Chapter 2
Phospholipase Signaling in Breast Cancer

Yu Jin Lee, Kyeong Jin Shin, Hyun-Jun Jang, Dong-Young Noh, Sung Ho Ryu, and Pann-Ghill Suh

Abstract Breast cancer progression results from subversion of multiple intra- or intercellular signaling pathways in normal mammary tissues and their microenvironment, which have an impact on cell differentiation, proliferation, migration, and angiogenesis. Phospholipases (PLC, PLD and PLA) are essential mediators of intra- and intercellular signaling. They hydrolyze phospholipids, which are major components of cell membrane that can generate many bioactive lipid mediators, such as diacylglycerol, phosphatidic acid, lysophosphatidic acid, and arachidonic acid. Enzymatic processing of phospholipids by phospholipases converts these molecules into lipid mediators that regulate multiple cellular processes, which in turn can promote breast cancer progression. Thus, dysregulation of phospholipases contributes to a number of human diseases, including cancer. This review describes how phospholipases regulate multiple cancer-associated cellular processes, and the interplay among different phospholipases in breast cancer. A thorough understanding of the breast cancer–associated signaling networks of phospholipases is necessary to determine whether these enzymes are potential targets for innovative therapeutic strategies.

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2.1 Introduction

Breast carcinoma is the most common malignancy worldwide after lung cancer, the fifth most common cause of cancer death, and the leading cause of cancer death in women [1]. The global burden of breast cancer exceeds that of all other cancers, and the incidence rates of breast cancer are increasing. Recently, mortality rates have exhibited a small decline, which more likely is a result of increased public awareness and early diagnosis, the implementing more affordable and effective screening programs, and advances in therapeutic techniques [2]. Nevertheless, the heterogeneity of breast cancers makes them both a fascinating and a challenging solid tumor to diagnose and treat. For example, patients with estrogen receptor (ER)-positive tumor can be treated with adjuvant endocrine therapy to suppress the growth-promoting actions of estrogen receptor alpha (ERα) [3]. Current ER-targeted pharmacological interventions include Tamoxifen and fulvestrant. Patients whose tumors express human epidermal growth factor receptor 2 (HER2) can benefit from treatment with specific antagonists of this receptor, such as Lapatinib and Trastuzumab (Herceptin) [4]. The majority of patients treated with adjuvant systemic therapy respond poorly to treatment, or go on to develop acquired resistance to hormonal therapies or HER2-targeted therapies, rendering the therapy ineffective. For the subset of patients with tumors that are ER-negative, progesterone receptor (PR)-negative, and HER2-negative (triple-negative, or basal-like cancers), there is no standard adjuvant intervention and they can be treated only with conventional chemotherapy [5]. Therefore, there is a critical need for new systemic therapies. Over the last decade, in-depth research has focused on the molecular biology of this disease, and study populations have been selected for clinical trials based on their molecular markers. Technological breakthroughs and high throughput approaches in particular have allowed researchers to probe deeply into the nature of breast cancer, revealing that this disease requires an interconnect-environment, and that the innate characteristics of the patient influence disease pathophysiology, outcome, and treatment response. Thus, focusing on personalized medicine to target disease manifestation on an individual basis will facilitate the development of more effective interventions, particularly for later stage malignancies with worse prognoses, and also in cases where resistance to existing therapies develops over time.

Phospholipases (PLC, PLD, and PLA) comprise a highly diverse group of enzymes that share the common property of hydrolyzing phospholipids, which are major components of cell membranes [6, 7]. Phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol, can be broken down into various intracellular signaling moieties, such as diacylglycerol (DAG), phosphatidic acid (PA), lysophosphatidic acid (LPA), and arachidonic acid (AA) [8]. Through inter- and intracellular

signaling, bioactive lipid mediators or second messengers regulate a variety of cellular physiological and pathophysiological functions, including proliferation, survival, migration, vesicle trafficking, tumorigenesis, metastasis, and inflammation [9, 10].

Each phospholipase regulates its own specific signaling pathways, but shares common signaling molecules with other members of its subfamily, acting as upstream regulators or downstream effectors. Recent findings indicate that phospholipases crosstalk with one another, which influences cell fate via the integration and fine-tuning of intracellular signals [8, 9]. To understand these complex signaling systems in the microenvironments of tumors, as well as in individual tumor cells, systematic analyses of phospholipase functions are required. In this chapter, we summarize current understanding of the various roles of phospholipases in breast tumor progression, with a focus on the signaling networks of phospholipases. We also discuss potential strategies for treating cancer by disrupting these networks, with a focus on their potential utility for aiding clinical management and prognostication, and for informing therapeutic options.

2.2 Review of Past Studies

2.2.1 Characteristics and Cellular Signaling of Phospholipases

Phospholipases are common enzymes present in a broad range of organisms, including bacteria, yeast, plants, animals, and viruses. Phospholipases can be categorized into three major classes—PLA (consisting of A1 and A2), PLC, and PLD—which are differentiated by the type of reaction that they catalyze [11, 12] (Fig. 2.1).

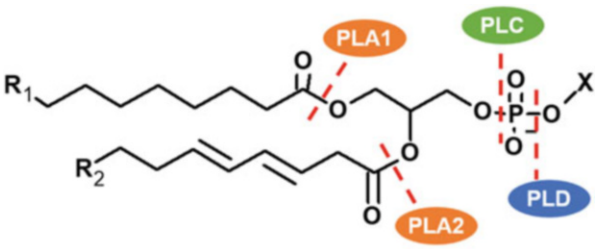

Fig. 2.1 Phospholipid structure and the site of actin of phospholipases. Phospholipids are composed of a glycerol-3-phosphate esterified at the sn-1 and sn-2 positions to nonpolar fatty acids (R1 and R2, respectively) and at the phosphoryl group to a polar head group, X. Phospholipase A1 and phospholipase A2 cleave the acyl ester bonds at sn-1 and sn-2, respectively. Phospholipase C cleaves the glycerophosphate bond, whereas phospholipase D removes the head group, X. *PLA* phospholipase A, *PLC* phospholipase C, *PLD* phospholipase D

2.2.1.1 PLC

Phosphoinositide-hydrolyzing PLC cleaves the glycerophosphate bond that links the polar head group to the glycerol backbone to produce inositol-1,4,5-triphosphate (IP3) and DAG in the cellular setting of ligand-mediated signal transduction (Fig. 2.1). DAG activates protein kinase C (PKC), whereas the binding of IP3 to its receptor triggers the release of calcium ions from intracellular stores into the cytosol [13]. Since the first report of PLC, 13 mammal PLC isozymes have been identified, and they can be divided into six subgroups: PLC-β [1–4], -γ [1 and 2], -δ [1, 3, 4, and], -ε, -ζ, and –η [1 and 2] [14] (Fig. 2.2). Interestingly, PLC isozymes have highly conserved X and Y domains which are responsible for PIP2 hydrolysis. Each PLC contains distinct regulatory domains, including the C2 domain, the EF-hand motif, and the pleckstrin homology (PH) domain [15]. Notably, each PLC subtype exhibits a unique combination of X-Y and regulatory domain, so that each PLC isozyme is regulated differently and has a different function and tissue distribution; thus, PLC-mediated signaling pathways regulate diverse biological functions [16].

The X and Y domains are usually located between the EF-hand motif and the C2 domain, and are composed of α-helices alternating with β-strands, with a structure that is similar to an incomplete triose phosphate isomerase α/β-barrel [17]. Conversely, the PH domain, although a membrane phospholipid-binding region like the C2 domain, has specific functions according to the type of isozyme. For example, he PH domain of PLC-δ1 binds PIP2 and contributes to the access of PLC-δ1 to the membrane surface [18]. In contrast, the PH domain specifically binds the heterotrimeric Gβγ subunit in PLC-β2 and PLC-β3 isozymes [19], and mediates interactions with phosphatidylinositol-3,4,5-trphosphate (PIP3) in PLC-γ1, where it is required to induce phosphoinositide 3-kinase (PI3K)-dependent translocation and activation [20]. As for the latter, it is worth noting that PLC-γ1 and PLC-γ2 isozymes contain an additional PH domain, which is split by two tandem Src homology domains, SH2 and SH3, for direct interaction with the calcium-related transient receptor potential cation channel, thereby providing a direct coupling mechanism between PLC-γ and agonist-induced calcium entry [21]. Finally, the C2 and EF-hand motifs are important for calcium regulation: the EF-hand motifs are helix-turn-helix structural domains that bind calcium ions to enhance PLC enzymatic activity [22, 23]. Interestingly, among the PLC isoenzymes, PLC-β subtypes also distinguish themselves by the presence of an elongated C-terminus, consisting of about 450 residues, which contains many of the determinants for the interaction with Gq alpha subunit as well as for other functions, such as membrane binding and nuclear localization [24–26].

The activation and regulation of PLC isozymes differ by subtype. For example, PLC-β subtypes are activated by G protein-coupled receptors (GPCRs) through several mechanisms. In contrast, PLC-γ subtypes are commonly activated by receptor tyrosine kinases (RTKs) via SH2 domain-phosphotyrosine interactions, and are subjected to phosphorylation by RTKs after the stimulation of growth factors like

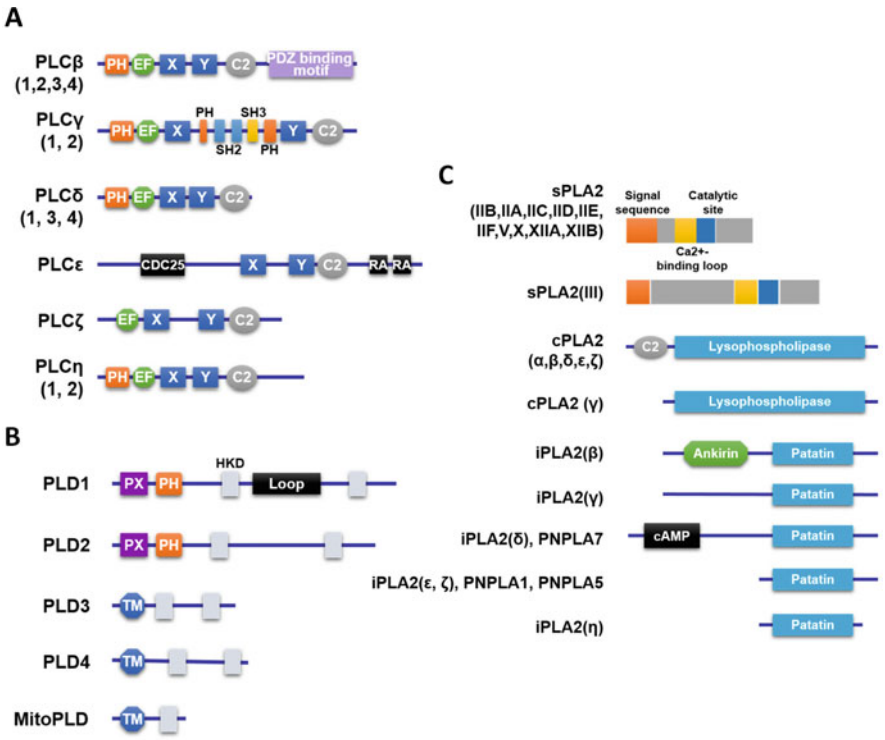

Fig. 2.2 Schematic structure of phospholipases. (**a**) Thirteen mammalian PLC isozymes are subdivided into six groups. All PLC isotypes have X and Y domains, which contain catalytic activity. Several isoforms have pleckstrin homology (PH) and a calcium-binding (C2) domain, which can regulate PLC activity. EF-hand domain is responsible for forming a flexible tether to the PH domain. PLCε has a RAS guanine nucleotide exchange factor (GEF) domain for RAP1A122 and the RA2 domain mediates interaction with GTP-bound *Ras* and RAP1A. PLCγ has SRC homology 2 (SH2) and Sh3 domains, which interact with many proteins. (**b**) In mammals, PLD1 and PLD2 hydrolyze phosphatidyl-choline (PC). PC-PLD has several conserved regions, including phox homology (PX) and PH domains, and two conserved catalytic domains (HKD). Non-PC-hydrolyzing PLD3, PLPD4, and mitochondrial PLD (mitoPLD) have recently been described. (**c**) The three major types of PLA2 include secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), and calcium-independent PLA2 (iPLA2). Eleven sPLA2, six cPLA2, and nine iPLA2 have been found in mammals. sPLA2 has a signal sequence to target the extracellular region, a Ca2+-binding loop, and a catalytic site. cPLA1α, cPLA1β, cPLA1δ, cPLA1ε, and cPLA1ξ have a C2 domain, and a lysophospholipase-like domain. iPLA2β has Ankyrin repeats, which may mediate its oligomerization. Both iPLA2δ and PNPLA7 also have a cyclic AMP-binding domain and a patatin domain that is implicated in enzymatic activity. PLA1 has not been well characterized and has few links to cancer. *DAG* diacylglycerol, *IP3* inositol 1,4,5-triphosphate, *PA* phosphatidic acid

epidermal growth factor (EGF) and fibroblast growth factor (FGF) [15, 27]. Interestingly, PLC-ε can be activated by both GPCR and RTK systems, via distinct activation mechanisms [28]. Indeed, several GPCR ligands, such as lipoprotein A, thrombin, and endothelin, can activate PLC- ε, but PLC- ε also associates with Rap

and translocates to the perinuclear area, where it interacts with activated RTKs [29]. It has been suggested that overall PLC activity may be amplified and sustained by both intracellular calcium mobilization and extracellular calcium entry. PLC-δ1and PLC-η1 are activated via GPCR-mediate calcium mobilization. In particular, the PLC-δ1 isozyme is one of the most sensitive of the PLC isozymes, suggesting that its activity is directly regulated by calcium. PLC-η1 specifically acts as a calcium sensor during the formation and maintenance of the neuronal network in the postnatal brain. Moreover, several studies have suggested positive feedback amplification of PLC signaling. Indeed, the overall PLC activity may be amplified and sustained by both intracellular calcium mobilization and extracellular calcium entry, through either a negative or a positive feedback amplification of PLC signaling [30, 31]. By these mechanisms, it has been suggested that PLC-β, PLC-γ, and PLC-ε may be primarily activated by extracellular stimuli. In contrast, activation of PLC-δ1and PLC-η1 may be secondarily enhanced by intracellular calcium mobilization serving to amplify PLC activity [32]. As for PLC-ζ, its activation and nuclear translocation mechanisms remain to be revealed.

2.2.1.2 PLD

Phosphatidylcholine-specific phospholipase D (PLD) hydrolyzes PC, the most abundant membrane phospholipid, to yield choline and the secondary messenger signaling lipid PA (Fig. 2.1). In mammals, two isoforms found in association with membrane surfaces in the cytoplasm, PLD1 and PLD2 [33, 34]. PLD3 and PLD4 are endoplasmic reticulum (ER) integral transmembrane proteins with a short N-terminal cytoplasmic tail, and the bulk of the protein, including the hypothetical catalytic domains, is present in the ER lumen [35, 36]. In contrast, PLD6 (MitoPLD) is anchored by an N-terminal transmembrane tail into the outer surface of mitochondria [37]. PLD5, on which there are no published studies, is most similar to PLD3 and PLD4, but is unlikely to have enzymatic activity because the canonical PLD enzymatic catalytic motif is not well conserved in its sequence. Enzymatic activities have also not been identified for PLD3 or PLD4, and it is possible that they have non-enzymatic functions instead. PLD6 has been reported to both hydrolyze cardiolipin, a mitochondrial-specific lipid, to PA, and to function as a endonuclease (phosphodiesterase) to generate a specialized form of micro-RNA known as piwi-interacting RNA (piRNA) [38]. For different reasons, therapeutic applications are not immediately apparent for PLD3–6; therefore, this review focuses on PLD1 and PLD2.

PLDs are ubiquitously expressed in almost all of tissues and cells of mice, and their activity is stimulated in response to various extracellular agonists, such as hormones, neurotransmitters, extracellular matrixes (ECM), and growth factors [39–41]. Clarification of the domain structure of PLDs has contributed to the elucidation of the activation mechanisms and physiological functions of PLD isozymes. Both PLD1 and PLD2 has several conserved regions, including phox homology (PX) and PH domains that are important for binding various lipids and proteins, and two

conserved catalytic domains (HKD), which are essential for enzymatic activity [42, 43]. However, it has been reported that the PH domains of PLD1 and PLD2 are not required for PLD activation. One interesting domain is the "loop domain," which is found in PLD1, but not PLD2. The loop domain seems to be involved in auto-inhibition of enzymatic activity of PLD1, because deletion of this region increases basal activity, and insertion of the loop domain into recombinant PLD2 significantly reduces its basal activity [44–46].

PLD1 and -2 are widely expressed in different tissues and cell types, and are activated by a variety of GPCRs and RTKs [47]. PA generated by PLDs functions locally as a signaling messenger to regulate diverse cellular functions, including endocytosis, exocytosis, membrane trafficking, cell proliferation, and actin cytoskeleton reorganization [48]. PA can also act as a lipid anchor, recruiting PA-binding proteins to localized sites of signal transduction, examples of which include the guanine nucleotide exchange factors (GEFs) DOCK2 and SOS, which activate Rac1 and Ras, respectively [49–51]. In some instances, PA additionally activates the proteins recruited, s uch as phosphorylating phosphatidylinositol 4-phosphate (PI4P), to generate phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and mammalian target of rapamycin (mTOR), which regulate many processes including cell hypertrophy, differentiation, and survival [52]. Finally, PA also functions as an intermediate for the production of bioactive DAG or LPA [53, 54]. Therefore, aberrant expression or activation is closely linked to human diseases including cancer, diabetes, neurodegenerative disorders, and myocardial disease.

2.2.1.3 PLA

PLA hydrolyzes the carboxylic esters at the sn-1 (PLA1) or sn-2(PLA2) positions on glycerol backbones of phospholipids to produce free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively (Fig. 2.1). PLA1 can be divided into two groups according to cellular localization: intracellular and extracellular PLA1. Three members of the mammalian intracellular phospholipase A1 subfamily have been identified: PA-preferring phospholipase A1, p125, and KIAA0725p [55, 56]. These enzymes commonly contain a lipase consensus sequence. There are 10 mammalian extracellular phospholipase A1 enzymes: phosphatidylserine-selective phospholipase A1 (PS-PLA1), membrane-associated PA-selective phospholipase A1α (mPA-PLA1 α), mPA-PLA1β, pancreatic lipase, lipoprotein lipase, hepatic lipase, endothelial lipase, and pancreatic lipase–related proteins-1–3 (Fig. 2.2). These PLA1s share multiple conserved motifs, including a lipase consensus sequence, a catalytic Ser-Asp-His triad, cysteine residues, and a lipid-binding surface loop [55]. In contrast to other phospholipases, the physiological roles of PLA1 remain largely unknown, especially in mammalian.

The PLA2 family of enzymes catalyze the hydrolysis of the sn-2 bond of membrane phospholipids to release AA and lysophospholipid secondary messengers under the influence of various stimuli, including circulating hormones and growth factors. The first PLA2 was identified in snake venom, while other enzymes were

discovered in other organisms. The growing superfamily of PLA2s is categorized into 14 groups based on amino acid sequences and these 14 groups are subdivided into 4 classes in mammals (Fig. 2.2). PLA2s are classified into several major types: secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), calcium-independent PLA2 (iPLA2), platelet-activating factor acylhydrolases (PAF-AHs), lysosomal PLA2s, and adipose-specific PLA2s. They differ from each other in terms of substrate specificity, calcium requirement, and lipid modification [56, 57]. The ubiquitously expressed cPLAα 2 has high selectivity for membrane phospholipids that contain AA, which can be metabolized to growth-promoting eicosanoids. This has resulted in a number of studies that link cPLA2α activity to tumorigenesis. cPLA2α has a cytoplasmic distribution when inactive, but translocates to intracellular membranes once activated by concurrent Ca^{2+} binding and phosphorylation at serine residue 505 [58]. cPLA2α -released AA is a potent cytotoxic compound, inducing cell death through stimulation of mitochondrion-mediated apoptosis and sphingomyelin phosphodiesterase (SMase)-ceramide pathways, unless the AA is subjected to further metabolism [59]. The iPLA2 family is important for membrane homeostasis and energy metabolism, and the sPLA2 family modulates extracellular phospholipid environments.

2.2.2 Phospholipases Signaling in Cancer

Phospholipases can be activated by multiple extracellular signals, including hormones (e.g., insulin and growth hormones), growth factors (e.g., EGF and vascular endothelial growth factor [VEGF]), and lipids (e.g., LPA and sphingosine 1-phosphate [S1P]; Fig. 2.3) [14, 60–62]. These extracellular cues stimulate phospholipases through the direct activation of RTKs or GPCRs [15, 63]. Phospholipases act as key mediators of many cellular functions by generating bioactive lipids that transmit signals to a variety of downstream molecules and interactions with their binding partners. As illustrated in Fig. 2.3, phospholipases and their lipid mediators underlie complicated, multilayered signaling networks. Furthermore, lipid mediators are major participants in a variety of cellular processes related to tumorigenesis and/or metastasis, such as matrix metalloproteinase (MMP) secretion, actin cytoskeleton reorganization, migration, proliferation, growth, inflammation, and angiogenesis [14, 55, 56, 64]. The importance of phospholipases and their products (that is, lipid mediators) in key cellular functions has been characterized by cell-based analyses, and by studies using transgenic and knockout mice. Studies using transgenic and knockout mice have demonstrated that phospholipases are crucially involved in various phenotypes. Specifically, many studies on phospholipase transgenic and knockout mice have demonstrated tumor-related phenotypes, such as tumorigenesis, metastasis, and angiogenesis, in a variety of organs, including the intestine, colon, lung, and ovary (Table 2.1). The following sections discuss what have been learned from studies of cell lines and mouse models regarding the

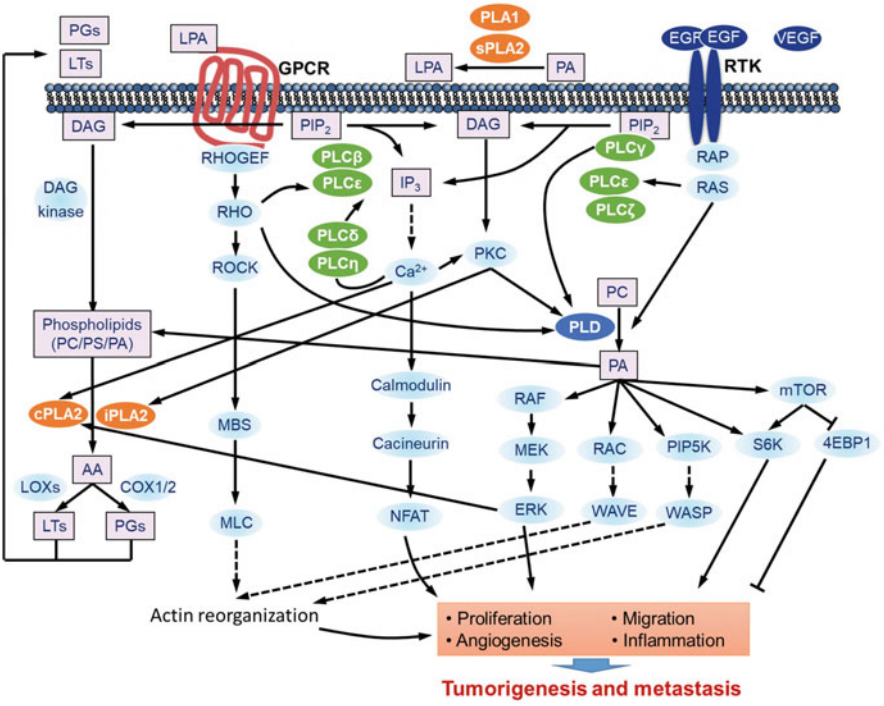

Fig. 2.3 Overview of phospholipase signaling pathways and networks in cancer. Phospholipases (PLA, PLC, PLD)-related signal pathways are closely connected with each other and essential in various tumor processes (e.g., growth, differentiation, and migration). Among PLC isozymes, PLCβ and ε are activated by G protein or small GTPase in GPCR signaling. Activity of PLCδ and η is controlled by calcium signaling induced by GPCR. PLCγ is directly phosphorylated by RTK activated by growth hormones such as EGF and VEGF. Activated PLC can cleave PIP_2 into DAG and IP_3 which are important second messengers in cellular functions. PLC-mediated signaling, IP_3-induced calcium release, and PKC activation can stimulate other phospholipases activity, PLA, and PLD. Cytosolic PLA2(cPLA2) and intracellular calcium-independent PLA2 (iPLA2) can generate AA by hydrolyzing various phospholipids (PC, PS, PA). AA is further modified into eicosanoids, including PGs and LTs by COX and LOX, respectively. PGs and LTs are released from the cell and act as autocrine and paracrine factors. In extracellular environment, membrane-associated PA-selective PLA1(mPA-PLA1) and secretory PLA2 (sPLA2) hydrolyze PA into LPA, which induces GPCR signaling in an autocrine/paracrine manner. PLD, activated by PKC, converts PC into PA, which can stimulate multiple downstream signal molecules. *PL* phospholipase, *GPCR* G-protein-coupled receptor, *RTK* receptor tyrosine kinases, *EGF* epidermal growth factor, *PIP_2* phosphatidylinositol-4,5-bisphosphate, *DAG* diacylglycerol, *IP_3* inositol-1,4,5-trisphosphate, *PKC* protein kinase C, *AA* arachidonic acid, *PC* phosphatidylcholine, *PS* phosphatidylserine, *PA* phosphatidic acid, *PGs* prostaglandins, *LTs* leukotrienes, *COX* cyclooxygenase, *LOX* lipoxygenase, *LPA* lysophosphatidic acid, *4EBP1* 4E binding protein 1, *CASP* caspase, *GEF* guanine nucleotide exchange factor, *MBS* myosin binding subunit, *MLC* myosin light chain, *NFAT* nuclear factor of activated T cells, *PIP5K* phosphatidylinositol 4-phosphate 5-kinase, *ROCK* RHO kinase, *S6K* S6 kinase, *VEGF* vascular endothelial growth factor, *WASP* Wiskottt–Aldrich syndrome protein, *WAVE* WASP family protein member

Table 2.1 Cancer-related phonotypes of phospholipase transgenic and knockout mice

Gene	Types of mutation	Tissue affected	Phenotype	Refs
sPla2	Spontaneous/ Transgenic	Intestine	Increased tumor susceptibility in $Apc^{min/+}$ mice Reduced tumorigenesis in $Apc^{min/+}$ mice	[128, 131]
	Spontaneous	Colon	Inverse correlation of Pla2g2a expression level with susceptibility to carcinogen-induced colon tumor	[129]
cPla2	Knockout	Intestine	Decreased tumor number in small intestine of $Apc^{min/+}$mice	[118]
	Knockout	Lung	Decreased number of carcinogen-induced lung tumor	[127, 145]
	Knockout	Colon	Increased colonic injury and number of colon tumor by carcinogen Impaired colonic eicosanoid production	[117, 123]
	Knockout	Angiogenesis	Tumor regression and attenuated vascularity	[130]
iPla2b	Knockout	Lung metastasis	Decreased lung metastasis	[132]
	Knockout	Ovary	Reduced tumorigenesis and ascites formation from injected ovarian cancer cells	[135]
Plcb3	Knockout	Hematopoietic cells	Developed myeloproliferative disease, lymphoma, and other tumors	[86]
Plcg	Transgenic (dominant negative)	Lung metastasis	Decreased number of lung metastases in PyVmT and TRAMP models	[74]
Plcg2	Knockout	B cells	Lymphoma development in $Plc\gamma2^{-/-}$; Eμ-Myc transgenic mice	[65]
Plcd1	Knockout	Skin	Developed spontaneous skin tumor	[84]
Plce1	Knockout	Skin	Delayed onset and markedly reduced incidence of carcinogen-induced skin squamous tumors	[85]
	Knockout	Colon	Alleviates the colitis and suppresses tumorigenesis	[66]
Plcz1	Transgenic	Ovary	Developed benign ovarian teratomas	[67]
Pld1	Transgenic/ Knockout	Intestine	Accelerates tumorigenesis in $Apc^{min/+}$ mice Loss of PLD1 suppresses the intestinal tumorigenesis in $Apc^{Min/+}$ and AOM/DSS mice models	[110, 109]
	Knockout	Tumor microenvironment	Suppressed tumor growth, metastasis, and angiogenesis	[94]
Pld2	Knockout	Angiogenesis	Reduced tumor growth and tumor blood vessel formation	[111]
	Knockout	Lung metastasis	Inhibited invadopodia formation in breast cancer cells	[106]

APC adenomatous polyposis coli, *Pl* phospholipase, *PyVmT* polyomavirus middle T antigen, *TRAMP* transgenic adenocarcinoma of the mouse prostate

functions of various phospholipases in breast cancer–associated processes and signaling pathways.

2.3 Current Evidence and Concepts

2.3.1 PLC and Breast Cancer

A role for PLC has recently been identified in the regulation of a number of cellular behaviors, and in the promotion of tumorigenesis by regulating cell motility, transformation, and cell growth, partly by acting as signaling intermediates for cytokines such as EGF and interleukins in cancer cells [65–67]. Aberrant expression and activation of PLC isozymes are observed in a variety of human cancers, and are related to tumor progression.

Previous studies have highlighted alteration in PLC expression levels in breast tumor cells. It has been reported that PLC-β2 is abnormally elevated in breast cancer and correlates with poor clinical outcomes, suggesting its role as a marker for breast cancer severity [68]. In addition, PLC-β2 provokes the transition from G0/G1 to S/G2/M cell cycle phase, which is important in cancer progression and inositol lipid–related modifications of the cytoskeleton architecture occurring during tumor cell division, motility, and invasion [69]. PLC-β isozymes can be activated by GPCRs, indicating that most chemokines secreted in the tumor microenvironment can activate PLC-β to increase cell migration and invasion; indeed, gain- and loss-of-function studies in tumor cells have demonstrated the functional importance of PLC-β in tumor cell migration and invasion. Recently, PLC-β1 was shown to be highly expressed in breast cancer tissues in comparison with normal mammary gland tissues. Also, there are significant differences in PLC-β1 expression between metastasis and recurrence tumor tissue, which may indicate its role in promoting migration in breast cancer [70, 71]. However, further experimental verification is necessary.

Among the PLC isozymes, PLC-γ is important because it plays a specific and key role in cell proliferation, and in migration and invasion, therefore contributing to tumorigenesis and/or metastasis [72–74]. Compared with normal mammary gland tissue, moderately or poorly differentiated breast tumors (grade 2 or 3) express higher levels of PLC-γ1. Expression is at marginally low levels in low-grade tumors compared with normal tissues. A significant association was found between PLC-γ1 expression and the risk of metastatic relapse in T1/T2, N0-stage breast cancer patients treated with chemotherapy [75]. As expected from its expression pattern, PLC-γ1 is involved in the migration and metastatic potential of breast cancer [76]. Growth factor receptors (e.g., EGFR and HER2) and their downstream molecules are associated with increased cancer proliferation and motility. The epithelial growth factor receptor (EGFR)/ErbB family is among the most notable cancer molecular targets in many epithelial tumors. ErbB2 (also known as HER2/neu) in particular is overexpressed in approximately 25% of breast cancers, and trastuzumab (Herceptin), a well-established breast cancer drug, targets ErbB2. Major downstream

signaling pathways of ErbB are the mitogen-activated protein kinase (MAPK) pathway, PI3K pathway, and PLC-γ1 pathway, which lead to gene expression changes.

EGF-induced migration of breast cancer cells mainly depends on the transient activation of PLC-γ1 via ErbB2 activation. Correlatively, downregulation of PLC-γ1expression blocked Rac1 and CDC42 GTPases via IP3-induced calcium release activation, resulting in the suppression of human breast cancer cell–derived lung metastasis in a mouse model [77, 78]. In addition, PLC-γ1 has been shown to mediate the cell motility effects of growth factors including PDGF, EGF, insulin-like growth factor (IGF), and hepatocyte growth factor (HGF). A dominant-negative PLC-γ1 fragment reduced the metastatic potential of breast cancer in a transgenic mouse model. Metastasis assays also demonstrated that nude mice with PLC-γ1 knockdown exhibited inhibition of breast cancer–derived lung metastasis [79]. This result suggests that PLC-γ1 is a potential therapeutic target in the clinical treatment of tumor metastasis.

Moreover, PLC-γ1 is a target of the micro RNA (miR)-200bc/429 cluster that suppresses EGF-driven cell invasion, viability, and cell cycle progression in breast cancer [80]. The miR-200 family consists of five members. They are expressed as two separate polycistronic pri-miRNA transcripts, with miR200b-200a-429 at chromosomal location 1p36 and miR-200c-141 at chromosomal location 12p13. This shared seed sequence suggests that the clusters may share some common target genes. The miR-200 family is downregulated to undetectable levels in breast cancer cell lines with invasive and generally mesenchymal phenotypes compared with well-differentiated breast cancer cell lines. Consistent with its expression in breast cancer cell lines, the levels of the miR-200 family are approximately 10- to 22-fold lower in mesenchymal sarcomatoid regions of human primary breast cancers compared with epithelial epithelioid regions [81], and loss of the miR-200 family contributes to breast cancer progression [82]. In breast cancer, position 4915–4921 on the 3-′-untranslated region (UTR) of PLCG1 is a direct target of miR-200bc/429, and the downregulation of PLC-γ1 by miR-200bc/429 inhibits EGF-driven cell invasion. These reports suggest the mechanism by which PLC-γ1 is overexpressed in breast cancer (Table 2.2).

Furthermore, PLC-δ4 is upregulated in breast tumor cells, and its overexpression enhances cell proliferation in breast cancer cells with lower oncogenicity [83]. Patients with tumor metastasis expressed higher levels of PLC-δ4 than those with local recurrence. Significantly, breast cancer patients with higher expression levels of PLC-δ4 experience a shorter disease-free survival period, which may indicate a correlation between PLC-δ4 and recurrence in breast cancer patients.

Unlike PLC-γ and PLC-ε, the PLC-β and PLC-δ isoforms are known tumor suppressors [84, 85]. Loss of PLC-β3 in mice can result in myeloproliferative diseases, lymphoma, and other types of cancer through the regulation of signal transducer and activator of transcription 5 (STAT5) phosphorylation. Consistent with this, PLC-β3 downregulation has been observed in human chronic lymphocytic leukemia samples [86]. Furthermore, monoallelic deletion of *PLCB1* (which encodes PLC-β1) increases the risk of developing acute myeloid leukemia in patients

Table 2.2 Aberrant expression and mutation of phospholipases in breast cancer

Gene	Expression	Correlation	Refs
sPLA2	Increased	Poor prognosis	[112, 116]
cPLA2A	Increased	Poor prognosis Her2 subtype	[113, 114, 115]
PLCB1	Increased	Invasiveness	[71]
PLCB2	Increased	Poor prognosis with breast cancer malignancy	[68, 69]
PLCG1	Increased	ND	[72, 73, 75]
PLCD1	Decreased	With ER status and tumor grade	[89]
	Increased	ND	[88]
PLCD3	Increased	ND	[88]
PLCD4	Increased	ND	[83]
PLCE	Increased	ND	[71]
PLD1	Increased	ND	[91]
PLD2	Increased	ND	[93]

ND not determined, *PL* phospholipase

with myelodysplastic syndrome. The loss of PLC-δ1 expression is highly associated with its role as a tumor suppressor in esophageal squamous cell carcinoma (ESCC). In addition, decreased PLC-δ1 expression is correlated with poor clinical outcomes in patients with acute or chronic myeloid leukemia [87, 88]. Also, PLC-δ1 is downregulated via hypermethylation in breast cancer. PLC-δ1 suppressed cell migration by regulating cytoskeletal reorganization proteins [89]. Although some mechanistic details remain unclear, the position of PLCs in the vicinity of cell surface receptors that relay signals from the extracellular microenvironment may enable them to amplify downstream signals through the generation of second messengers, activating effectors such as PKC and other phospholipases to continue the propagation of mitogenic signals.

2.3.2 PLD and Breast Cancer

PLD-mediated signaling pathways are highly complicated; therefore, its physiological functions are diverse. Recently, increased expression of PLD enzymes, their subcellular mislocalization, and altered PLD catalytic activity have been implicated as contributing factors in several types of human cancer, such as colon, gastric, kidney, and thyroid cancers. PLD is increasingly recognized as a critical regulator of cancer progression and tumorigenesis. In malignant breast cancer, PLD activity is increased, as is the expression of PLD1 and -2 [90, 91]. PLD1 tends to be overexpressed in tumors that show high expression of cytokeratins 5/17, which are frequently associated with poor prognosis [92]. In addition, elevated PLD2 expression suppresses apoptosis and also promote tumor growth rate and chemoresistance in breast cancer [93]. PLD1 has a critical function not only in the cancer cell itself but also in the tumor microenvironment. Studies in PLD1-deficient mice showed that

PLD1 promotes tumor growth and metastasis through enhanced angiogenesis and decreased tumor cell-platelet interactions [94]. Recent genomic analyses of human cancers have revealed several unique PLD2 mutations in breast, stomach, and brain cancers, although most of the reported mutations remain to be functionally characterized [95, 96]. These studies provide initial evidence that increased PLD activity is linked to oncogenic signals and tumorigenesis.

Several mitogenic signals (such as EGF, EDGF, and FGF) and oncogenic activation (such as *v-ras*, *v-raf*, and *v-src*) stimulate PLD-mediated oncogenic signaling pathways [39–41, 97–99]. The oncogenic signaling network is mediated by the interaction between PLDs and Ras, and facilitates the activation of MAPK [100]. Furthermore, recent work has revealed that PLD2-generated PA recruits SOS1 to the plasma membrane and activates RAS, promotes cell proliferation and anti-apoptosis of cancer cells [50]. Another critical downstream target of PLD in cancer cells is the mTOR, a serine/threonine kinase known to be a key regulator in cell growth and survival signaling pathways. Because PA binds to and activates mTOR, overexpression of PLD1 or PLD2 stimulates mTOR activity, which was monitored by the phosphorylation of the mTOR enzymatic substrate S6 kinase in breast adenocarcinoma or rat fibroblasts, through PA production. PLD activation also induces c-Myc expression, which is regulated by mTOR activity, in breast adenocarcinoma, indicating the involvement of PLD-mTOR signaling pathway in cancer cell growth and survival signals [101, 102]. The mTOR inhibitor rapamycin has been used as an anti-cancer drug. However, rapamycin-based therapeutic strategies are unsuccessful in some cancer patients. Interestingly, it has been demonstrated that PA competes with rapamycin in mTOR regulation, and activation of PLD inhibits the effect of rapamycin in human breast cancer cell line. Therefore, inhibition of PLD may provide the strategy for suppressing the survival signal of rapamycin-resistant cancer cells. In normal proliferating cells, DNA-damaging agents cause apoptosis through a mechanism that involves increased expression of p53. In rat fibroblasts and MDA-MB-231 breast cancer cells, overexpression of PLD1 decreased p53 levels and apoptosis after treatment with DNA-damaging agents, suggesting that PLD activity promotes p53 degradation [103].

Many studies have detected a positive correlation between PLD activity and invasive potential. Overexpression of PLD in breast, glioblastoma, or lymphoma cells stimulates invasion, whereas expression of dominant-negative PLD prevents invasion [104]. Similarly, small-molecule PLD inhibitors (FIPI: 5-fluoro-2-indolyl des-chlorohalopemide;NOPT:N-[2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4,5]dec-8-yl) ethyl]-2-naphthalenecarboxamide) and PLD siRNA also decrease tumor size and breast cancer cell metastasis formation in vivo [94]. PLD2 stimulates cell protrusion in *v-src*-transformed cells and is required for EGF-induced membrane ruffling. Elevated PA levels can reorganize actin by its regulation of RAC complexes and phosphoatidylinositol 4-phosphate 5-kinase (PIP5K). In addition to lipid-mediated activation of downstream effectors, the PX domain of PLD2 shows RHO GEF activity, which induces actin reorganization. Thus, PLD2 induces stress fiber formation by mediating nucleotide exchange for RHOA [105]. A recent study showed that PLD2 knockout inhibited lung metastases in the mammary tumor virus

(MMTV)-*Neu* transgenic mouse breast cancer model [106]. PLD2-generated PA binds to and regulates the motor protein KIF5B, which controls membrane type1 metalloproteinases (MT1-MMP, also known as MMP14) surface localization and invasion. Furthermore, increased PLD activity enhanced the ability of MDA-MB-231 breast cancer cells to migrate and invade matrigel, and PLD2 overexpression increased the invasion and metastasis of EL4 mouse lymphoma cells. In contrast, inactive PLD2 inhibited metastasis in a syngeneic mouse model [107, 108]. Taken together, these results demonstrate that PLD1 and PLD2 promote tumor progression through distinct mechanisms.

PLD2-dependent cancer metastasis is intrinsic to cancer cells, whereas PLD1 is critical for both cancer and stromal cells [94, 109, 110]. Phenotypic analysis of *PLD1* knockout mice, which are otherwise viable and normal, revealed that PLD1 expression in tumor microenvironment plays important roles in tumor growth metastasis. The tumor microenvironment consists of various types of cells, such as vascular and lymphatic endothelial cells, mesenchymal cells, and immune cells. The soluble factors, signaling cues, ECM, and mechanical cues provided by tumor microenvironmental cells can promote tumor progression by supporting tumor growth and invasion, and by protecting the tumor from host immune system attack. Angiogenesis, which is required to supply oxygen and nutrients, is one of the major aspects of tumor microenvironment contributing to tumor progression; inhibition of angiogenesis in tumors prevents tumor growth. Ghim et al. found that the ablation of PLD2 from endothelial cells led to the suppression of hypoxia-induced HIF-1α expression and VEGF secretion, and also reduced proximal tumor neovascularization [111]. Additionally, when mouse melanoma or lung cancer cells were implanted into wild-type or PLD1 knockout mice exhibited a much lower density of microvascular cells. When VEGF-coated matrigel plugs were inserted into the same mice, endothelial cells failed to migrate to the plugs in the PLD1 knockout mice, suggesting inherent defects in the migration of PLD1 knockout-derived endothelial cells. Consistent with this observation, PLD1 knockout mice showed impaired integrin signaling, manifested in a failure to properly adhere to ECM integrin ligands, such as fibronectin, vitronectin, and collagen. Therefore, PLDs in the tumor microenvironmental cells are required for both primary tumor growth and metastasis.

2.3.3 PLA and Breast Cancer

Phospholipase A2 has a role in many biological processes, including inflammation, cell growth, and cancer development. Yamashita et al. were the first to report that PLA2 levels were highly elevated in patients with various malignant tumors, and especially in breast cancer [112]. Their study indicated a possible role of PLA2 in breast cancer progression. In particular, the role of EGFR/HER2 transactivation in estrogen-induced cPLA2α activation in breast carcinoma cell lines suggests that cPLA2α activity and expression may be coupled with HER2 over-expression in

tumor cells [113, 114]. Previous investigations found a correlation between the expression of intermediates in the eicosanoid signaling pathway, particularly COX-2, and the abundance of HER2 in breast carcinomas. cPLA2α expression was correlated with worse prognostic indicators, which also characterize more invasive tumors of the HER2-positive and basal-like subtypes. Elevated cPLA2α expression was associated with decreased survival in patients with luminal breast cancers, and also correlated with a reduced efficacy of endocrine therapy. This study found that cPLA2α expression was an independent predictive marker of a poor response to endocrine therapy over the first 5 years of post-treatment follow-up [113, 115]. In addition, PLA is synchronously overexpressed, and participates, in tumorigenesis by producing sufficient substrates for the metabolic cascade of COX2/PEG2 and other pathways, and is significantly correlated with a poor prognosis. Recently, higher plasma PLA2 and sPLA2 activity was detected in patients with breast cancer, particularly at late disease stages, than in healthy controls [112, 116]. Thus, plasma PLA2 activity may be a potential prognostic biomarker for patients with breast cancer. However, the functions and underlying molecular mechanisms of PLAs in breast cancer remain to be elucidated.

PLA2 has been shown to have both growth-inhibiting and growth-promoting effects [117, 118]. Its metabolite, AA, also has opposing functions in different tumor microenvironments. AA can be converted into various biologically active eicosanoid mediators including prostaglandins (PGs), hydroxyeicosatetraenoic acids (HETEs), and epoxyeicosatrienoic acids (EETs) by cytochrome P450 monooxygenase, COX isoforms, and lipoxygenases (LOXs) [119, 120]. The metabolism of AA by 15-LOX produces 15-S-hydroxyeicosatetraenoic acid (15-(S)-HETE) and prevents the proliferation of cell in culture [120, 121]. In contrast, PGE2 contributes to cell proliferation; consequently, the AA-based eicosanoid signaling pathway has been implicated in the development and progression of cancer in different human tissues, including the breast [121–123]. PGE2 stimulates the expression of growth-promoting genes, such as *c-fos* and VEGF [124], and promotes COX-2 expression in colorectal cancer, breast cancer, and normal epithelial cells [124, 125]; this leads to a positive feedback effect on downstream growth-promoting signaling. PGE2 can functions in both autocrine and paracrine manner to stimulate aromatase expression in breast cancer and normal tissue [126]. Consequently, COX-2 upregulates the production of the most biologically active estrogen 17-β-estradiol (E2), and the subsequent stimulation of proliferative signaling pathways. cPLA2α can generate AA to produce PGEs and enhance tumorigenesis, but sPLA2 has tumor-suppressive functions [127–129]. Thus, the requirement to balance PLA2 activity with the metabolism of its products may be responsible for some inconsistencies in published data regarding whether PLA2 supports or suppresses breast carcinoma progression.

The PLA family may promote tumor progression via extracellular regulation of the tumor microenvironment, to trigger cell migration and invasion [130–132]. The lipid mediators of PLAs involved in tumor metastasis and angiogenesis are LPA, AA, leukotrienes, and prostaglandins [133–135]. Serum LPA is a well-established indicator of tumor initiation and progression in breast cancer [136], ovarian cancer [137] and multiple myeloma [138]. LPA receptors, which show deregulated

expression in cancer cells and tissues [139–141], activate RHO family small GTPases to drive cell migration and invasion. Furthermore, AA induces the expression and surface exposure of GalT-1, which acts as a membrane receptor for ECM proteins and cell-to-cell interactions in MDA-MB-231 breast carcinoma cells, providing another mechanism by which PLA2 activity impacts the invasive capacity of breast carcinoma cells [142].

The altered metabolism of AA by COX and LOX in cancer cells has also been shown to play a role in cancer progression. In a mouse xenograft model, breast cancer cells overexpressing LPA1 has enhanced subcutaneous growth and bone metastasis [143]. Tumor cells stimulated LPA release from circulating platelets. The resulting pro-inflammatory PGs and leukotrienes are key mediators of intracellular crosstalk between tumor cells and stromal cells, and they induce the migration and proliferation of stromal cells such as immune cells, tumor-associated fibroblasts, and endothelial cells, which produce additional inflammatory cytokines and chemokines to establish the tumor microenvironment [144, 145]. The cooperation of phospholipases is important for angiogenesis because cell-cell communication must be tightly integrated and regulated. Malignant tumor cells express high levels of PLA2 and AA metabolic enzymes, resulting in the production of eicosanoid metabolites. These molecules mediate endothelial cell recruitment, proliferation, migration, and tube formation. Various studies have shown a correlation between COX2 overexpression and enhanced production of PGE2 by cancer cells. Through autocrine and paracrine pathways in tumor cells and stromal cells, PGE2 stimulates the production of VEGF and the chemokines CXCL1 to recruit endothelial cells. Moreover, cPLA2α-deficient endothelial cells are defective in tumor vascularization [134, 146, 147]. Therefore, the role of LOX signaling in proliferation, metastatic invasion and angiogenesis is emerging. The balance between COX and LOX activity in determining the nature of the AA metabolites produced is not only important establishing their respective and interacting roles in breast cancer progression, but also for potential novel therapeutic interventions.

2.3.4 A Multicellular Phospholipase Network

Invasion and metastasis is a multicellular and multistep process, and phospholipases contribute to this process by affecting both inter- and intracellular signal. First, overexpressed PLA2 and eicosanoid metabolic enzymes generate PGs and leukotrienes, which can activate stromal cells to migrate towards tumor cells. The recruited stromal cells secrete growth factors, cytokines, chemokines, and eicosanoids that coordinate the tumor microenvironment. Second, factors that are secreted from stromal cells probably go on to potentiate tumor cell migration and invasion by activating PLC and PLD (Fig. 2.3), as well as many other factors. This suggests that the phospholipase signal circuit could have crucial inter- and intracellular roles during metastasis.

Although many reports have suggested the functional association of phospholipases in physiological angiogenesis, the precise mechanism underlying tumor-associated angiogenesis remains unclear. The majority of such studies have used in vitro experiments, which do not consider the tumor microenvironment or cell-cell communication. As noted above, tumor microenvironments are complex and dynamically regulated by intracellular signaling evets. However, further investigation is needed to fully understand the roles of phospholipases in the context of tumor microenvironment.

2.3.5 Phospholipases as Anticancer Drug Targets

Despite strong evidence implicating phospholipases in tumorigenesis and progression, developing effective therapeutic strategies to inhibit phospholipases has been difficult for a number of reasons. In general, phospholipases are considered "undruggable" targets [148]. One of the major concerns that phospholipases regulates many key cellular processes, and therefore their inhibition would inevitably lead to severe side effects. Some phospholipases, such as iPLA2s, control normal brain and heart functions by remodeling phospholipids [149, 150]. On the other hand, abnormal hyperactivity, which is induced by the dysregulation of phospholipases, may be a potential therapeutic targets in cancer. Therefore, current challenges include developing therapeutics with optimal pharmacokinetic parameters that minimize side effects and maximize anticancer effects. In addition, isoform-specific inhibition of phospholipases has proven difficult. Historically, compounds that were structurally unrelated to PI(4,5)P2, such as aminosteroid U73122, were identified as potential candidates, but they showed great non-specificity. In fact, U73122 was suggested to have other targets, including calcium pumps and unrelated enzymes regulating lipid metabolism [151–153]. Furthermore, depending on the environmental stimulus, some phospholipase isozymes have oncogenic roles and others have tumor-suppressive roles. Therefore, the development of isozyme-specific inhibitors may improve our ability to target these enzymes. Second, although many reports have addressed the prognostic value of phospholipases in different tumor types, the number of studies has been small and detection methods have been limited. Additionally, breast cancer is a complex disease with very distinct clinical, morphological, and molecular entities. This heterogeneity cannot be explained only by clinical parameters like tumor size, histological grade, and ages. To evaluate the clinical and prognostic value of phospholipases as anticancer therapeutics, more careful clinical studies and integrated research approaches are needed [154]. Third, there are no reports on constitutively active mutations of the phospholipases in specific cancers, and few spontaneous animal models for cancer have been developed. In other words, phospholipases may be modulators of tumorigenesis and cancer progression by interacting each other. Finally, because lipid second messengers generated by phospholipases are quickly converted to the next metabolite, measuring the activation status of phospholipases in cancer tissue has proven impossible. Moreover,

downstream targets of lipid mediators are not specific to phospholipase-mediated signaling. Therefore, identification of predictive biomarkers is crucial for drug development.

Although phospholipases themselves are not strong oncogenes or tumor suppressors, phospholipases and lipid mediators strongly interact with their binding partners, including oncogenes and tumor suppressors, in a complex tumor microenvironment. Furthermore, phospholipases can interact with other signaling pathways depending on the surrounding environment or cell type, implying that specific drugs could potentially be designed to target tumor-associated phospholipases. In this respect, blocking the eicosanoid signaling pathway through the deactivation of COX enzymes has been tested in clinical studies. The inhibition of COX enzymes using non-steroidal anti-inflammatory drugs (NSAIDs) had therapeutic effects on several tumors [155]. However, their therapeutic efficacy is insufficient because NSAIDs cannot block the generation of leukotrienes by PLA2. Therefore, the use of PLA2 inhibitors might be considered an attractive alternative. Varespladib, a sPLA2-specific inhibitor, was under clinical evaluation as an anti-inflammatory agent; unfortunately, this trial was halted in 2012 due to inadequate efficacy [156]. Thus, inhibitors of other PLA2 isozymes have to be developed as anticancer drugs, and their efficacy improved to reduce side effects [157]. Further development of isozyme-specific inhibitors of PLA2 may lead to novel therapeutic strategies.

Interest in targeting PLD isozymes with small-molecule inhibitors has grown steadily since PLD family members were implicated in a variety of human diseases, including cancer. The dual-PLD inhibitors FIPI and halopemide (more effective against PLD2) effectively block PA production and several biological processes that have been known to be mediated by PLD activation, such as cytoskeleton reorganization, cell spreading, and chemotaxis, in vitro [158]. Although isozyme selectivity remained elusive, this discovery represented an important advance. Recent advances in the development of isozyme-selective PLD inhibitors, and in molecular genetics, have suggested that PLD isozymes in mammalian cells and pathogenic organisms may be valuable targets for the treatment of several human disease. Isozyme-selective inhibitors of PLD have been generated that inhibit the migration of breast cancer cell lines [159, 160]. In different settings, it may be advantageous to use PLD1-specific or PLD2-specific inhibitors rather than a dual PLD1/2 inhibitor, depending on the extent of redundancy of the individual PLD isoforms in the process that is being inhibited. However, this remains an unexplored topic that will be important to address as therapeutic approaches are developed, in particular in the context of cancer.

Pharmacological inhibitors of PLC activity, selective small molecules, or other selective probes are crucial for elucidating physiological and aberrant functions of specific proteins in cells and whole organisms. Notably, however, PLCs not only lack potential drug molecules but also appear to lack even a reliable, direct small-molecule inhibitor. Based on structural insights and a detailed understanding of the catalytic mechanism of PIP2 hydrolysis, PLC proteins are not intrinsically intractable. The main limitations to inhibitor development have been related to a lack of

suitable high-throughput screening, difficulties in generating chemical probes based on PIP2 substrate, and insufficient evidence linking changes in PLC function to disease development.

As mentioned above, several binding proteins of phospholipases and their lipid mediators may determine the role of phospholipases in cancer, and whether they act as cancer-promoting genes. For example, PLC-γ1 interacts with SOS1 to activate RAS, thereby increasing cell proliferation, and PLC-γ1 induces cell migration by interacting with the GUT1-Beta-Pix complex [161, 162]. These interactions are mediated by specific motifs and domains, suggesting that interaction blockers could be used as more specific anticancer therapies. However, no such blockers have developed to date. Therefore, understanding the mechanism of action of a specific domain-containing superfamily, as well as the roles of specific phospholipase isozymes in cellular signaling, metabolism, and cellular function, is paramount for the development of optimal therapeutic compounds.

Although some reagents that can block phospholipase signaling are available, we are far from developing anticancer therapies. By using integrated information (e.g., genomics, proteomics, and lipidomics) and animal experiments, the functional roles and regulatory mechanisms of phospholipases in tumorigenesis will be further defined. These efforts may lead to the generation of phospholipase-specific anticancer therapies.

2.4 Future Research Direction

Lipid signaling in pathology is an emerging field of investigation, and metabolite intermediates are a major lipid class involved in all of the crucial cell signaling pathways. Although phospholipases can regulate the pathways involved in tumorigenesis and cancer progression, and the signaling mechanisms of each phospholipase have been fairly well established, the functional roles of phospholipases in breast cancer are poorly understood. One of the major challenges to overcome this gap is to understand the complexity of the tumor microenvironment and intracellular signaling pathways. Tumor microenvironments generate various extracellular signals depending on the surrounding situation, which can trigger multiple signaling pathways, and different phospholipases can be simultaneously activated. Furthermore, phospholipases distributed throughout the signaling network can interact with one another and regulate each other's activities. Therefore, understanding the phospholipase-mediated signaling network within tumor microenvironments may be helpful for evaluating their functional importance in cancer. As shown in Fig. 2.3, phospholipases and their lipid mediators induce hierarchical pathways as well as complex networks that have feedback loops and crosstalk. For example, PLC is located in the immediately adjacent to the signaling receptors (e.g., RTKs and GPCRs) and generates two major second messengers (DAG and IP3) on activation. Thus, PLC serves as "generator" of second messengers and functions during the early stages of signaling transduction. Additionally, PA and PLD comprise a

complex network with a variety of binding partners, and they have dynamic interrelationships with their binding partners that can, in turn, simultaneously or sequentially interact [31]. Hence, PLD act as a "signal mediator," which can finely regulate multiple signals as they pass downstream. PLAs generate LPA, an extracellular ligand for receptors, and AA, the intracellular precursor of extracellular PG and leukotriene ligands. On the basis of these characteristics, PLA2 is a "signal amplifier" that can transmit signals into the extracellular environment in an autocrine and/or paracrine manner. In this viewpoint, these signaling roles of phospholipases are supported by the localization of phospholipase binding partners, as well as the localization of the phospholipases themselves.

For the development of future therapeutic strategies, one of the main contributions of this breakthrough in cancer research is the integration of molecular studies into clinical trials. Despite evidence demonstrating the involvement of phospholipases in tumor-associated signaling in cells, there are few clinical studies presenting phospholipases as oncogenes or tumor suppressors. Recently, many bioinformatics data sets have been made available, including those derived from genomic and transcriptomic studies, as well as from the interactomes of phospholipases and their-associated signaling pathways. These combined analysis data can be used to assess the overall involvement of phospholipases in cancer. Interestingly, most experimental animal tumor models involving phospholipases were not established by single knockout or the overexpression of a single phospholipase molecule, but rather by a combination of knockouts or transgenic animals expressing different oncogenes or tumor suppressors. These results suggest that drugs that target phospholipases may be effective when combined with other drugs that target different cellular signaling pathways. It is possible that inhibitors of phospholipases could be developed to improve the efficacy of other targeted therapies, and to diminish toxicity arising from the inhibition of a physiologically important housekeeping enzyme. Thus, this integrated approach has provided valuable information on the nature of the disease, explaining in part the different responses to treatment and the disparate prognoses. Knowing the pathways regulating the processes involved in neoplastic development should help in the design of clinical trials aimed at patients with specific characteristics that are candidates to benefit from specific treatment.

Although many key questions remain regarding the development of isozyme-specific inhibitors and signal pathway blockers, phospholipases are considered as attractive targets for anticancer therapy. Targeting distinct phospholipases may have broad therapeutic potential, and it is likely that small molecule inhibitors of phospholipases will be tested for efficacy in diseases for which there is currently an unsatisfactory conventional therapy. The absence of toxic effects in animal models is highly encouraging. With current advances in mass spectrometry-based metabolomics, lipidomics, and phosphoproteomic analyses, new participants in established signaling and metabolic pathways are being revealed, which provide exciting opportunities for therapeutic targeting. The challenges for the future will be elucidating the complexity and variability of the phospholipase network in the tumor microenvironment, and understanding the tumor-specific roles of each phospholipase and its corresponding regulatory mechanisms.

2.5 Summary

- Phospholipases are essential mediators for many physiological processes; however, aberrant signaling is involved in carcinogenesis and cancer progression.
- Phospholipases are the link between two major pathways, HER2/HER3/PI3K and EGFR/HER2/PLC, and play an active role in breast cancer cell proliferation, migration, invasion, and angiogenesis.
- Phospholipases are not an easy target for therapy, so attention needs to be given to interacting partners, or cross-talk signaling pathways in tumor microenvironment.
- The integrated analyses of phospholipases are important for developing innovative therapeutic strategies or the comprehension of new molecular processes.

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