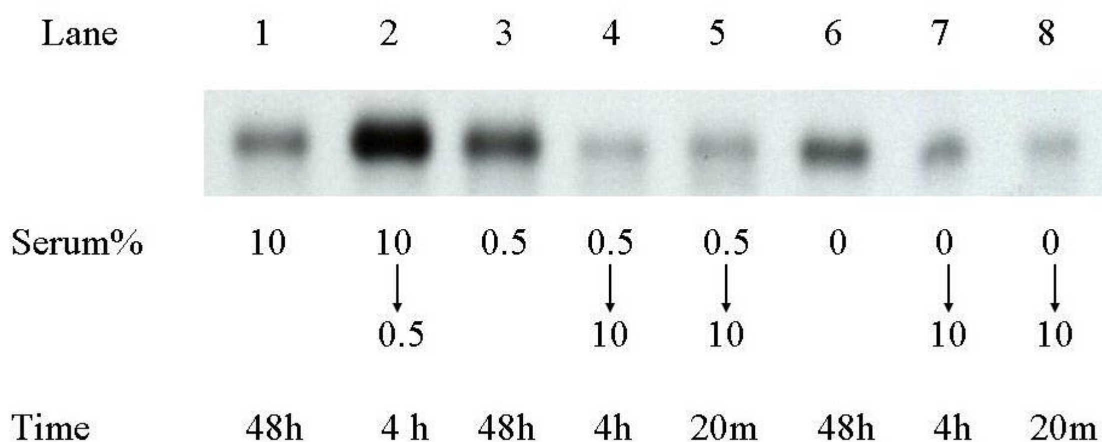


**Figure 4.9 Effects of prolonged serum withdrawal on the PLD activity of MDA-MBA-231 cells.** MDA-MB-231 cells were placed in DMEM with 10% serum for 24 hours, then the medium were switched to fresh DMEM with 10% (lane 1,2), 0.5% (land 3,4,5), or 0% (lane 6,7,8) serum for 48 hours. Then at 4 hours or 20 minutes before the PLD activity assay, the medium was switched back to 10% serum (lane 4,5 and lane 7,8) or to 0.5% serum (lane 2). PLD activity assay was performed as described in Methods and Materials. PLD activity was represented by the PtBt band which indicates the production and accumulation of phosphatidylbutanol. Data represents results from two separate experiments.

### 4.2.3 Low serum condition dramatically elevates migration and invasion of MDA-MB-231 cells

The correlation between PLD activity and invasiveness among MDA-MB-231 and MCF7 cells (Figure 4.6) and our findings that serum can suppress PLD activity in MDA-MB-231 cells led us to speculate that serum might also affect the migration/invasion ability of these cancer cells. We tested the effect of different serum concentrations on the migration/invasion of MDA-MB-231 cells (Figure 4.10). In a typical Boyden chamber assay cell suspensions are placed into the upper chamber and medium containing certain chemoattractant are placed into the bottom chamber. In the migration/invasion assay shown before in Figure 4.6, we used 10% serum as chemoattractant. However, as shown in Figure 4.10, MDA-MB-231 cells have highest migration/invasion rate when 0.5% serum are present in both upper and bottom chambers, about 10-20 folds higher than with the presence of 10% serum in both chambers. 0% serum in both chambers yielded the lowest (close to none) migration and invasion rate. These results correlate well with the PLD activity observed under the same serum conditions observed in Figure 4.9, suggesting that like the elevated PLD activity, the elevated cell migration and invasion can be triggered by serum withdrawal, although a minimal amount of serum (0.5% in this case) is still required for the elevated migration and invasion. Thus, a complete depletion of serum (0% serum) would abolish the cell migration and invasion. The other two serum concentration combinations, 0.5% upper/10% bottom, and 10% upper/0.5% bottom, result in intermediate migration/invasion rates which are closer to the one yielded by 10%/10% serum. These intermediate results may be due to the mix of the upper and bottom medium over the

relatively long assay period (24 hours assay time), which would result in a roughly 4-7% serum mix that could probably still impede cell migration and invasion. In essence, other than upregulating PLD activity, which can provide survival signals, serum withdrawal (discounting the extreme parameter-0% serum) can also enhance MDA-MB-231 cells' migration/invasion ability.

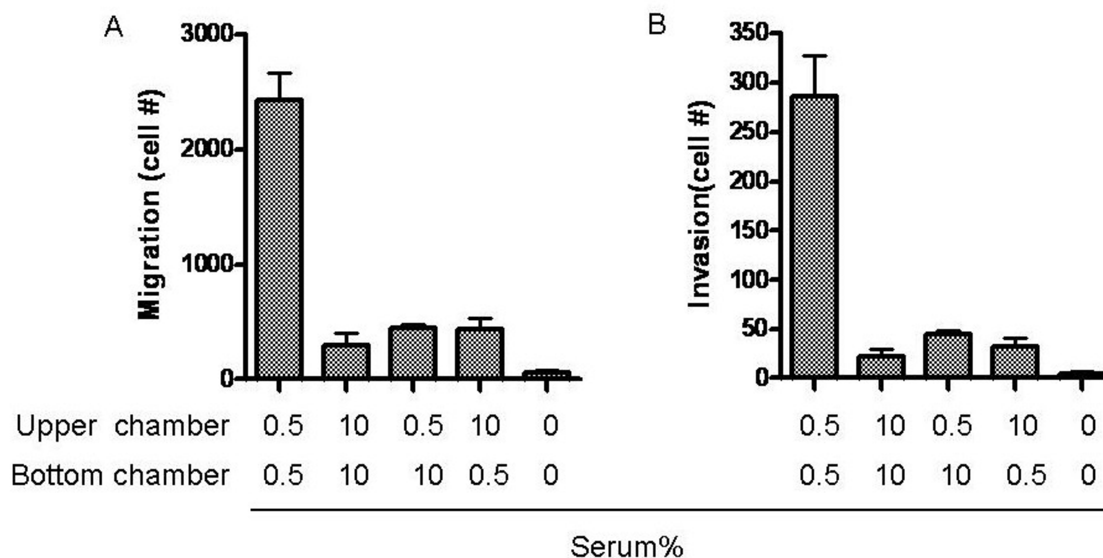


**Figure 4.10 Low serum condition dramatically enhances migration and invasion of MDA-MB-231 cells.** Cell migration and invasion abilities were examined by Boyden chamber assay as described in Chapter 2. 20,000 cells were placed into each chamber, with DMEM containing indicated serum% in upper and bottom chambers, with the exception of 0% serum treatment because cells have adhesion difficulty in 0% serum. So, for the 0% serum treatment, cells were first placed with 10% serum for 6 hours (allowing attachment), then carefully changed to fresh plain DMEM after washing twice by DMEM. After the assay, data was normalized to the control. Data represents results from many experiments done separately.

We did similar assays to test the effect of serum on the migration/invasion potential of MCF7 cells. Not surprisingly, MCF7 cells remain non-migration/non-invasive regardless of serum concentrations (Data not shown). To account for a possible effect of serum on MDA-MB-231 cell, as attributed by the total protein level in serum, we added albumin into medium with 0.5% serum to make its total protein level comparable to medium with 10% serum. Subsequently, we compared the MDA-MB-231 PLD activity and migration/invasion in these mediums and found that albumin did not suppress the enhanced PLD activity and migration/invasion in 0.5% serum (Data not shown). These results further correlate the elevated PLD activity and migration/invasion of MDA-MB-231 cells induced by serum withdrawal, and both responses are possibly due to the lack of serum growth factors.

### 4.2.4 Enhanced migration/invasion of MDA-MB-231 cells is dependent on PLD

We then examined whether the enhanced migration/invasion of MDA-MB-231 cells are dependent on the elevated PLD activity. To suppress PLD activity, we introduced catalytically inactive PLD2 mutant (which function as dominant negative) into MDA-MB-231 cells. The transient expression of this PLD2 mutant suppressed the PLD activity to 50% of vector control in these cells (Figure 4.11A). The mutant PLD2 also greatly suppressed migration and invasion of MDA-MB-231 cells (Figure 4.11B&C). For an unknown reason, the mutant PLD2 seems to have a greater suppression effect on cell invasion than migration. We also performed an alcohol trap assay, using primary butanol to block the production of phosphatidic acid by PLD, and therefore, its physiological functions. As shown in Figure 4.12, the primary butanol suppressed migration and

invasion of MDA-MB-231 cells, while the control tertiary butanol did not. The PLD activity assay and migration/invasion assays in Figure 4.11 and Figure 4.12 were done in medium containing 0.5% serum since MDA-MB-231 cells have highest PLD activity and migration/invasion rate under this condition. These results suggest that PLD activity is required for the enhanced migration and invasion observed in MDA-MB-231 cells.

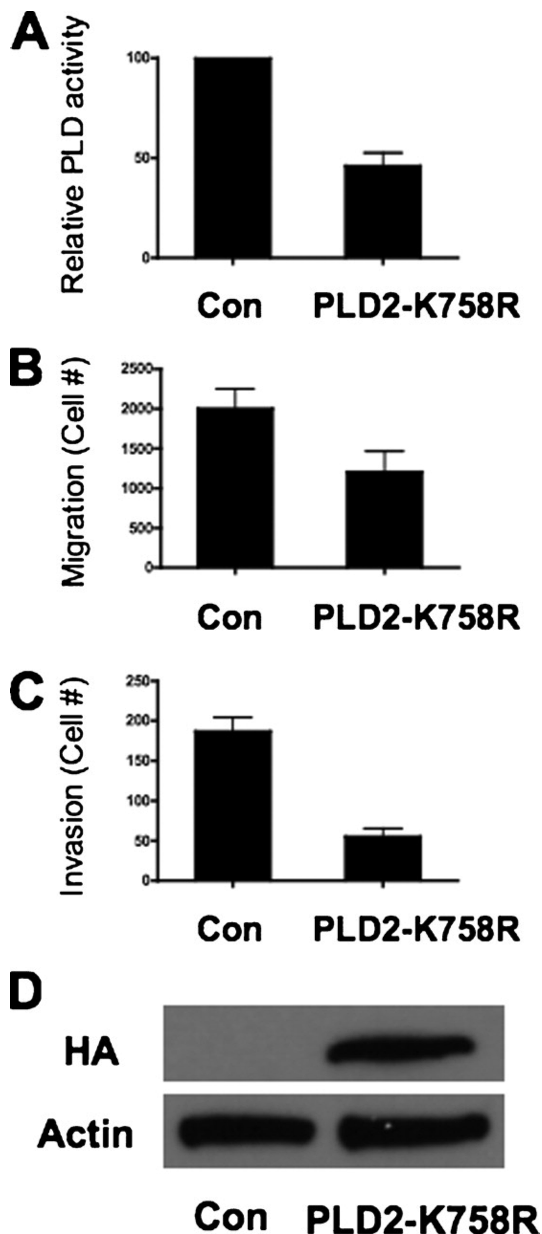


**Figure 4.11 (190) Catalytically inactive PLD2 mutant suppresses migration and invasion of MDA-MB-231 cells.** Catalytically inactive mutant PLD2-K758R with HA tag was transiently expressed in MDA-MB-231cells. Assays were performed 24 hours after transfection. (**A**) PLD activity assay was performed as described in Chapter 2. Results were normalized to PLD activity of control cells transfected by empty vector only. (B)&(C) Migration and invasion were examined by Boyden chamber assay as described in Chapter 2. (D) Expression of PLD2-K758R was detected by HA-antibody. All experiments were done under 0.5% serum condition. Error bars represent variation between separate experiments.

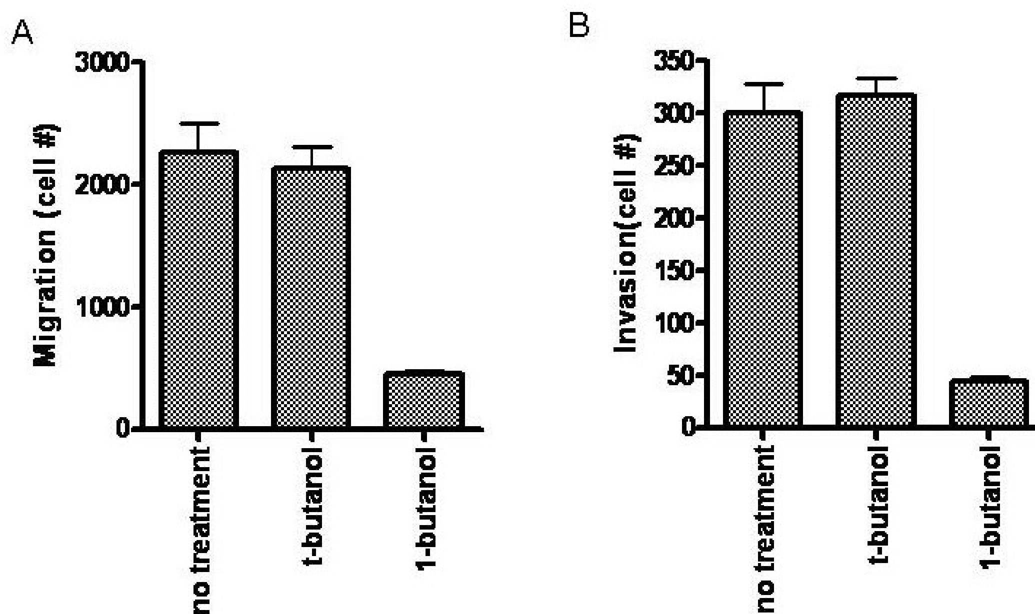


**Figure 4.12 1-butanol but not t-butanol suppresses migration and invasion of MDA-MB-231 cells.** Boyden chamber assays as described in Chapter 2 were performed in DMEM with 0.5% serum, either with no treatment or with 0.8% (v/v) t-butanol or 1-butanol. Cells were first allowed 6 hours for establishing attachment, then t-butanol or 1-butanol was added carefully.

### 4.2.5 Enhanced migration/invasion of MDA-MB-231 cells is dependent on mTOR

As discussed in chapter 3, we have shown that PLD generates survival signals through mTOR in MDA-MB-231 cells (22). We therefore examined the effect of mTOR inhibitor rapamycin on the migration and invasion of MDA-MB-231 cells. Rapamycin suppressed both migration and invasion of these cells (Figure 4.13), suggesting that PLD-

induced cell migration and invasion, like the PLD-induced survival signals, is dependent upon mTOR.

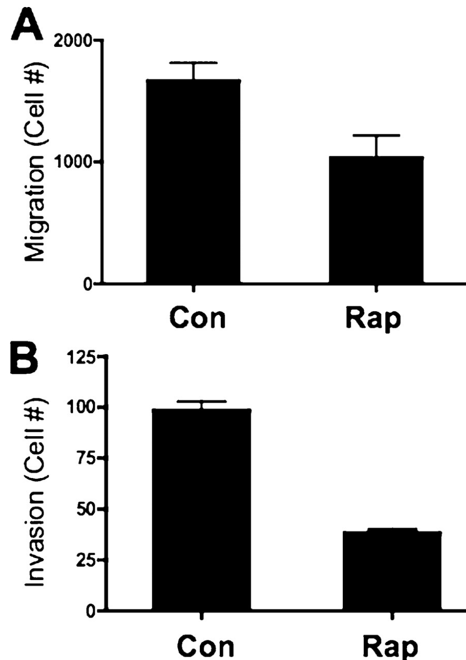


**Figure 4.13 (190) Rapamycin suppresses migration and invasion of MDA-MB-231 cells.** Boyden chamber assay as described in Chapter 2 was performed in DMEM with 0.5% serum, with either $Me_2SO$ control (Con) or15μM rapamycin (Rap). Error bars represent standard deviation from three independent experiments.

### 4.2.6 Elevated PLD activity in MCF7 cells enhances cell migration

MCF7 cells were less aggressive in vivo and non-invasive in Boyden chamber assays as compared to MDA-MB-231 cells, and PLD activity of MDA-MB-231 cells is as much as 50 fold higher than that of MCF7. Since the enhanced migration and invasion of MDA-MB-231 cells are dependent on PLD activity, we examined the effect of PLD on migration and invasion of MCF7 cells. MCF7R is a fast-growing MCF7 variant with elevated PLD activity as compared to parental MCF7 cells (22), and MCF7R cells can also migrate and invade the Matrigel™ (Figure 4.14). This further correlates the PLD activity and cell migration/invasion ability. MCF-7 cells were stably transfected with

PLD2, and pools of clones (MCF7-P2 cells) were selected. MCF7-P2 cells have significantly higher PLD activity relative to the vector-transfected control MCF7v cells. MCF7-P2 cells can migrate better than MCFv control cells, although the enhanced migration ability is still not comparable to that of MDA-MB-231 or MCF7R cells (Figure 4.14). MCF7-P2 cells do not have enhanced invasion through Matrigel™. The minimal increase in migration and complete absence in invasion, by introducing PLD2 alone into MCF7 cells, indicates that there are additional differences between MDA-MB-231, MCF7R and parental MCF7 cells other than PLD activity levels.

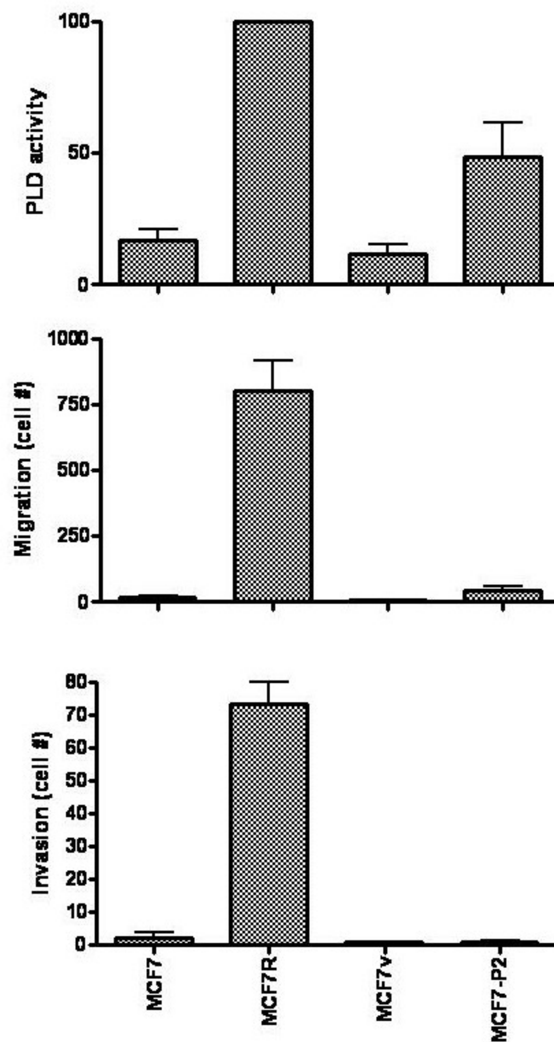


**Figure 4.14 Elevated PLD activity in MCF7 cells enhances cell migration.** MCF-7 cells were stably transfected with PLD2 and pools of clones (MCF7-P2 cells) were selected. PLD activity assay and Boyden chamber assay were done as described in Chapter 2. PLD activities were normalized to PLD activity of MCF7R cells. Experiments were done in 0.5% serum condition. Error bars represent standard deviation of three independent experiments.

### 4.2.7 The effects of reduced serum on PLD activity/migration/invasion of MDA-MB-231 cells may due to the downregulation of PI3-Kinase.

As it has been reported that PLD activity can be activated by mitogenic signals, it was surprising to see that serum withdrawal can actually lead to huge increase of PLD activity in MDA-MB-231 cells. We have previously reported that elevated PLD activity in MDA-MB-231 cells generates survival signals through mTOR independent of PI3-

Kinase/AKT pathway (22) and a model was generated based on the results (Figure 3.3). In the meantime, PI3-Kinase can be activated by mitogenic signals such as growth factors present in the serum to transfer PIP2 into PIP3, which then activate AKT and lead to downstream targets essential for cell survival.

The hypothesis is that when MDA-MB-231 cells are under the stressful condition of serum withdrawal and are lacking the growth factors required for activation of PI3-kinase, the PIP2/PIP3 ratio could shift in the direction favoring PIP2, which in turn is required for the activation of PLD. While PIP2 can also be generated by other means, the temporary accumulation of PIP2, due to the downregulation of PI3-Kinase activity under serum withdrawal could at least partially account for the initiation of PLD activation. Note that PIP2 can also be generated by PI-4-P 5-kinase, which is a downstream target of PLD itself. Thus once the PLD activation is initialized, a positive feedback loop could be established to keep the PLD signaling pathway functioning under the stressful condition of serum withdrawal.

To test this hypothesis, we first examined the effect of serum on the PI3-Kinase activity of MDA-MB-231 cells by checking the phosphorylation of its downstream target AKT. As shown in Figure 4.15A, switching medium from 0.5% to 10% serum immediately (as fast as 10 minutes) elevated P-AKT level, while downregulation of P-AKT by switching medium from 10% to 0.5% serum took about 4 hours to reach its maximum effect (Figure 4.15B). These results are nicely correlated with the effect of serum on PLD activity with a similar time course pattern (Figure 4.8), where the 10%

serum immediately suppressed the PLD activity, while the maximum induction of PLD activity by 0.5% serum took about 4 hours.

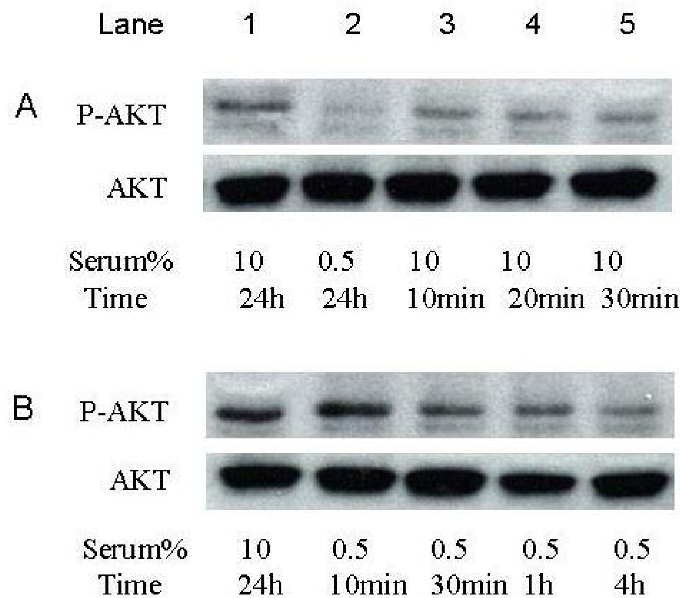


**Figure 4.15 Serum upregulates PI3-Kinase activity (A); and serum withdrawal downregulates PI3-Kinase activity (B) in MDA-MB-231 cells.** (**A**) Cells were placed in DMEM with 10% serum for 24 hours, then switched to DMEM with 0.5% serum for 24 hours (lane 2,3,4,5). Then 10% serum was added back 10, 20, or 30 minutes (lane 3,4,5) before cells were collected for western blot preparation. Control cells were always placed in DMEM with 10% serum (lane 1). (**B**) Cells were placed in DMEM with 10% serum for 2 days, then switched to DMEM with 0.5% serum 10 minutes, 30 minutes, 1 hour or 4 hours (lane 2,3,4,5) before harvest for western blot preparation. Control cells were always placed in DMEM with 10% serum (lane 1). Phosphorylated AKT and total AKT level were detected by western blot with antibodies specific to P-AKT or AKT respectively. Shown Data represent the results of 2 independent experiments.

We then examined the effect of PI3-Kinase inhibitor LY294002 and wortmannin on PLD activity of MDA-MB-231 cells. If the aforementioned hypothesis was correct, inhibition of PI3-Kinase would lead to the accumulation of PIP2 which in turn can stimulate PLD activity. Since PLD activity of MDA-MB-231 cells are lower in 10% and 0% serum than in 0.5% (Figure 4.9), we first tested the effect of LY294002 and wortmannin upon PLD activity under these condition (Figure 4.16A). Both LY294002 and wortmannin can significantly upregulate PLD activity in 10% serum or 0% serum. The upregulation of PLD activity in 10% serum by PI3-Kinase inhibitors suggests that the downregulation of PI3-kinase could be involved in the activation of PLD in MDA-MB-231 cells, which would support our hypothesis. On the other hand, LY294002 suppressed PLD activity of MDA-MB-231 cells in 0.5% serum (Figure 4.16B), suggesting that a minimal PI3-kinase activity is necessary for the elevated PLD activity induced by low serum condition. Consistent with this piece of data is the observation shown in Figure 4.9 where a complete depletion of serum resulted in lower PLD activity than in 0.5% serum. Since PI3-Kinase can be activated by serum (Figure 4.15), it is possible that the minimal requirement of serum represent a requirement of minimal PI3-Kinase/AKT signaling. MDA-MB-231 cells have relatively weak PI3-Kinase signaling (22), so either under complete serum depletion (0%) , or in low serum (0.5%) plus LY294002, PI3-Kinase signaling may be suppressed to the extent that it is unable to meet the minimal requirement for PLD activation. This would explain why low PLD activity was observed under these conditions. It is unclear why inhibition of PI3-kinase would lead to elevation of PLD activity in a complete serum depletion condition.

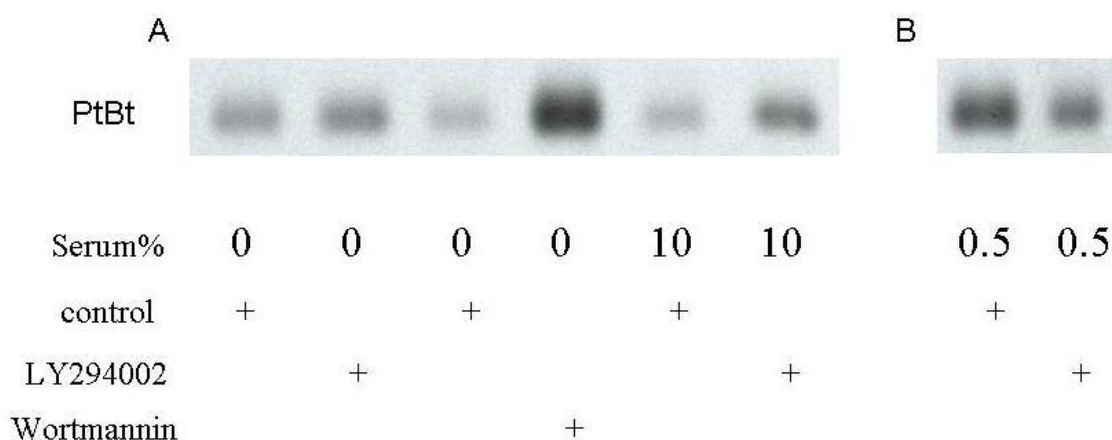


**Figure 4.16 Effects of PI3-Kinase inhibitors on the PLD activity of MDA-MB-231 cells.** (**A**) PI3-Kinase inhibitor LY294002 and wortmannin upregulate PLD activity of MDA-MB-231 cells in 0% and 10% serum. (**B**) LY294002 downregulates PLD activity of MDA-MB-231 cells in 0.5% serum. PLD activity assay was performed as described in Chapter 2. Cells were placed in DMEM with 10% serum for 2 days, and media was switched to fresh DMEM with indicated serum% 24 hours before cells were collected for PLD assay. Solvent control (methanol) or LY294004 (10nM) or wortmannin (25nM) was added at the time of switching medium. PLD activities are represented by the PtBt bands which indicate the production and accumulation of phosphatidylbutanol. Shown Data represent the typical result of two separate experiments.

Next we wanted to know what effect inhibition of PI3-Kinase has on migration and invasion of MDA-MB-231 cells. As shown in Figure 4.17, LY294002 suppressed the migration and invasion in both 0.5% and 10% serum. The result is not surprising even though we hypothesized that the downregulation of PI3-Kinase may lead to elevated PLD

activity which is required for the enhanced cell migration and invasion, because PI3-Kinase itself was implicated in cell migration and invasion by numerous studies (7, 92, 109, 151, 185). Thus while PLD activity is required for the migration and invasion of MDA-MB-231 cells, PI3-Kinase and its downstream effectors may also be required for the cell migration. A direct inhibition on PI3-Kinase would lead to impaired cell migration/invasion ability. On the other hand, we also found that the complete depletion of serum would result in lower PLD activity and almost no migration and invasion (Figure 4.9, Figure 4.10), suggesting a minimal serum requirement for such activities. As discussed before, the minimal serum requirement may represent a requirement for minimal PI3-Kinase activity. LY294002 in 0.5% serum may abolish the required minimal PI3-Kinase signaling, thus impairing PLD activation and cell migration/invasion.

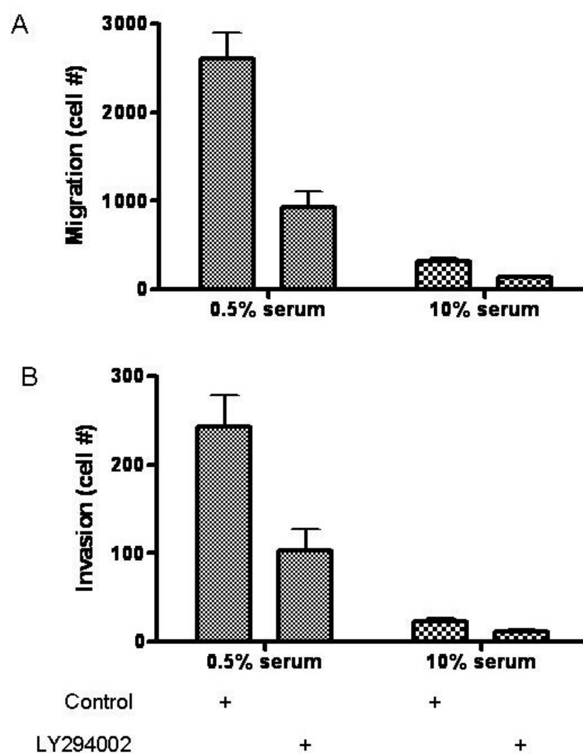


**Figure 4.17 LY294002 suppress migration and invasion of MDA-MB-231 cells.** Boyden chamber assay were performed as described in Chapter 2. Cells were first placed into upper chamber with indicated amount of serum for 6 hours to allow the cells to establish attachment, then control (DMSO 1/1000 v/v) or LY294002 (10nM in DMSO) were added. Error bars represent the standard deviation of 3 separate experiments.

**4.2.8 Elevated cell migration/invasion by reduction of serum is general in aggressive human cancer cell lines, many of these elevated cell migration/invasion correlates with an elevated PLD activity.**

Although the mechanism remains elusive, it is very clear that the serum withdrawal (not to the extreme of serum-free condition) has two significant effects upon MDA-MB-231 human breast cancer cells: highly elevated PLD activity and highly enhanced cell migration/invasion. Also, the enhanced MDA-MB-231 migration/invasion is dependent on the elevated PLD activity through a PLD/mTOR pathway. To determine whether the serum withdrawal effects is a general phenomenon in other cancers or is restricted to MDA-MB-231 cells, we examined a panel of human cancer cell lines with differing properties to check the serum effects on their PLD activity and cell migration/invasion ability (Figure 4.18). The cell lines examined included the breast cancer cell lines MCF-7, MDA-MB-468, MDA-MB-435s, BT-549, T47D, and SK-BR3. We also examined T24 bladder carcinoma and Calu-1 lung carcinoma cells which have highly elevated levels of PLD activity. These cell lines were categorized into three groups according to their PLD activities (Figure 4.18A). The first group included the T24, Calu-1 and MDA-MB-231 cells, all with elevated PLD activity as a stress response when serum was reduced, substantially higher than in 10% serum. The second group included BT-549, MDA-MB-468, and MCF-7 cells that all had detectable PLD activity in the presence of serum but did not have increased PLD activity as stress response when serum was reduced (Figure 4.18A). In the third group, several cancer cell lines have very low levels of PLD activity in both low and high serum, including MDA-MB-435s, T47D, and SKBR3 (Figure 4.18A).

We next evaluated the effect of serum withdrawal on the ability of these cancer cell lines to migrate and invade Matrigel$^{TM}$. Five of the cell lines exhibited strong migration and invasive behavior. Interestingly, all five of these cell lines migrated (Figure 4.18B) and invaded Matrigel$^{TM}$ (Figure 4.18C) far more efficiently in 0.5% serum than in 10% serum. The five cell lines with enhanced migration and invasive behavior included not only all three cell lines where PLD activity was elevated in low serum (MDA-MB-231, T24, and Calu-1) but also the MDA-MB-435s and BT-549 cells (Figure 4.18, B and C). The MDA-MB-435s cells had very little PLD activity, and the BT-549 cells had detectable PLD activity but no significant increases in PLD activity in low serum. Thus, although there was a correlation between a stress-induced increase in PLD activity and increased cell migration, there were also cases where there was a stress-induced migration that was apparently independent of PLD activity. In all cases, the ability to migrate and invade Matrigel$^{TM}$ was enhanced under the stress of serum withdrawal, indicating that the migration and invasive properties of these cancer cells was a stress response.

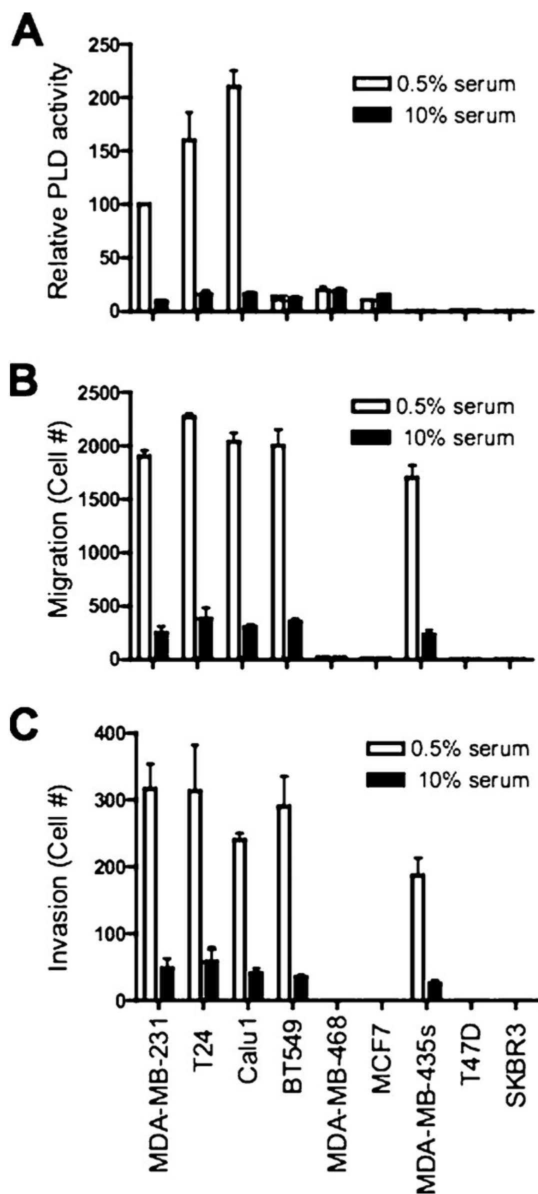


**Figure 4.18 (190) Effect of serum withdrawal on PLD activity and cell migration/invasion ability of various human cancer cell lines.** PLD activity assay and Boyden chamber migration/invasion assay were performed as described in Chapter 2. (**A**) Different cell lines were placed in DMEM with 10% serum at various initial densities to allow 70-80% confluence after 2 days, and then the medium were changed to fresh DMEN with either 0.5% or 10% serum for 24 hours before the PLD activity assay. The results were normalized to PLD activity of MDA-MB-231 cells in 0.5% serum. Error bars represent variations of at least 3 independent experiments. (**B**)&(**C**) 20,000 cells from each cell line were added to chambers with indicated amount of serum. Error bars represent variations of three separate experiments and duplicates.

### 4.2.9 Serum reduction increases expression of HIF1α in MDA-MB-231 cells

As discussed above, in some cancer cells serum reduction can cause stress responses including activated survival pathways and elevated cell migration/invasion ability. In MDA-MB-231 cells these responses are dependent on elevated PLD activity induced by serum reduction. In a growing tumor, an environment with limited serum renders limited access to blood supply which consequently, limits oxygen, a condition called hypoxia. In response to the stress of hypoxia, cells increase expression of HIF1α (146, 147). HIF1α is a component of the HIF1 transcription factor that stimulates transcription of several genes that promote angiogenesis such as vascular endothelial growth factor (VEGF) and the vascular endothelial growth factor receptor (VEGFR) (146). Because of the link between oxidative stress and the stress of serum withdrawal in a growing tumor, we examined the effect of serum withdrawal on HIF1α expression in the MDA-MB-231 cells. As shown in Figure 4.19, there was a substantially higher level of HIF1α in cells in 0.5% serum than in cells maintained in 10% serum. The dependence of HIF1α on PLD activity was assessed using the "alcohol trap assay". As shown in Figure 4.19, the elevated HIF1α seen in low serum was suppressed by primary but not tertiary butanol, indicating a dependence upon PLD activity. Consistent with PLD dependence, the dominant negative PLD2-K758R mutant also suppressed the increase in HIF1α expression induced by serum withdrawal Figure 4.19. This data indicates that the elevated PLD activity in MDA-MB-231 cells also contributes to elevated expression of HIF1α as a stress response induced by serum reduction.

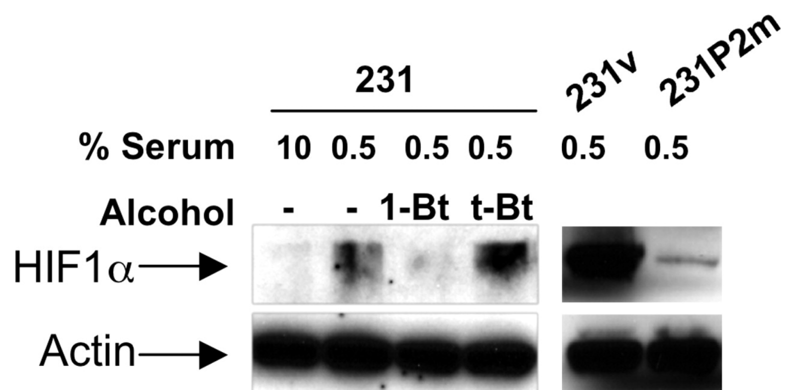


**Figure 4.19 (190) Serum reduction elevates HIF1α in MDA-MB-231 cells.** MDA-MB-231 cells (*231*) were plated in media containing 10% serum. 24 h later, the cells were placed in 0.5% serum, as indicated. The cells were treated with the indicated alcohol, either 1-butanol (*1-Bt*) or tertiary-butanol (*t-Bt*), 4 h prior to harvest. Alternatively, MDA-MB-231 cells stably transfected with either vector control (*231v*) or pCGN-PLD2-K758R were placed in media containing 0.5% serum for 24 h. All cells were harvested and the level of HIF1α was determined by Western blot analysis. Blots were reprobed for actin as a loading control. The experiment is representative of one repeated three times.

# Chapter 5 Answers to the cancer progression puzzle

**--------Serum reduction induced stress responses is the key: enhanced cell survival and migration/invasion ability.**

## 5.1 Two possibilities

Tumorigenesis is a multi-step process which requires mutation at each of these steps to allow the cancer cells to overcome the automatic anti-cancer defense mechanisms. From the evolutionary point of view, tumorigenesis is also a process in which the cancer cell clones with the most proliferation advantages are selected for at each stage of cancer development. The proliferation advantages include the elevated capabilities to divide, to avoid cell death, to obtain resource and space, and all other potential abilities which help the cells to increase in numbers. Certain growth-limit physiological barriers are present at each stage of tumorigenesis, the cancer cell clone with the “right” mutation (which enable the cancer cells to overcome these barriers at that stage) would be selected for. These clones will outgrow other cancer cell clones in the colony to provide a whole new population of cancer cells ready for the next step of tumorigenesis. Since metastasis appears to be the final stage of cancer phenotypically, it is possible that the mutations specifically responsible for the metastasis are also acquired at the last stage of cancer. Much research and medical practice have been based on this possibility, including the search for the “metastasis gene” and early cancer detection with the hope of locating and removing tumors prior to metastasis.

However, the reasoning above leads to a problem conceptualized by Weinberg as “progression puzzle of cancer”. If the “metastasis mutation” does occur at the late stage

of cancer after a primary tumor was well-established, there is no reason to believe such a mutation would give proliferation benefits to these cancer cells in the primary tumor. Thus, the rare “metastatic cells” would remain rare in the whole cancer cell population of the primary tumor. Would these rare metastatic cells initiate the metastasis process? Yes, if they are fortunate to find their way into the circulation system. However, considering how rare the tumor cells could survive the circulation and form secondary tumors at the distant site (estimated from 1 in 10,000 to 1 in millions) (158, 178), it would be very difficult for metastasis to occur given only a handful of released cells with the “metastasis mutation”. Thus, tumors that occured via this route may become benign tumors; it would take a very long time for the metastasis to happen, or not happen at all.

A few pieces of evidence are also counter the possibility that malignant cancers acquire metastasis ability at the late stage. First, despite decades of intensive investigation, a specific “metastasis gene” has not been found. Second, DNA microarray analysis has shown that secondary tumor cells are often strikingly similar to the primary tumor cells, suggesting that the dominant population of primary tumor cells is identical to the metastasis cells (12). Third, the early detection methods for breast cancer, breast self exam and mammograms, have been scrutinized for their effectiveness on survival benefits (163); (9, 54, 114, 115, 124, 125).

The logical reasoning and evidence above lead to the hypothesis that the malignant cancers actually obtained the metastatic trait at the earlier stage of tumorigenesis. In other words, tumors devoid of these mutations and tumors which obtained these mutations after primary tumor was established would likely remain benign

throughout a lifetime. In contrast, tumors which obtained this type of mutations at the early stage would be metastatic ever since, and would be able to develop into malignant cancers.

But how exactly do cancer cells acquire the metastasis ability at the earlier stage of tumorigenesis? From an evolutionary point of view, there are two possibilities.

(1) Cancer cells acquire the metastasis trait without being specifically selected for it. In this case, the mutation responsible for metastasis happens to be one of the early mutations which give a proliferation advantage in the early stage of tumorigenesis. In another word, the metastatic property of cancer is only an unfortunate byproduct of selection for certain “proliferation mutations”.

(2) Cancer cells acquire the metastasis trait by natural selection. Metastasis and the invasion of the local tissues are closely related process in that they both enhance abnormal cell motility inside the body. So, the selection for the metastasis trait actually means the selection for abnormally enhanced cell motility. In a growing tumor, however, enhanced cell motility does not necessarily provide a proliferation advantage because cells do not divide effectively at high level of motility. How is enhanced cell motility selected among cancer cells? The answer may lie in another important aspect of tumorigenesis: angiogenesis, the growth of new blood vessels.

Access to blood supply is essential for the survival of both normal tissue cells and tumor cells. Tumor cells may be hardier than the normal cells, but they still require nutrients and oxygen for survival. Thus, as the incipient unvascularized tumor reachs a

certain size, the cells at the core would gradually be subject to harsher and harsher conditions, while the cells at the edge may still survive and proliferate. As the proverb goes, “If the mountain won't come to Mohammed, then Mohammed must come to the mountain”, there are also two possibilities for further tumor development.

First, bring blood to the cells. If a mutation that confers angiogenesis ability occurs before this point, the cells with the “angiogenesis mutation” would be selected for and the tumor would probably develop into a benign tumor, because now with the ability to bring blood to the cells, even the future mutation for metastasis do occur, they won’t provide proliferation advantage anymore, thus the majority cells in the tumor would still be without the “metastasis mutation” and remain benign.

The second possibility involves bringing the cells to blood. If some cancer cells at the dying core have the mutation which can facilitate the cell survival and cell motility under the harsh condition, these cells may be able to migrate out to new locations where blood supply can be accessed. With abundant blood supply, the mechanism facilitating cell motility is deactivated, and these cancer cells can establish new colonies, multiply in numbers and be selected for in the long run. This might explain how abnormally enhanced cell motility thus the potential for metastasis is selected in the early stage of a malignant cancer. The mutation which confers the angiogenesis could, and most likely, would happen later, but the “angiogenesis mutation” would then be layered on top of the “metastasis mutation”, thus allowing the vascularized tumor cells to retain the metastatic ability. The data in this thesis supports this hypothesis. In short, we showed that serum reduction leads to the stress response in cancer cell lines, elevating PLD activity which in

turn provides survival signals and results in enhanced cell migration/invasion; in the presence of abundant serum, cancer cells have low PLD activity and are much less mobile. A model is illustrated in Figure 5.1. We also showed that serum reduction in MDA-MB-231 cells induces elevated expression of HIF1α, which in turn could lead to angiogenesis, and the elevation of HIF1α is dependent on PLD (Figure 4.19). In MDA-MB-231 cells, PLD not only provide survival signals and enhanced cell motility but also helps angiogenesis, which might have happened later, with another mutation, ultimately resulting in a highly malignant tumor with angiogenesis ability for fast growing but still retaining the invasion ability.

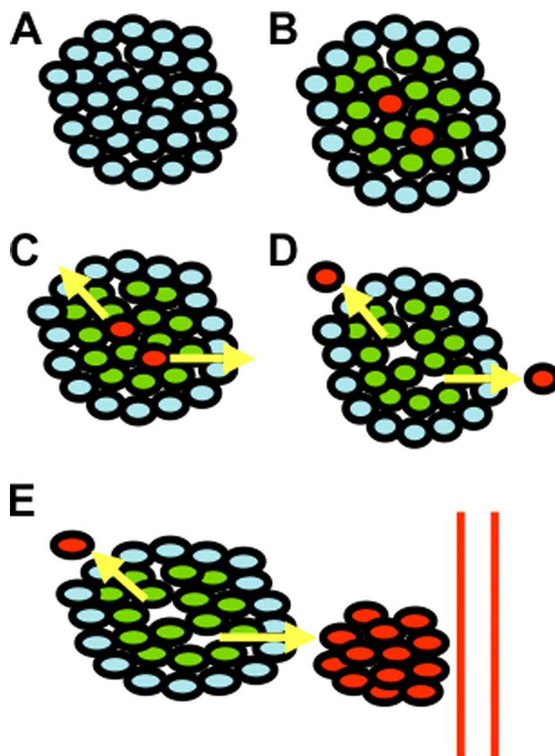


Figure 5.1 (190) **Model for enhanced survival and migration signals in a developing tumor.** In a developing tumor mass (*A*), cells inside the mass (*green cells*) were subjected to hypoxia, nutrient, and growth factor deprivation (*B*). It is proposed that cells that elevate their PLD activity (*red cells*) in response to this stress will not only survive (*C*) but also gain the ability to migrate (*D*). Cells that find a site where vasculature can provide nutrients, oxygen, and growth factors will form secondary metastatic tumors (*E*).

To complete the discussion, we must consider what happens when cancer cells at the edge of the incipient tumor, that is where the blood is still accessible, acquire a

mutation that confers elevated mobility in the presence of blood supply. First, similar to what was discussed above, the cells with this type of mutations do not have a proliferation advantage over their neighbors because while other cancer cells proliferate with the supply of blood, these cells would spend time and energy moving around. Second, our data showed that all 5 highly invasive human cancer cell lines are much more invasive with reduced serum than with abundant supply of serum. Thus this may not be the route for the rise of highly metastatic cancers.

In summary, there are two possible answers to the cancer progression puzzle. (1) “Metastasis mutations” are not specifically selected for, and are the same mutations which confer proliferation benefits in the early stage of tumorigenesis. (2) “Metastasis mutations” are selected for in the early stage of tumorigenesis, the key selection factor being the lack of blood supply in early unvascularized tumors. The data presented in this thesis support the second possibility but does not exclude the first one. There are multiple pathways for cancers to develop and to achieve malignancy, an example of which is MDA-MB-435s cells as they have very low PLD activity but are highly invasive (Figure 4.18), indicating a PLD independent invasion/metastasis pathway. But it is obvious that PLD plays an important role in tumorigenesis, especially in the rise of the highly malignant type of cancers.

## 5.2 Practical importance

Understanding how and when cancer acquires metastatic ability has fundamental importance for cancer prevention and detection, and especially treatment strategies. Early detection is based on the hope of finding and eliminating tumors before the onset of metastasis. But how early is early enough? Breast self exams (BSE) have long been hailed as the first preventive step in early detection. However, studies involving tens of thousands of women over decades showed that BSE does not ensure a survival benefit, meaning that BSE will not lower one's chance of dying from breast cancer (9, 163). Mammogram, another breast cancer early detection method broadly thought to be more reliable, has also been scrutinized for its survival benefits (54, 114, 115, 124, 125). Seven randomized trials about the mammograms were reviewed and were found with severe flaws except for the Canadian trial which showed that mammograms did not provide statistically significant survival benefits. As for the other trials that claimed benefits of mammograms, these benefits would not be there if certain flaw factors were considered (54, 114, 115, 124, 125). For example, in some of these trials, women with pre-existing breast cancer were preferentially excluded from the screening group. Excluding women with breast cancer is not unreasonable, but the numbers excluded from both the screening and control groups would be about the same had there been no bias. The problem was most severe in the New York trial in which 853 women in the screened group but only 336 in the control group were excluded at the time of randomization. If these women had been included, the breast cancer mortality rate would have been higher in the screened group than in the control group (13).

The reason for the unexpected ineffectiveness of these cancer early detection methods may be that the metastasis actually happens before the primary tumors could be detected by these methods. Addressing the problem objectively is essential for developing more advanced early detection methods with higher sensitivity and accuracy.

Anti-angiogenesis therapy has been a prime field of interest in cancer research. However, if the cancers are like the invasive cancer cell lines studied in this thesis, anti-angiogenesis treatment by itself may lead to the stress response and trigger the migration and invasion of tumor cells, thus making it difficult to eradicate the cancer, and could even result in more metastasis. One example can be found in the study of the ID1 gene. It has been recently reported that ID1 gene is overexpressed in melanoma, breast, head and neck, brain, cervical, prostate, pancreatic and testicular cancers, resulting in increased tumor angiogenesis by downregulating the expression of thrombospondin-1 (TSP-1), a naturally occurring angiogenesis suppressor (10, 29, 106, 173). In the earlier mouse model research on ID1, tumor transplants were used, and the tumor growth were resisted in the ID1-knockout mice by inhibiting angiogenesis, while the transplanted tumor grew well in the wild type ID1+/+ mice. But people do not get cancer by tumor transplant, and clinical trials of agents targeting Id1 have proved disappointing. To mimic the spontaneous tumorigenesis, the same group of researchers designed the new experiments in which mice were exposed to carcinogens placed on their skin and allowed to gradually develop cancer. To their surprise, the results showed a completely opposite outcome with respect to ID1: ID1-/- mice are more susceptible to skin tumorigenesis and developed more and larger tumors compared to wild-type ID1+/+ mice (152). Their preliminary data

suggested that an impaired T-cell function in the ID1-/- mice may be responsible for this otherwise bizarre result (152).

Actually, their results coincide well with our hypothesis. The transplanted tumor cells would have limited growth when the angiogenesis pathway is blocked in ID1-knockout mice, as compared to the tumors in the wild type mice. However, in the spontaneous tumorigenesis (although hastened by the carcinogens), when the angiogenesis was blocked, only the aggressive cancer cell clones emphasized by this thesis and illustrated in Figure 5.1 would be selected. These cells spread and proliferate fast, once securing other means to induce angiogenesis while still remaining highly malignant, they would increase in number and size as aggressive tumors faster than the tumors in the wild type mice. In the wild type mice, on the other hand, during the tumorigenesis, the cancer cells that acquired angiogenesis ability first would also be selected, resulting in benign tumors with smaller number and size.

This is not to say that anti-angiogenesis therapy is detrimental for patients. As a general cancer therapy, anti-angiogenesis makes a lot of sense. But because of the complexity of cancer, when applying antiangiogenesis therapy, like any other cancer therapy, a corresponding strategy needs to be considered when dealing with different cancers with different profiles. For example, if our hypothesis is correct, when treating highly invasive cancers with highly elevated PLD activity by anti-angiogenesis therapy, it would be a good idea to apply complementary drugs that could either block PLD activity, or induce cell death in low serum condition, or inhibit the cell motility.

In this regard, it is worth mentioning honokiol, a natural compound found in the bark and seed cones of the Magnolia tree. The magnolia tree has been used as a traditional herb medicine for thousands of years by Chinese and Japanese. For two decades, honokiol has been studied as a potential anticancer drug (59, 94, 120, 175, 183). Recently, our collaborator, Dr. Jack Arbiser's group, found that honokiol inhibits angiogenesis in vitro and tumor growth in vivo (6), and they along with other groups found that honokiol also induce apoptosis in cancers (8, 71, 83, 175, 183).Our preliminary data showed that honokiol effectively kills various human cancer cells including MDA-MB-231, MCF7, T24, Calu1 under low serum conditions, but even a five times higher dose of honokiol does not kill these cancer cells with sufficient serum supply. Interestingly, we also found that honokiol blocks the induced PLD activity by serum reduction, while it has no effect on the basal PLD activity of MDA-MB-231 and Calu1 cells in the presence of serum. Our data suggest that the honokiol's anticancer effect may reside in its ability to interfere with both angiogenesis and cell survival mechanisms, probably by blocking PLD activity.

Other than helping to choose the right strategy when treating different cancers, our hypothesis also means that not only a highly upregulated PLD activity, but any mutation that gives both survival benefits and enhanced cell motility in the lack of blood supply, could potentially result in highly malignant cancers. Thus, with current advancing technologies and knowledge about cancer, future study could focus on looking for the individual genes whose mutation could contribute to both survival and invasion of cells under low serum conditions.

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