2011

Expression of the cytosolic phospholipase A2 isozymes in the human breast cancer cell line models MDA-MB 231 and MCF-7 and the normal breast cell line model MCF-10A

Debolina Ganguly
West Virginia University

Follow this and additional works at: <https://researchrepository.wvu.edu/etd>

Recommended Citation

Ganguly, Debolina, "Expression of the cytosolic phospholipase A2 isozymes in the human breast cancer cell line models MDA-MB 231 and MCF-7 and the normal breast cell line model MCF-10A" (2011).

Graduate Theses, Dissertations, and Problem Reports. 4716.

<https://researchrepository.wvu.edu/etd/4716>

This Thesis is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Thesis has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

Expression of the cytosolic phospholipase A₂ isozymes in the human breast cancer cell line models MDA-MB 231 and MCF-7 and the normal breast cell line model MCF-10A.

Debolina Ganguly

Thesis submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Biological Sciences

Dr. Philip Keeting, PhD., Chair
Dr. Ashok Bidwai, PhD.
Dr. Clifton Bishop, PhD.

Department of Biology
Morgantown, West Virginia
2011

Keywords: Breast Cancer, Arachidonic acid, cPLA₂, MDA-MB 231, MCF-7, MCF-10A

ABSTRACT

Expression of the cytosolic phospholipase A₂ isozymes in the human breast cancer cell line models MDA-MB 231 and MCF-7 and the normal breast cell line model MCF-10A.

Debolina Ganguly

Breast cancer remains one of the leading causes of cancer related death among women worldwide. Numerous reports have provided evidence of a role of inflammation in the onset and progression of many cancers, including breast cancer. A mechanism that leads to inflammation involves the arachidonic acid (AA) pathway, which leads to the production of a group of pro-inflammatory bioactive lipids, collectively known as the eicosanoids. Despite evidence of AA and the eicosanoids in cancer, limited work has been done on the enzyme cytosolic phospholipase A₂ (cPLA₂) that provides the unesterified AA that can be used for eicosanoid generation. The present studies were undertaken to investigate the expression of the cPLA₂ isozymes cPLA₂α, -β, -γ, in the human breast cancer cell lines MDA-MB 231 and MCF-7, and in the immortalized non-tumorigenic human breast cell line MCF-10A.

Additionally, surgically removed cancerous human breast tissue was also assessed for cPLA₂ isozyme expression. The present study indicates the presence of the cPLA₂ isozymes -α, -β, -γ in the tumorigenic breast cancer cell lines MDA-MB 231 and MCF-7. The isozymes were not detected in the non-tumorigenic MCF-10A cell line. Stimulation of the cells by Ca²⁺ ionophore A23187 elicited a redistribution of the Ca²⁺ dependent cPLA₂ isoforms -α and -β within the MDA-MB 231 and the MCF-7 cells. AACOCF₃, a cPLA₂α inhibitor, pretreatment caused a decreased cPLA₂α signal, observed also in assays of cPLA₂β and cPLA₂γ. Assays of cPLA₂ isozyme expression in the human breast tissues were unable to demonstrate the presence of any of the cPLA₂ isozyme proteins. The results of this research indicate that the tumorigenic human breast cell lines MDA-MB 231 and MCF-7 express the cPLA₂α, cPLA₂β and cPLA₂γ proteins, and that the cPLA₂ proteins could not be detected in the non-tumorigenic human breast cell line, MCF-10A. The A23187 elicited redistribution of cPLA₂α and cPLA₂β proteins within the cells, and decreased signal in AACOCF₃ pretreated specimens provides evidence supporting antisera specificity. Although cPLA₂α is recognized to be the principal enzyme responsible for AA release leading to eicosanoid biosynthesis, cPLA₂β and cPLA₂γ, which share ~30% homology with cPLA₂α, may also participate in releasing AA and could support pro-inflammatory eicosanoid biosynthesis. Further investigations will be necessary to fully define the regulation of the cPLA₂ isozymes in the MDA-MB 231 and MCF-7 cells. The expression of the cPLA₂ isozymes in cancerous human breast tissue remains undescribed.

ACKNOWLEDGEMENTS

During the course of my Master's study, I have been fortunate to get the support and encouragement of many people. None of this work could have been accomplished without the guidance and encouragement of my advisor, Dr. Philip Keeting. I would like to thank him for giving me the opportunity to work in his laboratory, and also for providing positive criticism when things did not work in the way they had been supposed to. I feel deeply indebted to him in this regard. I would also thank him for his patience in reading through the drafts of this thesis. I would like to thank my committee members, Dr. Ashok Bidwai and Dr. Clifton Bishop for their guidance and suggestions. I thank Dr. Bidwai for the academic support and encouragement he has offered me throughout the course of my study.

I thank past and present members of Dr. Bidwai's laboratory for their constant support and for providing me with an amicable environment to work in. I thank all graduate students of the Department of Biology as well as Marlene, Diana and Micky. I am indebted to Katrina for her support during times when things were going against me.

Finally, I am grateful to my parents whose belief in me has been the force that has kept me going. I would like to thank all my friends who have been my biggest support in all these years. I am indebted to my husband, Bhaskar whose guidance and support throughout these years has helped me stay aboard on this rewarding but often difficult journey.

Table of Contents:

Section	Page
Introduction	1-4
Background	5-36
Materials and Methods	37-44
Results	45-49
Discussion	50-58
Figures	59-84
Bibliography	85-104

List of Figures

Figures	Page
Fig. 1. Schematic diagram of the homologous protein domains of the cPLA ₂ isoforms.	26
Fig. 2. Characterization of cPLA ₂ α antisera.	60-61
Fig. 3. Basal expression of cPLA ₂ isoforms in MDA-MB 231, MCF-7 and MCF-10A cells.	62-63
Fig. 4. Effects of TNFα and IL-1β on cPLA ₂ isozyme expression in MDA-MB 231 cells.	64
Fig. 5. Effects of TNFα and IL-1β on cPLA ₂ isozyme expression in MCF-10A cells.	65
Fig. 6. Immunohistochemical detection of cPLA ₂ α and cPLA ₂ βI in MDA-MB 231 cells.	66-67
Fig. 7. Immunohistochemical detection of cPLA ₂ βII and cPLA ₂ γ in MDA-MB 231 cells.	68-69
Fig. 8. Immunohistochemical detection of cPLA ₂ α, cPLA ₂ β and cPLA ₂ γ in MCF-7 cells.	70-71
Fig. 9. Immunohistochemical assay of cPLA ₂ α, cPLA ₂ β and cPLA ₂ γ in MCF-10A cells.	72
Fig. 10. Modulation of cPLA ₂ isozymes in MDA-MB 231 cells by AACOCF ₃ .	73-74
Fig. 11. Modulation of cPLA ₂ isozymes in MCF-7 cells by AACOCF ₃ .	75-76

Fig. 12. Modulation of cPLA ₂ α and cPLA ₂ β isozymes by Ca ²⁺ ionophore (A23187) in MDA-MB 231 cells.	77-78
Fig. 13. Modulation of cPLA ₂ α and cPLA ₂ β isozyme distribution by Ca ²⁺ ionophore (A23187) in MCF-7 cells.	79
Fig. 14. Immunochemical assays of cPLA ₂ isozymes α, β and γ in human breast tumor sample I.	80
Fig. 15. Immunochemical assays of cPLA ₂ isozymes α, β and γ in human breast tumor sample II.	81
Fig. 16. Immunochemical assays of cPLA ₂ isozymes α, β and γ in human breast tumor sample III.	82
Fig. 17. Immunochemical assays of cPLA ₂ isozymes α, β and γ in human breast tumor sample IV.	83
Fig. 18. Immunochemical assays of cPLA ₂ isozymes α, β and γ in human breast tumor sample V.	84

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AACOCF ₃	Arachidonyl trifluoromethylketone
BCIP	Bromo-Chloro-Indolyl-Phosphate
BSA	Bovine serum albumin
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
cPLA ₂	Cytosolic phospholipase A ₂
DMEM	Dulbecco's Modified Eagle Medium
EGF	Epidermal growth factor
FBS	Fetal bovine serum
HETE	Hydroxyeicosatetranoic acid
IL-1 β	Interleukin-1 β
LOX	Lipoxygenase
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
NBT	Nitro Blue Tetrazolium
OA	Oleic acid
PA	Palmitic acid
PAGE	Polyacrylamide gel electrophoresis
PG	Prostaglandin
PGH ₂	Prostaglandin precursor
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator activated receptor

PDGF	Platelet-derived growth factor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TBST	Tris buffered saline Tween-20
TNF α	Tumor necrosis factor α
TGF β	Transforming growth factor β

INTRODUCTION

Breast cancer is one of the most widespread cancers among women worldwide. In the US, it is the most common cancer, and the leading cause of cancer-related deaths, among women between 45-64 years of age (Ward et al., 2008, American Cancer Society, 2010). Early detection and improved treatment procedures have resulted in a gradual decline in the number of deaths caused by the disease since 1990 (Ward et al., 2008, American Cancer Society, 2010). Nevertheless, in 2010, the estimated death toll in the USA for the disease was 39,840 (American Cancer Society, 2010). The gravity of the disease, therefore, demands further research focused on its early diagnosis, its treatment, and for evaluating the prognosis of the disease course.

Breast cancer is a heterogeneous disease characterized by a complex interplay of molecular, genetic, and physiological factors. The identification of some common characteristics, such as *c-erb-b2/her-2/neu* oncogene expression (Jimenez et al., 2000; Kallioniemi et al., 1991), mutations in the BRCA-1 (Miki et al., 1994; Welch et al., 2002), or BRCA-2 genes (Venkitaraman, 2002), as well as assays of the tumor ER/PR status (Ferno et al., 1990; Warnberg et al., 2001; Xu et al., 2003) have provided approaches for possible intervention. However, the totality of the molecular factors that contribute to the onset and progression of breast cancer remains undefined. Continuing investigations are expected to identify additional molecular pathways involved in the onset and progression of the disease, and to guide the exploration of novel therapeutic approaches.

Numerous reports (Coussens and Werb, 2002; Kim et al., 2009; Mantovani et al., 2008) indicate that inflammation may be associated with the onset and progression of many cancers, including cancers of the breast. Metastatic potential is also linked with an inflammatory environment (Kim et al., 2009). A mechanism contributing to an inflammatory

state is the arachidonic acid (AA) cascade producing the eicosanoids, a group of bioactive lipids that are often pro-inflammatory.

There have been numerous reports linking AA and the eicosanoids to cancer biology (Markaverich et al., 2007; Nie et al., 2001; Pan et al., 2008; Sveinbjornsson et al., 2008). AA is typically esterified in the membrane glycerophospholipids, and in that state the fatty acid is not useful to the eicosanoid synthesizing pathways. However, AA can be released from the glycerophospholipids by enzymes of the phospholipase A₂ (PLA₂) family, and the free AA can be converted into the eicosanoids. Enzymatic utilization of AA by the cyclooxygenases (COXs) and the lipoxygenases (LOXs) are chiefly responsible for initiating the synthesis of the eicosanoids from free AA. Elevated levels of COX-2 have been identified in archived breast cancer tissues, and studies indicate that COX-2 overexpression is sufficient to induce mammary tumor formation in transgenic mice (Denkert et al., 2004; Liu et al., 2001). Cancerous human breast tissues reveal a 3- to 30- fold higher level of 12-LOX mRNA when compared to paired normal breast tissue (Ding et al., 2003; Natarajan et al., 1997). The linkage of the COX and LOX enzymes with cancer has been frequently studied, but fewer investigations have focused on the enzyme cytosolic phospholipase A₂ (cPLA₂), the PLA₂ principally responsible for releasing AA necessary for eicosanoid synthesis (Fujishima et al., 1999; Six and Dennis, 2000). AA release is the obligate first step in the biosynthesis of eicosanoids.

The PLA₂'s are a family of esterases that catalyze the hydrolysis of the *sn*-2 bond of cellular glycerophospholipids. AA is frequently found esterified at the *sn*-2 position. Of the five PLA₂ categories (reviewed below), the cPLA₂ group is principally responsible for the initial release of AA in response to cell-stimulation by proinflammatory cytokines and

hormones. In the 1990's, it was determined that cPLA₂ consisted of several related proteins, designated as cPLA₂ α , cPLA₂ β and cPLA₂ γ . Additional enzymes have since been identified (Schaloske and Dennis, 2006), but only cPLA₂ α , cPLA₂ β and cPLA₂ γ will be considered further in this report. Expressed sequence tag (EST) analyses using cPLA₂ α sequences identified the β and γ isoforms of cPLA₂. We hypothesize that the cPLA₂ isozymes α , β and γ play significant, though possibly distinct roles in breast cancer by contributing to AA release and, thereby, to eicosanoid generation. Furthermore, distinctive variations in the levels in one or all of the isozymes in normal and transformed breast cancer cells may exist and this may be significant in terms of assessing breast cancer risk, onset, progression, proclivity to metastasize, and patient prognosis. Preliminary results from this laboratory (Maricica Pacurari, 2006) demonstrated cPLA₂ β and cPLA₂ γ mRNA expression in the human breast cancer cell line MCF-7 by real time RT-PCR assays. However those experiments left it uncertain if cPLA₂ α mRNA was also expressed in those cells, and the expression of the encoded proteins was only addressed in those studies, in a preliminary manner.

In those studies an antisera specific to cPLA₂ β and cPLA₂ γ were generated using gene specific antigenic amino acid sequences corresponding to the respective cPLA₂ isoforms. The present studies were undertaken to produce a cPLA₂ α antiserum and to determine if the cPLA₂ isozymes, - α , - β and - γ , were expressed in the breast cancer cell lines MDA-MB-231 and MCF-7, and in the immortalized non-tumorigenic human breast cell line MCF-10A. Additionally, surgically removed cancerous human breast tissues were to be screened for cPLA₂ isozyme expression.

BACKGROUND

Cancer is characterized by several distinct features including uncontrolled cell division, resistance to growth inhibitors, evasion of apoptosis, invasion of distant tissues, the ability to induce angiogenesis, and immortality (reviewed in Hanahan and Weinberg, 2000). The recognition and evaluation of oncogenes with a dominant gain of function and the tumor suppressor genes with a recessive loss of function has provided valuable information on the emergence and progression of the disease (Bishop and Weinberg, 1996).

Breast cancer represents one of the most common cancers among women in the US, representing approximately 1 in 4 cancers diagnosed. The American Cancer Society (2010) has classified breast cancer as in situ when it is localized in the ducts or in the lobules of the breast and invasive when the disease has spread out of the ducts of the glandular walls thereby invading the surrounding tissue. It was estimated that there will be 207,090 new cases of invasive breast cancer and an additional 54,010 new cases of in situ breast cancer in 2010. Assignment of stages in breast cancer depends on tumor size and the extent of its spread through the breast tissue and the neighboring organs (T), as well as the involvement of the lymph node (N) and presence or absence of distant metastases (M). The assigned stages are I, II, III and IV, where Stage I represents the early disease and Stage IV the highly advanced disease (American Cancer Society, 2010). Displaying a complex interplay of molecular, genetic and physiological factors, breast cancer is a heterogeneous group of diseases, presenting major challenges to physicians and scientists. Individuals with inherited mutations of the breast cancer susceptibility genes, BRCA1 and BRCA2 have a high risk of developing the disease and these mutations are estimated to be responsible for 5%-10% of breast cancer incidence (American Cancer Society, 2010). BRCA1 and BRCA2 are tumor

suppressor genes involved in transcriptional regulation and DNA damage repair (Welch and King, 2001).

Studies on animal and human cancer cells have revealed that tumorigenesis is a multistep process, and that each step correlates with genetic alterations that lead to the progressive transformation of normal human cells into highly metastatic derivatives (Bishop and Weinberg, 1996). An active proliferative state in normal cells is initiated by mitogenic growth signals and their processing by subsequent downstream cytoplasmic counterparts. Most of the growth signals are synthesized by one cell type to stimulate the proliferation of another, a process known as heterotypic signaling. However, many cancer cells acquire the ability to generate or activate their own growth signals, thereby becoming independent of stimulation from exogenous mitogenic stimuli (Fedi et al., 1997). Examples of self-stimulation are the platelet-derived growth factor (PDGF) and transforming growth factor (TGF)- α production by glioblastomas and sarcomas (Fedi et al., 1997). Moreover cell surface receptors responsible for the transmission of the growth stimulatory signals into the cell interior may also be dysregulated in cancers. The epidermal growth factor receptors (EGF-R/erbB) are upregulated in some stomach, brain and breast tumors, and the HER2/*neu* receptor is often overexpressed in stomach and breast cancers (Yarden and Ullrich, 1988). Cancer is sometimes characterized by ligand independent signaling through the structural alteration of receptors. Truncation of the major part of the cytoplasmic domain of EGF receptor has been shown to constitutively transmit signals (Fedi et al., 1997) leading to metastasis.

The intricate signaling networks that drive normal cell division and restrict cellular proliferation are thus altered and utilized in cancer cells promoting uncontrolled replication

and survival. Mitogenic signals are transmitted to the interior of the cell by a series of distinct and often crosstalking signaling pathways. The acquisition of growth factor autonomy can be achieved by the accumulation of alterations in the downstream members of the cytoplasmic network that receive and process the signals transmitted by the growth factor receptors. The following section comprises a review of some of the principal signaling networks operating in cancers. This content is provided as a frame of reference for transformation and metastasis because the various eicosanoids can mediate similar responses and effects to those described for the polypeptides discussed.

SIGNALING PATHWAYS AND CANCER:

Ras-Mitogen Activated Protein Kinase (MAPK) Pathway:

The Ras-MAPK pathway plays a significant role in regulating normal cell division and malignant transformation. The activation of receptor tyrosine kinases (RTKs) by growth factors leads to the occupancy of Ras by GTP as mediated by Son of Sevenless (SOS). The GTP-bound Ras is the active form of Ras. Neurofibromin 1 (NF1) is a negative regulator of Ras activation by GTP. Activated Ras activates the serine/threonine kinase Raf that, in turn, phosphorylates and activates a MAPK/ ERK kinase (MEK), which then leads to the activation of ERK. ERK 1/2, upon translocation to the nucleus, leads to the activation of the AP1 transcription factors. The stimulation of these transcriptional regulators causes the expression of the D-type cyclins enabling the cell to progress through the G1 phase of the cell cycle and enter S-phase. Several human tumors exhibit activating mutations in the Ras genes (Bos, 1989). Most of these mutations result in constitutive pathway activation leading to hyperproliferation. Ras pathways have also been implicated in the induction of angiogenic

factors and ERK-mediated expression of matrix metalloproteinases (MMPs) (reviewed in Downward, 2003). The MMPs are capable of degrading all components of the extracellular matrix and are therefore associated with several aspects of tumor metastasis such as malignant tumor growth, tumor-induced angiogenesis and tumor invasion (Vihinen and Kahari, 2002). 45% of colon cancers and 90% of pancreatic cancers exhibit Ras mutations (Katz et al., 2007). The Ras-MAPK signaling cascade cross-talks with other pathways, and thereby enables the Ras activating signals to have multiple biological effects.

Phosphatidylinositol 3- kinase (PI3K) Pathway:

The PI3K cascade can be activated by Ras and also by the RTKs. PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP₂), to produce phosphatidylinositol-3, 4, 5-triphosphate (PIP₃). PIP₃ leads to the activation of several downstream signaling pathways. One principal downstream mediator of PIP₃ is AKT, a serine/threonine kinase. Activated PI3K/AKT signaling is associated with cellular energy metabolism, cell proliferation, and survival (reviewed in Dreesen and Brivanlou, 2007). p53, a transcription factor heavily involved in DNA damage surveillance, can be negatively regulated by AKT through the activation of MDM2 by AKT dependent phosphorylation. The tumor-suppressor PTEN opposes the PI3K/AKT pathway by dephosphorylating PIP₃. PTEN mutations have been implicated in glioblastomas, lung carcinomas, and melanomas (Vivanco and Sawyers, 2002). AKT overexpression has been found in breast, ovarian and thyroid cancers (Vivanco and Sawyers, 2002). Activated AKT can directly and indirectly inhibit the pro-apoptotic mediators BAD and pro-caspase 9.

Janus Kinases (JAK)/ Signal Transducers and Activators of Transcription (STAT)

Pathways:

In response to certain growth factors and cytokines, the JAK/STAT signaling pathways stimulate cellular proliferation, differentiation, migration, and apoptosis (Rawlings et al., 2004). A wide array of ligands stimulate JAK, a soluble tyrosine kinase that acts on STAT and other substrates. Phosphorylated STATs enter the nucleus and bind to specific regulatory DNA-sequences to activate or repress transcription of target genes. STAT can also be activated by the EGF or PDGF (Darnell, 2002). Mutations of the JAK/STAT signaling components, such as HER2/neu (breast and stomach cancers) and EGF-R (breast, brain and stomach cancers), can lead to hypersensitivity towards mitogenic signals and, thereby, promote proliferation (Slamon et al., 1987; Yarden and Ullrich, 1988).

Notch signaling pathway:

The Notch signaling pathway is associated with several cell fate specification events and is associated with many developmental defects and cancer. Notch can inhibit cellular differentiation due to lateral inhibition, or can promote adjacent cells to adopt the same cell fate by lateral induction (Ehebauer et al., 2006). Binding of Notch ligands such as Delta, Serrate or Lag2 to their cognate Notch receptor leads to the cleavage of the intracellular domain of Notch (NICD). NICD translocates to the nucleus and forms a transcriptional activation complex with the DNA-binding factors CSL (Suppressor of Hairless), and the Mastermind like protein family (MAML), causing the activation of the Hairy Enhancer of Split (HES) family. Aberrant Notch signaling has been implicated in breast tumors, melanoma progression, medullablastoma, and ovarian cancers (Roy et al., 2007). The

presence of ligands such as Jagged I and the amplification of Notch receptors have been shown to be involved with an aggressive form of breast cancer (reviewed in Dreesen and Brivanlou, 2007). It has been proposed that Notch signals possibly increase cell proliferation by the activation of a downstream target, the transcription factor c-myc. Deregulated c-myc expression is evident in many cancers.

Transforming growth factor (TGF)- β :

TGF- β is a ubiquitously expressed cytokine that plays significant roles in cellular functions such as cell cycle arrest, apoptosis, homeostasis, immune regulation, and wound healing (Bierie and Moses, 2006b; Massague, 2008). Although TGF- β is a tumor suppressor, there is ample evidence that it acts as a pro-metastatic cytokine in advanced stage tumors (reviewed in Dreesen and Brivanlou, 2007). The activation of the TGF- β mediated pathways involves binding of TGF- β to the extracellular domain of the Type I and Type II TGF- β receptors. This leads to the phosphorylation and activation of the latent cytoplasmic transcription factors, the SMADs. The phosphorylated SMADs (1, 2, 3, and 5) form a complex with SMAD 4, thereby eliciting its translocation to the nucleus where it regulates the transcription of target genes. These include down-regulating c-Myc and the Id family of transcription factors, and upregulating p15^{INK4b} and p21^{CIP2,8} expression. The latter proteins inhibit cell cycle progression. Decreased expression of the SMAD proteins, as well as mutations of the TGF- β receptors, have been reported in a number of human cancers including pancreatic, colorectal, and gastric cancers, thereby supporting the role of TGF- β as a tumor suppressor (Derynck et al., 2001; Massague, 1998, 2008). However, breast cancers, melanomas and prostate cancers display active TGF- β signaling (Siegel and Massague,

2003). In the highly metastatic breast cancer cell line MDA-MB 231, transcriptional repression of *c-Myc* and *Id* genes by TGF- β is selectively inactivated (Chen et al., 2001). This supports the continuous growth of these tumor cells, even in the presence of high levels of TGF- β . TGF- β can augment epithelial tumor malignancy by promoting epithelial-mesenchymal cell transition (EMT), an effect that enhances migration and invasion in tumor cells, a process resembling the highly coordinated cellular movements that happen during embryogenic development (Oft et al., 1996).

Nuclear factor-kappa (NF- κ)B:

NF- κ B is a transcription factor that plays significant roles in proliferation, stress response, innate immunity, and inflammation (Courtois and Gilmore, 2006; Ghosh et al., 1998). Studies indicate that NF- κ B plays an important role in cancer development (reviewed in Grivennikov et al., 2010). NF- κ B is activated by a wide range of extracellular factors including tumor necrosis factor- α (TNF α), interleukin-1, some growth factors, bacterial or viral infections, and oxidative stress. In the absence of stimulation, inactive NF- κ B dimers are located in the cytoplasm. The inactive state of the NF- κ B dimers is caused by its interaction with the I- κ B inhibitory proteins. In response to stimuli, the I- κ B proteins are rapidly phosphorylated by I- κ B kinase (IKK), which elicits its release from the complex with the NF- κ B dimers. The free NF- κ B can translocate to the nucleus and activate transcription of several target genes, including *cyclin D1*, *c-myc*, and *c-myb*, thereby demonstrating that NF- κ B can act as a positive regulator of cell cycle progression. Activated NF- κ B can oppose apoptosis by increasing transcription of anti-apoptotic members of the Bcl-2 family and

cIAPs (Karin et al., 2006). NF- κ B driven responses inhibit p53-induced apoptosis (Tergaonkar et al., 2002).

NF- κ B is also associated with several aspects of invasive cell growth and survival. Its activity leads to increased angiogenesis by the upregulated expression of vascular endothelial growth factor (VEGF) (Li et al., 2002), COX-2, and inducible Nitric Oxide synthase (iNOS) (Greten and Karin, 2004). It promotes invasive growth by elevating the expression of the matrix metalloproteinases (MMP), plasminogen activator, and heparanase (Andela et al., 2000; Bond et al., 1999). NF- κ B also regulates the effects of several chemokines, thus promoting cell migration (Johnson, 1999). The activation of NF- κ B during chronic inflammation and its constitutive expression in some cancers suggests a significant role played by NF- κ B in the initiation and promotion of cancer. NF- κ B can contribute to the production of reactive oxygen species (ROS) in macrophages and neutrophils through the induction of the production of iNOS and other enzymes (Forman and Torres, 2001). The ROS produced can diffuse internally and enter the surrounding cells and cause DNA damage (Karin et al., 2002). Elevated expression of NF- κ B during chronic inflammation can prevent the elimination of genetically altered cells by inhibiting apoptosis. The human REL gene, which encodes one of the five NF- κ B transcription factor family members, is amplified in Hodgkin's lymphoma, and non-Hodgkin's lymphoma (reviewed in Dreesen and Brivanlou, 2007).

Cancers in general exhibit characteristics such as uncontrolled cell division, resistance to growth inhibitors, evasion of apoptosis, invasion of distant tissues, the ability to induce angiogenesis, and immortality; however, cancerous tissues can differ widely in the

paths taken to acquire the malignant phenotype. Within a given cancer type, mutation of particular target genes may be found in only a subset of histologically identical tumors.

INFLAMMATION AND CANCER:

Rudolf Virchow, in the year 1863, proposed that cancer originates at sites of chronic inflammation. Cell proliferation and inflammation are normal characteristics of wound healing. In a normal tissue, wound healing is accomplished through the production of pro-inflammatory cytokines but they are closely followed by the anti-inflammatory cytokines, and normal inflammation is self-limiting (reviewed in Coussens and Werb, 2002).

Proliferating cells undergo and sustain mutagenic assaults in the proinflammatory microenvironment. Their continued survival may be due to the persistent presence of the proinflammatory mediators, which is sensitive to the failure of factors required to resolve the inflammatory response (Dvorak, 1986).

Inflammatory conditions can by themselves elevate cancer risk, as is evident in inflammatory bowel disease. However, genetic alterations in a tissue can also lead to inflammation and neoplasia (Mantovani et al., 2008). Persistent infections can induce chronic inflammation. Leukocytes and other phagocytic cells produced to fight such infections cause DNA damage in proliferating cells through the generation of reactive oxygen and nitrogen species. These products react to form peroxynitrite, a mutagenic agent. Repeated tissue damage and its subsequent regeneration can lead to permanent genetic alterations such as point mutations, deletions or rearrangements (Maeda and Akaike, 1998). DNA damage resulting from chronic inflammation caused by infections by *Helicobacter pylori* is the world's leading cause of stomach cancer (Ernst and Gold, 2000).

Pro-tumorigenic actions of the inflammatory cells include the release of several growth and survival factors, the promotion of angiogenesis and lymphangiogenesis, causing DNA damage, the remodeling of the extracellular matrix (ECM), thereby facilitating invasion (reviewed in Coussens and Werb, 2002). A developing tumor microenvironment comprises a diverse array of leukocytes such as neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes, all of which can lead to the production of cytokines, ROS, MMP, TNF α , interleukins (ILs), and interferons (IFs) (Kuper et al., 2000; Wahl and Kleinman, 1998). Infiltrating macrophages in breast cancer can lead to TNF α production, which may regulate thymidine phosphorylase, an angiogenic enzyme (Leek et al., 1998). Neoplastic tissue infiltrates indicate the presence of tumor associated macrophages (TAMs), COXs (responsible for the production of the prostaglandins, leukotrienes and thromboxanes), chemokines, and dendritic cells (reviewed in Balkwill and Mantovani, 2001). The TAMs play a dual role of killing neoplastic cells upon activation by IL-2, IF, and IL-12 and producing angiogenic and lymphangiogenic growth factors, cytokines and proteases which can lead to tumor progression (Mantovani et al., 1992).

Members of the Ras-Raf signaling pathway are frequently mutated in cancers and can lead to the production of tumor promoting chemokines and cytokines (Guerra et al., 2007; Sparmann and Bar-Sagi, 2004; Sumimoto et al., 2006). Deregulated expression of the transcription factor Myc can favor the remodeling of the tumor microenvironment through the recruitment of inflammatory mediators. Tumor suppressor proteins such as TGF- β and PTEN can also regulate the production of inflammatory mediators (Balkwill, 2004; Brierie and Moses, 2006a; Phillips et al., 2005). The transcription factor NF- κ B can be activated during genetic alterations and can regulate the activity of genes encoding inflammatory

cytokines, adhesion molecules, COX-2, NOS, and various angiogenic factors. NF- κ B can promote cell survival by inducing the expression of anti-apoptotic genes (Bcl-2) and inhibiting p53 induced apoptosis (Karin et al., 2006). NF- κ B is involved in the initiation and progression of cancers of the GI-tract and liver (Greten et al., 2004; Pikarsky et al., 2004). The STAT3 transcription factor is also associated with oncogenesis by remaining constitutively activated in immune and tumor cells. STAT3 is also involved with the inhibition of apoptosis (Bromberg et al., 1999). The multiple signaling pathways disrupted in cancer can provide an ambient environment for tumor growth by promoting inflammation.

A critical mechanism leading to pain and inflammation is the arachidonic acid (AA) pathway. AA serves as the precursor of the group of bioactive lipids collectively known as the eicosanoids. The eicosanoids include the prostaglandins (PGs), thromboxanes (Tx_s), hydroxyeicosatetraenoic fatty acids (HETEs), leukotrienes (LTs), and the lipoxins (Brash, 1999; Smith et al., 2000). There have been several reports that AA and its products, the eicosanoids, are intimately linked to cancer biology. Some cancer cells depend on the overexpression of the AA metabolizing enzymes, the COXs and the LOXs for survival (Gao et al., 1995). Inhibitors of these enzymes have antineoplastic characteristics (Wong et al., 2001).

EICOSANOIDS:

The eicosanoids are potent lipid mediators that can act in the nanomolar range (reviewed in Funk, 2001). The COX, LOX and the cytochrome P450 monooxygenase pathways are responsible for the committive steps in eicosanoid generation, but can aberrantly metabolize AA during chronic inflammation and carcinogenesis. The eicosanoids regulate

numerous physiological processes including multiple aspects of inflammation. Common diseases like arthritis, ischemic cardiovascular disease, and asthma display an inflammatory role of the eicosanoids (Serhan, 2005). The eicosanoids have also been implicated as mediators of cell proliferation, transformation, angiogenesis, cancer progression, and metastasis (Choy and Milas, 2003), in various tissues, including cancers of the mammary gland. The biosynthesis of the eicosanoids is rate-limited, partly, by the availability of free AA. Esterified AA is typically not a substrate for the COX, LOX or P450 enzymes, thus, AA release, must precede AA's subsequent metabolism by the eicosanoid producing enzymes from the glycerophospholipids by a phospholipase.

The prostanoids, specifically the prostaglandins (PGs), exert their biological effects through their cognate cell surface receptors. These belong to the G protein-coupled receptor (GPCR) family. Recent studies indicate that certain PGs can activate nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family. The expression of the cognate receptors varies in a cell type specific manner, and this determines the specific action of the different PGs in that particular tissue. The prostanoids are synthesized in most tissues, and they commonly function in an autocrine or paracrine manner.

Among the PGs, PGE₂ is the most abundant and is produced by different human cancers, including colon, lung and breast. Murine models of colorectal cancer have shown that PGE₂ is necessary for tumor growth (Park et al., 2007; Wang et al., 2004). PGE₂ promotes tumor growth in small and large intestinal adenoma as indicated by studies in *Apc*^{Min/+} mice. The usage of a PG receptor antagonist has been shown to reduce chemically induced breast cancer development in mice (Kawamori et al., 2001).

The 5-LOX enzyme converts AA to the unstable LTA₄ through the intermediate product 5-HPETE. LTA₄ is converted into 5-HETE, or is hydrolysed into LTB₄. In some tissues 5-HPETE is converted into the cysteinyl leukotriene, LTC₄, by the addition of the tripeptide glutathione. LTB₄ is upregulated in some human colon and prostate cancers (Larre et al., 2008). Inhibiting LTB₄ synthesis by treatment with LTA₄ hydrolase inhibitor, bestatin, reduced tumor incidence in murine models of esophageal adenocarcinoma (Chen et al., 2003).

Numerous animal studies, clinical trials and population studies have revealed the significance of the prostanoid and leukotriene biosynthetic pathways in cancer (reviewed in Greenhough et al., 2009). The eicosanoids operate through different mechanisms that can modulate tumor initiation and progression. Eicosanoids regulate cell proliferation by activating the receptors on tumor epithelial cells; they can induce the secretion of growth factors and angiogenic factors. They can regulate apoptosis, migration and invasion, thus comprehensively causing a shift of the normal cellular environment into a tumor supporting one (reviewed in Wang and Dubois, 2010).

ARACHIDONIC ACID (AA):

Arachidonic acid is an essential polyunsaturated fatty acid (20:4, n-6), typically found esterified at the *sn*-2 position of cellular glycerophospholipids (Chen et al., 2008b; Irvine, 1982). As stated, it serves as the precursor for the bioactive lipids known as the eicosanoids. Each of these metabolites is formed by the enzymatic mono- or bis-oxygenation of AA, or, rarely, of dihomo- γ -linolenic acid or eicosapentaenoic acid. Three different groups of enzymes primarily utilize free AA as a substrate leading to bioactive lipid synthesis. They

include the cyclooxygenases (COXs), the lipoxygenases (LOs) and the cytochrome P450 monooxygenases. The esterified form of AA is not a substrate for most eicosanoid producing enzymes, thus, the release of AA must precede its subsequent metabolism by the COXs and the LOX's.

In the recent years, studies on bioactive lipid regulation have gained prominence. In unstimulated cells, free AA is held at low concentrations, thereby limiting the basal rate of eicosanoid production (Fitzpatrick and Soberman, 2001). Hence, the availability of AA serves as the rate limiting factor in eicosanoid generation. The mobilization of AA from the cellular membrane glycerophospholipids is accomplished by esterases of the phospholipase A₂ (PLA₂) family (Kudo and Murakami, 2002). PLA₂ is activated in response to a variety of stimuli including the cytokines, growth factors, hormones or even simple mechanical stimulation. Because free AA can be reincorporated into cellular lipids by acyltransferase and transacylase reactions (Snyder et al., 1992), eicosanoid production is a function of a competition between the acylation/reacylation pathways, and the enzymes leading to eicosanoid synthesis.

CYCLOOXYGENASES (COX):

The COX enzymes, COX-1 and COX-2, catalyze the dioxygenation of AA. This leads initially to PGG₂ endoperoxide production. This is then reduced by COX's peroxidase activity into PGH₂ endoperoxide. PGH₂ can then be used by isomerases, reductases, or synthetases, to produce the PGs (PGD₂, PGE₂, and PGF₂), prostacyclin, or TX. Of the COX enzymes, COX-1 is constitutively expressed in tissues and contributes to tissue homeostasis. COX-2, whose expression is inducible, is principally associated with the regulation of

inflammation. There is evidence that COX-2 plays vital roles in cancerous tissues of the breast, colon and the pancreas (Lupulescu, 1978; Muller-Decker and Furstenberger, 2007). Carcinogenesis can be induced in cultured mouse fibrosarcoma cells by increasing the amount of the available PGs. There is evidence that the usage of COX-2 inhibitors can reduce cell growth in cancers of the skin, colon, esophagus, gall bladder, pancreas, and breast (Barry et al., 2009; Chen et al., 2004; Fegn and Wang, 2009; Ye et al., 2005). Increased expression of COX-2 has been observed in adenomas and adenocarcinomas of the colon (Eberhart et al., 1994).

Cyclooxygenases and Breast Cancer: An elevated level of the PGs in breast tumors, with even further higher levels in the metastatic disease, was the first evidence of the involvement of COX-2 in the disease (Bennett et al., 1977; Rolland et al., 1980). Mammary tumors are sensitive to treatment by both specific and non-specific COX-2 inhibitors (Schreinemachers and Everson, 1994). Studies on transgenic mice have indicated that the overexpression of the COX-2 enzyme is sufficient to induce mammary tumor formation (Liu et al., 2001). These tumors were sensitive to treatment by COX-2 inhibitors. A decline in the levels of COX-2 and PGs in mice led to an elevation of the incidence of apoptosis in tumorous tissues indicating an inverse relationship of PGs to apoptosis (Liu et al., 2001).

LIPOXYGENASES (LOX):

The LOXs are a family of dioxygenases whose products have been described to be involved in processes like cell differentiation, inflammation, and cancer (Brash, 2001). The LOXs are placed into different classes based on their positional specificity of AA oxygenation. The principal members of the LOX family include the 5-, 12- and 15- LOXs.

LOX products such as 5- and 12-HETE and the leukotrienes (LT) appear to mediate proliferative, adhesive, and metastatic properties in tumor cells (reviewed in DuBois, 2003). 5- and 12-HETE have also been shown to modulate apoptosis, and studies have indicated that the inhibition of 5- and 12-LOX causes an elevation in apoptosis in various tumor models displayed by responses in *bcl-2* and *bax* expression (Chen et al., 2008a; Vincent et al., 2008). 12-HETE plays a crucial role in modulating several steps during metastasis and cancer cell adhesion. 12-HETE can stimulate PKC activity (Liu et al., 1991), which can cause the release of the cysteine protease cathepsin B; cathepsin B is involved in tumor metastasis and invasion (Honn et al., 1994). The LT's, LTA₃ and LTA₄, are also produced in inflammatory cells such as the polymorphonuclear leukocytes, macrophages, and mast cells (reviewed in Funk, 2001). Solid tumors of the human breast, prostate, colon, and the human breast cancer cell line MCF-7, display elevated levels of 12-LOX mRNA (Endsley et al., 2007; Natarajan et al., 1997; Natarajan and Nadler, 1998; Steele et al., 1999). LOX inhibitors have been shown to prevent rat mammary gland cancers and carcinogen-induced lung adenomas (Matsunaga et al., 1998; Moody et al., 1998; Rioux and Castonguay, 1998).

Lipoxygenases and Breast Cancer: Human cancerous breast tissues display a 3- to 30-fold higher 12-LOX mRNA level compared to normal breast tissue (Ding et al., 2003; Natarajan et al., 1997). 12-LOX expression is elevated in the breast cancer cell lines MCF-7, MDA-MD 231, COH-Br1, and T47D (Natarajan and Nadler, 1998). The same study also identified an increase in 12-LOX expression in the MCF-7 cell line after treatment with EGF. 12-LOX expression has also been shown to be elevated in breast cancer tissues (Natarajan et al., 1997). The use of LOX inhibitors inhibited the proliferation of MCF-7 cells (Natarajan and Nadler, 1998).

PHOSPHOLIPASES (PLA₂):

The phospholipases A₂ are a family of esterases that catalyze the hydrolysis of the *sn*-2 bond of cellular glycerophospholipids, leading to the release of arachidonic acid. PLA₂ catalysis yields lysophospholipids, which can serve as lipid mediators (Murakami and Kudo, 2002; Six and Dennis, 2000). The PLA₂s play significant roles in the maintenance and turnover of membrane phospholipids, signaling, tissue repair, and in the inflammatory response (Dong and Matsumura, 2008; Jurivich et al., 1996; Murakami and Kudo, 2002; Six and Dennis, 2000). However, certain members of the phospholipase family have been implicated specifically in inflammation and tissue repair, thereby raising the possibility of a pathological role. There are reports that the expression of PLA₂ is elevated upon treating cells with pro-inflammatory cytokines and growth factors (Capper and Marshall, 2001).

A diverse array of PLA₂ enzymes are found in mammalian tissues, each with a distinct mechanism of regulation. These enzymes have been classified into 19 forms (Schaloske and Dennis, 2006; Six and Dennis, 2000), based on gene nucleotide sequences, and have been characterized, purified and cloned. The PLA₂ superfamily is divided into 5 families of enzymes (Schaloske and Dennis, 2006), including the secretory PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂), the Ca²⁺-independent PLA₂ (iPLA₂), the platelet-activating factor acetylhydrolases (PAF-AH), and the lysosomal PLA₂'s.

Secretory PLA₂ (sPLA₂):

The sPLA₂ family includes 17 different members, and these require the presence of μM Ca²⁺ for catalysis. They have a conserved catalytic site and display 5 to 8 disulfide bonds (Schaloske and Dennis, 2006). Some of the sPLA₂ enzymes are involved with

releasing AA from the intracellular membranes, while others operate on the outer surface of the plasma membrane. sPLA₂'s play vital roles in inflammatory diseases and have been shown to be present at high concentrations in patients suffering from rheumatoid arthritis, inflammatory bowel disease, and pancreatitis, among others (Bidgood et al., 2000; Schaloske and Dennis, 2006). sPLA₂'s are also elevated in prostate cancers (Sved et al., 2004) and in colorectal adenocarcinomas (Mounier et al., 2008).

Cytosolic PLA₂ (cPLA₂):

A second family of the PLA₂'s, the cPLA₂ enzymes, includes 6 members, which are grouped by virtue of sequence homologies. These display a conserved catalytic site that includes a conserved serine residue and requires $\mu\text{M Ca}^{2+}$ for function (Kim et al., 1991). cPLA₂ will be discussed extensively in a subsequent section.

Ca²⁺-independent PLA₂ (iPLA₂):

The iPLA₂ family functions in the release of AA from the membrane glycerophospholipids in unstimulated cells and do not require Ca^{2+} for their activity. There are 7 identified members in the iPLA₂ family, which are similar to cPLA₂ in that they utilize a catalytic serine for catalysis. The rate of AA release due to iPLA₂ activity does not exceed the rate at which AA is reincorporated into membrane lipids and, hence, appears to be unconnected with eicosanoid biosynthesis. However, activation of iPLA₂ during cardiac ischemia has been reported to lead to lethal ventricular tachyarrhythmias (Mancuso et al., 2003).

Platelet-activating factor acetylhydrolases (PAF-AH):

The PAF-AH family consists of 2 groups of serine PLA₂'s that hydrolyze an acetyl group from the *sn*-2 position of the PAF precursors, a group of phosphatidylcholine based glycerophospholipids in which the *sn*-1 chain is linked by an ether linkage and the *sn*-2 position is occupied by an acetyl group (Six and Dennis, 2000). The PAF-AH family consists of 4 members ranging in size from 26-45 kDa. PAFs exhibit numerous pro-inflammatory properties, such as eliciting platelet aggregation, stimulating the production of ROS, and increasing LT biosynthesis (Izaki et al., 1996). The hydrolysis of the *sn*-2 acetyl group abolishes the pro-inflammatory effects of PAFs (Peplow, 1999).

Two distinct pathways, the *de novo* and the remodeling pathways, have been identified for the biosynthesis of PAF. In the latter pathway, cPLA₂ is involved in PAF biosynthesis, and its actions closely parallel AA release and eicosanoid generation in diverse cell types, as implicated by studies in cPLA₂ deficient mice (Snyder, 1995). Stimulation by calcium ionophore (A23187) has been shown to cause the production of PAF in peritoneal cells (Ninio et al., 1983), and stimulation by A23187 or formyl-methionyl-leucylphenylalanine has a similar effect on eosinophils. PAF expression has been reported in the estrogen dependent MCF-7 and T47D human breast cell lines, and also in the estrogen independent cell line MDA-MB 231. However, PAF production has not been identified in the immortalized nontumor forming breast cell line MCF-10. *In vivo* studies have suggested that PAF is associated with the stimulation of tumor angiogenesis (Bussolati et al., 2000). This has been further supported by a study done by Montrucchio et al. (1998), in which PAF was detected in 18 primary breast carcinoma tissues and the amount of PAF correlated with tumor vascularization.

Lysosomal PLA₂'s:

The lysosomal PLA₂'s are a recent addition to the PLA₂ superfamily. They exhibit transacylase, Ca²⁺-independent PLA₂ activity, and 1-O acylceramide synthase (ACS) activity (Hiraoka et al., 2002). They have a size of 45 kDa. The deficiency of lysosomal PLA₂'s has been implied to be responsible for the pathologic condition, phospholipidosis (Abe et al., 2007).

CYTOSOLIC PHOSPHOLIPASE A₂ (cPLA₂):

In humans, the cPLA₂ family consists of 4 isoforms including cPLA₂α, cPLA₂β, cPLA₂γ, and cPLA₂δ. In a different nomenclature system, they are referred to as group IVA, IVB, IVC, and IVD respectively (Schaloske and Dennis, 2006). Each of the 4 isoforms contains a divided catalytic region consisting of domains A and B. These domains include conserved amino acids required for enzyme catalysis, and are linked by isoform specific amino acid sequences (Murakami and Kudo, 2002). The lipase consensus sequence (GXSGS) is located close to the N-terminal region of the A domain (Fig. 1). The N-termini of both cPLA₂α and cPLA₂β, have a Ca²⁺ binding domain (CaLB domain) (Fig. 1), that regulates enzyme association to the phospholipid membranes (Murakami and Kudo, 2002; Song et al., 1999).

cPLA₂γ shares 29% sequence homology with cPLA₂α but lacks the CaLB domain that is present in cPLA₂α and -β (Fig. 1). cPLA₂γ is constitutively bound to cell membranes, and unlike cPLA₂α and cPLA₂β, membrane attachment in cPLA₂γ is dependent on lipid modifications of the protein (Song et al., 1999). cPLA₂γ contains putative acylation sites and a C-terminal prenylation site that may regulate its membrane association (Pickard et al.,

1999). Northern blot analysis indicates that the mRNAs of the cPLA₂ α , - β , and - γ are widely distributed in human tissues, however expression levels do vary between tissues (Pickard et al., 1999). Although there are similarities in the catalytic domains of cPLA₂ α , - β , and - γ , initial biochemical analyses indicate that each of them may have different substrate specificity and, probably, activation characteristics (Pickard et al., 1999; Song et al., 1999).

Fig. 1: Schematic diagram of the homologous protein domains of the cPLA₂ isoforms.

The percentage of identical amino acids to cPLA₂ α is shown below the homologous domains in cPLA₂ β and cPLA₂ γ . The conserved serine in the lipase consensus sequence is shown in catalytic domain A of each cPLA₂ isoform. The positions of the phosphorylated serines in cPLA₂ α is shown. Adapted from Song et al., 1999.

cPLA₂α:

cPLA₂α has been mapped onto chromosome 1 and comprises 18 exons that encode 749 amino acids with a molecular mass of 85 kDa. cPLA₂α is a cytosolic protein that preferentially cleaves AA containing phospholipids, and displays Ca²⁺ dependent translocation to the perinuclear membranes (Hirabayashi and Shimizu, 2000). Select receptor stimulation elicits a rapid increase in free intracellular Ca²⁺, which triggers the translocation of the cPLA₂ isozyme (cPLA₂α) from the cytosol to the nuclear envelope, endoplasmic reticulum, and the golgi apparatus where its substrate is located (Grewal et al., 2002; Grewal et al., 2003). MAPK and MAPK-activated kinases are usually responsible for activation of cPLA₂α via phosphorylation at Ser⁵⁰⁵ and Ser⁷²⁷ respectively (Hefner et al., 2000); it can also be activated by phosphorylation via the calcium/calmodulin kinase II pathway at Ser⁵¹⁵ (Muthalif et al., 2001). Under such stimulated conditions the rate of AA release exceeds the rate of its reacylation, allowing free AA to accumulate and thereby rendering it available for eicosanoid production. In the majority of cells and tissues there is a constitutive expression of cPLA₂α, allowing it to play significant roles in both immediate and delayed eicosanoid biosynthesis (Brash, 1999; Fitzpatrick and Soberman, 2001; Fujishima et al., 1999; Murakami et al., 1999; Smith et al., 2000).

The Ca²⁺ dependent translocation of cPLA₂α to the intracellular membranes is a crucial step in the initiation of stimulus-coupled AA release. In fact, phosphorylation of cPLA₂α *per se* is insufficient to cause AA release without an increase in Ca²⁺, suggesting phosphorylation plays an augmentative role in cPLA₂α activation. In addition to transcriptional regulation, cPLA₂α also undergoes post-transcriptional regulation. As reported by Tay et al. (1994), in rat mesangial cells, phorbol myristate acetate, platelet

derived growth factor, serum and epidermal growth factors increase the half-life of cPLA₂ α mRNA (Clark et al., 1995). Studies on the isolated CaLB domain have indicated a preference of binding to phosphatidylcholine vesicles in the presence of μ M levels of Ca²⁺ (Nalefski et al., 1998). Several proteins have been shown to regulate cPLA₂ α activation. Vimentin (Nakatani et al., 2000), a protein abundant in the perinuclear region, and nuclear cPLA₂ α -interacting protein (PLIP) (Sheridan et al., 2001) positively regulate cPLA₂ α mediated AA release (Six and Dennis, 2000). Calpactin light chain (Wu et al., 1997) and annexin I (Kim et al., 2001) inhibit cPLA₂ α function. However the physiological relevance of these regulatory proteins requires further clarification (Murakami and Kudo, 2002).

The cPLA₂ α gene regulatory region includes potential binding sites for AP-1, AP-2, NF- κ B, C/EBP, PEA3, OCT, and GRE (Miyashita et al., 1995). The cPLA₂ α promoter does not include a TATA box or a GC rich region, or SP1 sites, making it distinct from typical housekeeping genes. The 5'-flanking region contains a 27-base pair motif that consists of a polypyrimidine sequence which reportedly is responsible for the basal expression of cPLA₂ α (Miyashita et al., 1995). In cultured endothelial cells, the intracellular distribution of cPLA₂ α depends on cell density. At high cell density the cPLA₂ α isozyme is primarily localized in the cytosol and cell junctions, whereas in subconfluent cell cultures it appears to reside in the nucleus (Hirabayashi and Shimizu, 2000).

Stimulation by cytokines and mitogens such as IL-1 β , TNF α , EGF, colony stimulating factor, c-Kit ligand, IFN γ antigens, UV light, and other agonists moderately induces the expression of cPLA₂ α in various cell models (Clark et al., 1995). Increased cPLA₂ α synthesis upon induction by IL-1 β correlates with PGE₂ production, while the glucocorticoid dexamethasone, suppresses both cPLA₂ α and PGE₂ production without

affecting the cyclooxygenases levels (Lin et al., 1992). In some cell models a coordinated upregulation of cPLA₂α and COX-2 has been observed (Clark et al., 1995).

Pathogenesis of cPLA₂α. The cPLA₂α isoform is involved with numerous pathologies including inflammatory disorders, intestinal ulceration, and brain injury (Bonventre and Sapirstein, 2002; Sapirstein and Bonventre, 2000; Sun et al., 2004). Several studies have found variation in expression levels and activity of cPLA₂α in cancers such as cholangiocarcinomas (Wu et al., 2002) and non-small cell lung squamous carcinomas (NSCLC) (Nakanishi and Rosenberg, 2006). In NSCLC, cPLA₂α expression is associated with the presence of oncogenic *Ras* mutations (Nakanishi and Rosenberg, 2006), and the association of *Ras* with cPLA₂α and COX-2 expression has been suggested to be a potential mechanism of maintaining a high level of PGE₂ production and, thereby, supporting a cancerous state.

cPLA₂α pathology and function has been analyzed in cPLA₂α deficient mice. cPLA₂α knockout mice display a compromised generation of eicosanoids and PAFs, and exhibit a marked reduction of airway anaphylactic response, adult respiratory distress syndrome, and ischemic brain injury when compared to the wild type mice (Bonventre et al., 1997; Nagase et al., 2000). However, perhaps the most important phenotypes observed were difficulties in embryogenesis and implantation, a characteristic attributed to the suboptimal production of the prostaglandins, primarily PGF₂α (Bonventre et al., 1997). Genetic evidence of cPLA₂α involvement in the expansion of intestinal polyps in familial adenomatous polyposis (FAP) came from studies by Takaku et al. (2000), who introduced a cPLA₂α deletion into *Apc*^{Δ716} mice, a model for human FAP. Mice with disrupted genes of

the COXs, 5-LOX, or PG receptors had phenotypes not entirely identical to those of the cPLA₂α knockout mice, suggesting the involvement of additional PLA₂ enzymes (Murakami and Kudo, 2004). The cPLA₂α paralogs cPLA₂β and cPLA₂γ may participate along with cPLA₂α, or independently in situations when cPLA₂α is absent, thus providing AA for eicosanoid generation. Moreover, sPLA₂ activity is dependent on cPLA₂ in several circumstances (Balsinde et al., 1998; Murakami et al., 1999).

cPLA₂α has been suggested to be involved in TNFα mediated apoptosis (Obeid and Hannun, 1995). Usage of AACOCF₃, a cPLA₂α inhibitor, protected colonocytes from TNFα induced apoptosis (Dong et al., 2003). The cPLA₂ deficient L929 cell line is resistant to TNFα-induced apoptosis (Hayakawa et al., 1993). The apoptotic response is restored when cPLA₂α is expressed in the cells by transfecting with cPLA₂α cDNA (Hayakawa et al., 1993). However, many cells are resistant to TNFα induced apoptosis (Voelkel-Johnson et al., 1996), and the role of cPLA₂α in TNFα induced apoptosis remains unclear.

cPLA₂β:

cPLA₂β was originally cloned from libraries of human brain and pancreas. It maps to chromosome 15, has 6 exons, and encodes a protein of 110-114 kDa (Pickard et al., 1999; Song et al., 1999). cPLA₂β mRNA is expressed in different human tissues, with higher expression in the pancreas, liver, heart, and brain (Song et al., 1999). The mRNA of cPLA₂β is found predominantly in an unspliced form (8.5 kb) rather than its completely spliced form (3 kb) (Song et al., 1999). The cPLA₂β protein is expressed at varying levels in different human tissues such as the liver, brain, lung, spleen, kidney, pancreas, heart, testis, and ovary (Ghosh et al., 2006a). Comparison of the amino acid sequences of cPLA₂α and cPLA₂β

showed approximately 30% homology (Pickard et al., 1999). There is a 242 amino acid (aa) sequence in the cPLA₂β amino (N) terminal domain, that has no homology to cPLA₂α or cPLA₂γ (Song et al., 1999). Subsequent sequence analysis revealed that this 242 aa sequence bears homology to the human JmjC domain (Clissold and Ponting, 2001). However this domain has no effect on cPLA₂β's enzymatic activity as its deletion did not affect the enzyme's specific activity as compared to the full length cPLA₂β protein (Song et al., 1999).

cPLA₂β regiospecificity and calcium sensitivity. *In vitro* studies performed by Song et al. (1999) and Pickard et al. (1999), showed that PLA₂ activity of cPLA₂β was much lower than that of cPLA₂α. cPLA₂β displayed a similar preference for 1-[¹⁴C]palmitoyl-2-arachidonyl-PC (*PAPC) and 1-palmitoyl-2-[¹⁴C]arachidonyl-PC (P*APC) as it did for 1-O-hexadecyl-2-[³H]arachidonyl-PC (O-H*APC). In the vesicle assays, cPLA₂β initially exhibited regiospecificity toward *sn*-1 palmitic acid (PA) from *PAPC, followed by the release of *sn*-2 AA from O-H*APC (Song et al., 1999). cPLA₂β enzymatic activity is Ca²⁺ dependent, as indicated by experiments in which the phospholipase activity was strictly dependent on the presence of Ca²⁺. Chelating Ca²⁺ with EGTA caused the loss of phospholipase activity (Song et al., 1999). It has been suggested that the membrane association of cPLA₂β is dependent on the CaLB domain (Channon and Leslie, 1990; Schievella et al., 1995; Song et al., 1999).

Pathogenicity of cPLA₂β. A recent study investigated the presence of the PLA₂ isoforms in immature blasts of patients with acute myeloid and acute lymphoid leukemia (Fiancette et al., 2009). qRT-PCR analysis indicated that cPLA₂α and cPLA₂β had similar mRNA expression levels in the leukemic blasts, while the expression of cPLA₂γ was the least among the 3

isoforms (Fiancette et al., 2009). No other reports are currently available on the involvement of cPLA₂β in disease conditions.

cPLA₂γ:

cPLA₂γ maps to chromosome 19, and is composed of 17 exons that code a 541 amino acid polypeptide with a predicted molecular mass of 61 kDa. Despite sharing about 29% homology with cPLA₂α (Hartmann et al., 2002; Pickard et al., 1999), as well as harboring the residues utilized in cPLA₂α for its catalytic activity, cPLA₂γ is structurally and functionally different from cPLA₂α. In contrast to cPLA₂α, whose activity is regulated by phosphorylation at Ser 505 by MAPK, cPLA₂γ has protein kinase C phosphorylation sites that may regulate its enzymatic activity (Underwood et al., 1998). Unlike cPLA₂α and -β, cPLA₂γ is constitutively bound to cell membranes. The membrane association of cPLA₂γ remains unaltered upon mutation of the C-terminal prenylation site, suggesting that this site individually is not responsible for membrane binding. cPLA₂γ contains myristoylation, several palmitoylation, and acylation sites (Tucker et al., 2005). It has been suggested that fatty acylation, together with farnesylation, may be responsible for the constitutive membrane association of cPLA₂γ (Ghosh et al., 2006b).

Studies with cPLA₂γ transfected cells demonstrated that the enzyme may be localized to the perinuclear membranes. When N-terminal GFP-tagged cPLA₂γ was overexpressed in HEK293 and CHO cells, the enzyme was associated with the endoplasmic reticular and golgi membranes (Asai et al., 2003; Murakami et al., 2003). However, when the untagged cPLA₂γ was overexpressed in skeletal muscle cells, lung fibroblasts, and COS7 cells, the enzyme was associated with the mitochondria (Tucker et al., 2005).

cPLA₂γ regiospecificity. Initial studies by Song et al. (1999) indicated that cPLA₂γ can liberate *sn*-1 PA and *sn*-2 AA with equal potency using *PAPC and O-H*APC as substrates. A follow up study using purified cPLA₂γ displayed that it can release *sn*-2 AA using 1-*O*-hexadecyl-2-arachidonyl-phosphatidylcholine as substrate but not *sn*-1 PA from 1-palmitoyl-2-*O*-hexadecyl-phosphatidylcholine (Stewart et al., 2002). However when 1-palmitoyl-2-arachidonyl-phosphatidylcholine was the substrate, cPLA₂γ first hydrolyzed *sn*-2 AA and then the *sn*-1 PA (Stewart et al., 2002). This suggests that cPLA₂γ sequentially hydrolyzes the *sn*-2 fatty acids followed by *sn*-1 hydrolysis (Hirabayashi and Shimizu, 2000; Murakami et al., 2003; Stewart et al., 2002; Underwood et al., 1998). In a separate study done by Murakami et al. (2003), the enzymatic efficiency of cPLA₂γ for *sn*-2 AA was 2-fold greater when compared to *sn*-2 oleic acid (OA). In HEK293 cells, cPLA₂γ released AA, OA, PA, and stearic acid in a serum dependent manner, suggesting cPLA₂γ may have a role in phospholipid remodeling (Asai et al., 2003; Murakami et al., 2003; Stewart et al., 2002).

cPLA₂γ is involved in H₂O₂-induced AA release in cPLA₂γ-overexpressing HEK293 cells, although the exact mechanism of this activation has not been determined (Asai et al., 2003). In the same study, the use of a PKC inhibitor, calphostin C, did not affect the release of AA. However a protein tyrosine kinase inhibitor, herbimycin A, inhibited release (Asai et al., 2003). Orthovanadate (Na₃VO₄), a protein tyrosine phosphatase inhibitor, surprisingly enhanced the AA release by cPLA₂γ. The induction of cPLA₂γ by H₂O₂, leading to AA release, indicates that cPLA₂γ may release AA in response to oxidative stress (Asai et al., 2003).

Although cPLA₂γ lacks the CaLB domain present in cPLA₂α and -β, stimulating cells that overexpress cPLA₂γ by Ca²⁺ ionophore A23187 increases free AA levels and COX-1

mediated PGE₂ production compared to control specimens (Murakami et al., 2003). It has been suggested that although cPLA₂γ is Ca²⁺ independent, increases in Ca²⁺ levels stimulates mitochondrial production of ROS, which may be responsible for the regulation of mitochondrial associated cPLA₂γ activity (Ghosh et al., 2006b). Most of the findings on cPLA₂γ discussed here have been obtained from studies on cells overexpressing the isozyme, thus the regulation and activity of endogenous cPLA₂γ is uncertain. In one study by Duan et al. (2001), the mRNA and protein of cPLA₂γ was elevated in human macrophages infected by *Mycobacterium tuberculosis*. cPLA₂γ may have varied physiological roles due to its ability to act as a PLA₂, transacylase, or lysophospholipase.

Among all of the PLA₂ categories, cPLA₂ appears to be the principal activator for the release of AA used in eicosanoid production, at least during the early stage of eicosanoid synthesis (reviewed in Balsinde et al., 1999). In some instances eicosanoid production persists over several hours and during these situations, sPLA₂, along with cPLA₂, may contribute substrate for the delayed production of eicosanoids (Balsinde et al., 1999). Interestingly, blockage of cPLA₂ has been shown to eventually shut off sPLA₂ production and therefore, sPLA₂-dependent biological responses (Balsinde et al., 1998). In mammalian cells, regulation of AA production takes place by the interplay of an array of PLA₂ enzymes in different tissues; therefore understanding the regulation, function and expression of each PLA₂ is important in order to decipher its role in any disease.

cPLA₂ and Breast Cancer:

Extensive evidence is available on the physiological and pathological implications of cPLA₂α. Differential expression of the cPLA₂ isoforms in various tissues, suggests the need

to investigate the roles played by the other members of the cPLA₂ family. cPLA₂ α expression is elevated in several human tumors, such as colorectal (Wendum et al., 2005), small bowel (Wendum et al., 2003), lung (Heasley et al., 1997), and prostate cancer (Patel et al., 2008). Estrogen activates the prostanoid signaling pathway through the activation of cPLA₂ α in the MCF-7 breast cancer cell line (Thomas W, 2006). In murine models, cPLA₂ α expression is considered to be pro-tumorigenic (Hong et al., 2001). Although numerous studies have implicated the eicosanoids in cancer onset and progression (Markaverich et al., 2007; Nie et al., 2001; Pan et al., 2008; Sveinbjornsson et al., 2008), limited work has been conducted on the cytosolic phospholipase A₂ (cPLA₂) isozymes that are believed to provide the AA utilized for eicosanoid synthesis (Adler et al., 2008). Little is known about cPLA₂ β and cPLA₂ γ , and any roles played by these isozymes in breast cancer are yet to be determined.

RESEARCH PROJECT OBJECTIVES:

Dysregulated lipid metabolism appears to be important in breast cancer biology. Despite an acknowledged fundamental role of cPLA₂ in eicosanoid biosynthesis, limited cancer related studies have been directed towards this phospholipase. cPLA₂ α is elevated in several human tumors (Heasley et al., 1997; Wendum et al., 2005). Whether cPLA₂ β or cPLA₂ γ can contribute to AA release as a substrate for the eicosanoid producing enzymes is yet to be demonstrated, and they have not been investigated in connection with breast cancer. There is abundant evidence that the AA metabolites, including prostaglandin E₂ and HETEs, play significant roles in the onset and progression of several cancers, including breast cancer (reviewed in Nie et al., 2001; Winer et al., 2002). Therefore, defining and characterizing the

enzymatic pathways responsible for AA release may be critical to the understanding of breast cancer biology, and may contribute to effectively treating the disease. The studies reported herein were undertaken to investigate the expression of the cPLA₂ isozymes - α , - β , and - γ in the breast cancer cell line models MDA-MB231 and MCF-7, and in the immortalized non-tumorigenic breast cell line MCF-10A. Additionally, surgically removed cancerous human breast tissue was also screened for the cPLA₂ isozyme protein expression, and is compared with the results from the cell lines.

The MCF-7 cell line is characterized by the presence of the estrogen receptors ER α and ER β (Fabbro et al., 1986; Hall et al., 1990) and is the best characterized, and most widely utilized, model of an estrogen-responsive breast cancer cell line. *In vivo* studies have demonstrated a low invasive capability of these cells (Thompson et al., 1992). The basic EGF receptor and several MMPs are absent in these cells. The MDA-MB-231 cell line is a highly invasive cell line and displays the loss of E-cadherin (Pierceall et al., 1995), and estrogen and progesterone receptors (Cailleau et al., 1978). The MCF-10A cells display the absence of a tumor-inducing capability in mice, show anchorage-dependent growth, and grow in culture only in the presence of hormones and growth factors (Soule et al., 1990). These characteristics appear to make MCF-10A useful as a control cell line model of human breast tissue.

MATERIALS AND METHODS

Reagents: Recombinant tumor necrosis factor (TNF α) and recombinant interleukin-1 β (IL-1 β) were purchased from R&D Systems (Minneapolis, MN). A23187, epidermal growth factor (EGF), hydrocortisone, and insulin were purchased from Sigma Aldrich (St Louis, MO) and arachidonyl trifluoromethyl ketone (AACOCF₃) from Calbiochem (EMD Chemicals Inc., New Jersey). Stock solutions were prepared according to supplier's instructions and stored at -20°C.

Cell Culture Conditions: Human mammary tumor cell lines MDA-MB 231 and MCF-7, and the normal human mammary cell line MCF-10A were purchased from ATCC (Manassas, VA). Cells were maintained in a 1:1 ratio of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 medium. The growth medium of both the MDA-MB 231 and MCF-7 cell lines was supplemented with 10% heat-inactivated fetal bovine serum (FBS), Penicillin: Streptomycin: Neomycin (PSN; 1%), and glutamine (2 mM; Biofluids, Rockville, MD). The MCF-10A growth medium was supplemented with 5% heat-inactivated FBS, PSN (1%), glutamine (2 mM), EGF (20 ng/ml), hydrocortisone (0.5 μ g/ml), and insulin (10 μ g/ml). Cell cultures were maintained in a 5% CO₂, 37°C humidified incubator. All experiments were performed with cells in passages 5-30.

Tissue: Breast cancer tumor specimens were obtained from the Tissue Bank of the West Virginia University Health Science Center. Samples were obtained in microfuge tubes containing 200 μ l lysis buffer (composition described later). The tubes were snap frozen and stored in -80°C until used. Five tumor samples were assessed in these studies. The pathologic (p) staging of tumors includes the letters T, N and M, where T stands for tumor, N for lymph nodes and M for metastasis. T is followed by a number between 0 and 4 and the

number assigned depends on the tumor size and spread to the skin or the chest wall under the breast. A higher T indicates a larger tumor and/or wider spread to the tissues near the breast. The letter N is followed by a number between 0 and 3 implying the spread of the cancer to the lymph nodes near the breast and the number of lymph nodes affected. The letter M is followed by 0 or 1, respectively, indicating the absence or presence of spread to distant organs. X indicates no assessment of any given criteria (American Cancer Society, 2009). Tumor sample I was from a 43 year old female diagnosed with invasive ductal carcinoma staged at pT2N1aMX. Tumor sample II was from a 42 year old female diagnosed with infiltrating ductal carcinoma staged at pT2N1aMX. Tumor sample III was from a 48 year old female diagnosed with infiltrating ductal adenocarcinoma staged at pT1b,pN0(i-). Tumor sample IV was from a 64 year old woman, diagnosed with invasive ductal carcinoma and staged at pT1aN0MX and Tumor sample V was from a 54 year old woman, diagnosed with invasive ductal carcinoma and staged at pT1c (sn)N0(i-)MX.

Construction of expression vectors for molecular cloning of cPLA₂ α fragments for the generation of the cPLA₂ α antibody: Amino acid sequences corresponding to cPLA₂ α (411-442) are gene specific (GenBankTM accession number CAB42689) and comprise an antigenic segment considered appropriate for antibody generation (Princeton Biomolecules, PA (Welling et al., 1985)). Gene specific primer sets containing BamHI and XhoI restriction enzyme sites (lower cases) were synthesized to amplify the above mentioned fragment of cPLA₂ α as follows: forward primer 5'-ggatccaaCAAAGCAGAGGCTCCACAATG-3' and the reverse primer 5'-ctcgagGGAGACAGTGGATAAGATGTA-3'. Reverse-transcription polymerase chain reaction (RT-PCR) was used to amplify the cPLA₂ α gene specific fragment using MCF-7 cell RNA. To overcome the difficulty of cloning for gene sequence specificity

conformation, larger fragments than those necessary were amplified for antibody generation. For gene fragment specificity determination, the RT-PCR amplified fragments were purified (Qiagen), followed by ligation into pBSIISK⁺ vector, and cloned in DH-5 α cells (the generous gift of Dr. Bidwai, West Virginia University, Morgantown, WV). The transformed cells were grown and the plasmids of two representative clones were isolated, sequenced (Retrogen Sequencing, CA), and gene fragment specificity confirmed (Blast, GenBank, NCBI).

Site Directed Mutagenesis: To ensure the expression of only the specific antibody fragments as GST-fusion fragments, a stop codon was introduced in the cPLA₂ α plasmid using the Quick-Change site directed mutagenesis kit (Stratagene). Both the forward and reverse primer sets contained the stop codon (underlined sequence). Only the forward primer sequence is shown: the Glu⁴⁴³ (GAA) was substituted with stop codon, 5'-GGACAGTGATGATGAATCACACTTAACCCCAAAGGCACTGAAAATGAAGAT---GCTGG-3'. The plasmid containing the cPLA₂ α antibody fragment was subjected to 20 cycles of polymerase chain reaction (PCR) using the primer sets described above. The PCR products were digested with the DpnI enzyme to eliminate the non-mutant plasmids that were used as templates. The reaction mixture was used to transform *E. coli* DH5 α cells.

The above described section on the generation of the cPLA₂ α construct for antibody generation was conducted previously in the lab (Maricica Pacurari, 2006). The pBSIISK⁺ plasmids of cPLA₂ α from two representative clones were sequenced using T7 primers to confirm the specificity of the gene fragment and also to confirm the presence of the stop codon (Blast, GenBank, NCBI). The cDNA fragments encoding the specific antibody

fragments were subcloned into BamHI-XhoI sites of pZEX vectors, cloned in *E. coli* (BL21-DE3) cells that possess the plasmid pT-TRX for expression and purification of GST-fusion proteins (gifts of Dr. Bidwai).

Purification of Glutathione S-Transferase (GST) fusion peptides: Cultures were grown in 2xYTA containing 150 µg/ml ampicillin and 10 µg/ml chloramphenicol to an OD₆₀₀ between 0.5 to 0.7. The culture was then induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 hours at 30°C with vigorous shaking (225 rpm). The cells were harvested and resuspended in phosphate buffered saline (PBS) containing 100 mM phenylmethylsulfonyl fluoride (PMSF), 0.8% 2-mercaptoethanol and mixed overnight at 4°C. The next day the cells were lysed by sonication until 95% of cells were lysed as indicated by phase contrast microscopy (Nikon). Triton X-100 was added to a final concentration of 1% and the cell homogenate was mixed for 3 hours at 4°C. Following centrifugation, the cell homogenate was mixed with 1 ml of glutathione-Sepharose beads (GE Healthcare) overnight at 4°C. The column was washed thrice with 10 bed volumes of PBS with 100 mM PMSF. The bound proteins were eluted with 2 ml of 10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0. The purity and relative concentrations of the eluted proteins was determined by SDS-polyacrylamide gel electrophoresis (PAGE) relative to standard bovine serum albumin (BSA) detected by Commassie Blue staining.

Production of polyclonal antibodies to cPLA₂α: Approximately 5mg of the purified GST-fusion peptide of cPLA₂α was sent to a commercial facility (Cocalico Biologicals Inc, PA) for antibody generation.

Maltose-binding protein fusion peptides expression: cPLA₂ α peptide was excised from the pZEX plasmid following ligation into PQE-82L plasmid, excised and ligated into pMAL-c2x plasmids (New England Biolabs Inc) for *in vitro* expression as maltose-binding protein (MBP) fusion peptides. The eluted fusion peptides were quantified by the Bradford Assay (BioRad) and their purity was determined by SDS-PAGE.

Dot blot analysis for crossreactivity/specificity analysis of cPLA₂ α polyclonal antibody:

Different amounts of MBP-fusion peptides ranging from 0.001 to 3 μ g along with MBP and GST alone were dot blotted onto nitrocellulose membranes. The nitrocellulose membranes were air dried at room temperature and subjected to standard Western blot analysis as described later. The molecular weight (MW) of MBP expressed from a derivative of pMAL-c2x, including the amino acid residues coded for by the polylinker up to the Xmn I site is 42.482 kDa (New England Biolabs Inc). The molecular weight of the cPLA₂ peptide was calculated from the peptide sequence using ExPASy (http://www.expasy.ch/tools/pi_tool.html). The calculated molecular weight (kDa) of the cPLA₂ α peptide (32 aa) was 3.56 and that of the MBP- cPLA₂ α peptide was 46.046.

Western Blot: The cell lines (MDA-MB 231, MCF-7 and MCF-10A) were trypsinized and subcultured into 6 well plates at 200,000 cells/well and allowed to attach and recover for 24 hours in 10% FBS supplemented media. Media were replaced with 2% FBS containing media for 96 hours with a refeeding at 48 hours. Following this preincubation period, TNF- α and/or IL-1 β were added to fresh 2% FBS media. Cells were washed once with 1x PBS, incubated for 10 mins in 1x PBS with 1 M EDTA at 37°C, scrapped, pelleted, and resuspended in lysis or homogenization buffer. For whole cell lysates, the cell pellet was

resuspended in 200 µl lysis buffer (20 mM Tris-Cl, pH 7.4, 2 mM Na₃VO₄, 1 mM NaF, 100 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 1 µl/ml Sigma's Protease Inhibitor cocktailTM, 1 mM PMSF), and for microsomal preparations, the cell pellets were resuspended in 200 µl of homogenization buffer (10 mM HEPES, 8% sucrose, 1 µl/ml Sigma's Protease Inhibitor cocktailTM, 1 mM PMSF). Cell debris was removed by microcentrifugation for 15 minutes at 13,500 rpm at 4°C. For microsomal preparations, cell supernatants were subjected to ultracentrifugation at 100,000 x G for 1 hour at 4°C (Sorvall). Cytosolic fractions were collected and the pellets were resuspended in homogenization buffer and recovered by titration. Protein content of the whole cell lysates and microsomal preparations were quantified using the Bradford Assay (BioRad). 50 µg of protein per sample was separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Protein transfer was visualized by Ponceau-S staining. The membranes were washed 3X with TBST (Tris-Buffered Saline, 0.1% Tween-20) and were blocked for 2 hours in 2.5 % non-fat dry milk TBST blocking buffer and were incubated overnight at 4°C with specific primary antibody. The primary antibody was removed and the membranes were washed 3X with TBST, following which they were incubated with an alkaline phosphatase conjugated secondary antibody and developed with Nitro-Blue-Tetrazolium (NBT): Bromo-Chloro-Indolyl-Phosphate (BCIP) solution. The reaction was stopped with 0.5 M EDTA and bands were analyzed using the ImageJ software (NIH). Positive and negative control experiments were performed using the MBP-cPLA₂ isoform antigenic peptide.

Western Competition Assays: To confirm antibody specificity for each of the cPLA₂ isoforms, *in vitro* expressed MBP-fusion peptides (30 µg peptide/ml of antisera) were preincubated with isoform specific polyclonal serum for 24 hours at 4°C.

Immunohistochemistry: MDA-MB 231, MCF-7 and MCF-10A cells were subcultured onto gelatin coated coverslips in 6 well plates at 100,000 cells per well and allowed 24 hours in 10% FBS supplemented media to recover from trypsinization and attach. Media were replaced with 2% FBS containing media for 48 hours with a refeeding at 24 hours. Following this preincubation period, the cells were washed twice in warm 1x PBS and A23187 (5 μ M) and AACOCF3 (10 μ M) treatments were applied to select specimens. Following treatment, the cells were fixed in 4% paraformaldehyde at 4°C. After fixation, cells were washed 4X in PBS buffer (1x PBS, 0.1X Triton-X and 1% BSA), and incubated overnight at 4°C with primary antibody. The cells were washed 4X with PBS buffer and Alexafluor conjugated secondary antibody was applied. Coverslips were mounted on slides with Vectashield mounting media (Vector Labs, CA) and visualized with the Olympus FluoView (FV1000) confocal microscope.

Tissue: Tumor tissue samples immersed in lysis buffer (composition described earlier) were sonicated on ice at low power (7) for three 10 second bursts with 30 second intervals between each burst. Cell debris was removed by microcentrifugation for 15 minutes at 13,500 rpm at 4°C. Protein from the tissue lysates was quantified using the Bradford Assay (BioRad) and separated on 10% SDS-PAGE gels. For tumor samples I, II, III, IV and V; 20 μ g, 30 μ g, 4.8 μ g, 4 μ g, and 8 μ g protein, respectively, was run in each lane. Western blotting was conducted for cPLA₂ isozyme expression. The amount of protein used in Western blots for each tumor varied and it depended on the total protein extracted from each tumor sample (Tumor I: 196 μ g, Tumor II: 55.6 μ g, Tumor III: 33 μ g, Tumor IV: 28 μ g, Tumor V: 61 μ g).

RESULTS

Determination of cPLA₂α antibody titer: Western blot analysis was performed to determine the useful antibody titer for cPLA₂α detection. For this analysis, different dilutions of the cPLA₂α antisera were used against the MBP-cPLA₂α (411-442) peptide (Fig 2B). An antibody titer that produced good immunodetection against the peptide was selected for further protein analysis. The specificity of the immunodetection was confirmed by Western competition analysis, in which the antiserum was preincubated with the specific competing peptide. For the specificity and crossreactivity analysis of the cPLA₂α antisera, a 1:500 antisera dilution was selected. Although Western blots were conducted on MDA-MB 231, MCF-7 and MCF-10A cell protein, no cPLA₂α (85 kDa) specific band was detected (Fig. 3A,B). Initial studies on the cell protein were conducted using a 1:500 dilution of antisera; however, in later studies a 1:50 antibody dilution was used.

Analysis of specificity and crossreactivity of cPLA₂α antisera: The cPLA₂α antisera was specific as indicated by dot-blot analysis shown in Fig. 2C. It did not crossreact with affinity purified MBP-cPLA₂β (1 - 57) designated as cPLA₂βI, MBP-cPLA₂β (808 - 829) designated as cPLA₂βII, MBP-cPLA₂γ (307 - 327), or with MBP alone as indicated by Western blot analysis as shown in Fig. 2D.

Expression of cPLA₂ isoforms in the MDA-MB 231, MCF-7 and MCF-10A cell lines by Western blot analyses: To examine the expression of the cPLA₂ isoforms in the human breast cancer cell lines MDA-MB 231 and MCF-7 as well as in the non-tumorigenic human breast cell line MCF-10A growing in normal growth medium, Western blots were conducted

using the cPLA₂ isoform specific antisera and the preincubated antisera. Antibody titers for cPLA₂βI, cPLA₂βII and cPLA₂γ were made as recommended (Maricica Pacurari, 2006). No bands showing the predicted mass of cPLA₂α (85 kDa), cPLA₂βI (110 kDa), cPLA₂βII (110 kDa), or cPLA₂γ (61 kDa) were detected in any of the 3 cell lines tested in three independent trials (Fig. 3A-H).

Effect of cytokine treatment on cPLA₂ isoform levels in MDA-MB 231 and MCF-10A

cell lines: Analyses of the effect of TNFα and/or IL-1β on the cPLA₂ isoform levels were guided by earlier studies in the laboratory conducted on MCF-7 cells. It was reported that TNFα increased the expression of the cPLA₂γ protein and IL-1β elicited an increase in cPLA₂β mRNA in those cells. Both TNFα and IL-1β have been reported by others to induce the expression of cPLA₂α in diverse cell models (Clark et al., 1995; Grewal et al., 2003). No bands of the predicted mass of the cPLA₂ isoforms were detected from either the MDA-MB 231 or MCF-10A cell lines upon stimulation with TNFα and IL-1β (Fig. 4A-F, Fig. 5A-F).

Distribution of cPLA₂ isoforms in the MDA-MB 231, MCF-7 and MCF-10A cell lines:

To further explore the possible expression of, and the distribution of the cPLA₂ isoforms in the above mentioned cell lines, immunohistochemistry techniques were employed using cPLA₂ isoform specific antisera and fluorescent secondary antibodies. Negative control experiments were conducted using antisera preincubated with the corresponding blocking peptide and the secondary antibody. In both MDA-MB 231 (Fig. 6A-E, Fig. 7A-E) and the MCF-7 (Fig. 8A-G) cells, fluorescence output was significantly attenuated in cells evaluated

with the preincubated antisera suggesting the presence of the cPLA₂ isoforms. These observations indicate a cytosolic localization of the cPLA₂ isoforms, although further investigations are necessary to identify their precise location within each of the cell lines. No detection of cPLA₂ isozymes was observed in the MCF-10A cells, and a representative figure of the cPLA₂ isozymes in the MCF-10A cells done in two independent trials is shown in Fig.9A-C. These results were independent of antiserum preincubation with the blocking peptides.

Effects of AACOCF3 on cPLA₂ isoforms - α , - β and - γ detection in MDA-MB231 and

MCF-7 cell lines: AACOCF3 is an arachidonic acid analogue and an inhibitor of cPLA₂ α , cPLA₂ β , and cPLA₂ γ (Meyer et al., 2005; Riendeau et al., 1994). The effect of AACOCF3 on the cPLA₂ isoforms was assessed in both the MDA-MB 231 and MCF-7 cell lines. The concentration and the time of treatment by AACOCF3 were determined as described in the literature (Fatima, 2002; Riendeau et al., 1994; Woo et al., 2000). As shown in Fig. 10A-F, the MDA-MB 231 cells, upon treatment with AACOCF3, demonstrated significantly mitigated fluorescence output when assessed for cPLA₂ α , cPLA₂ β I and cPLA₂ γ . Similar results were observed in the MCF-7 cells (Fig. 11A-F).

Relocalization of cPLA₂ α and cPLA₂ β in response to Ca²⁺ ionophore A23187 in MDA-

MB 231 and MCF-7 cell lines: cPLA₂ α and cPLA₂ β include a Ca²⁺ binding domain and require Ca²⁺ for functioning. In unstimulated cells, cPLA₂ α is reportedly localized in the cytosol and after Ca²⁺ stimulation translocates to the perinuclear membranes (Grewal et al., 2002; Grewal et al., 2003; Schievella et al., 1995). In the present studies, following the

elevation of cytosolic Ca^{2+} levels by the Ca^{2+} ionophore A23187, a relocation of $\text{cPLA}_2\alpha$ was observed in both the MDA-MB 231 (Fig. 12B, 12C) and the MCF-7 cells (Fig. 13B). In contrast to the unstimulated cells where the $\text{cPLA}_2\alpha$ signal was diffused in the cytoplasm, in the A23187 stimulated cells the signal was concentrated around the nucleus. Similar redistribution of the $\text{cPLA}_2\beta$ signal was also observed in both the cell lines upon treatment with A23187 (Figs. 12E, 12F, and 13D).

Expression of cPLA_2 isoforms in cancerous human breast tissue samples: To assess the expression of the cPLA_2 isozymes - α , - β and - γ in cancerous human breast tissues, Western blot analyses were performed on human breast cancer tissue samples using antisera generated against the respective cPLA_2 isozymes. Due to the differential amount of tissue obtained for each of the five samples, quantitative analyses could not be performed. No bands of the predicted mass of the cPLA_2 isozymes were obtained from any of the five tumor samples tested (Fig. 14-18). Preincubation with the respective blocking peptides did not provide evidence of the presence of any of the cPLA_2 isoforms (Fig. 14-18).

DISCUSSION

Arachidonic acid and its bioactive metabolites play significant roles in normal tissue homeostasis, inflammation and in several pathophysiological conditions, including cancer. The eicosanoids, primarily PGE₂, play fundamental roles in tumor development and progression and are abundantly expressed in several human cancers including colon, lung, and breast (Dohadwala et al., 2002; Schrey and Patel, 1995; Shao et al., 2003). A significant reduction in breast cancer development in mice follows the administration of a PGE₂ receptor (EP1) antagonist (Kawamori et al., 2001). PG production from free AA is a multistep process catalyzed by the COX-1 and COX-2 enzymes. COX-2 selective inhibitors limit tumor growth in the breast, colon, gall bladder, esophageal, and pancreatic cancers (Grossman et al., 2000; Higashi et al., 2000; Ristimäki et al., 2002).

Typically, free AA is the substrate used for prostaglandin biosynthesis. In unstimulated cells, AA is esterified at the *sn*-2 position of the cellular glycerophospholipids. The release of AA from the cellular glycerophospholipids by the phospholipase A₂ (PLA₂) enzymes rate limits PG biosynthesis (Capper and Marshall, 2001). The free AA is then acted upon by the COX enzymes in a two step conversion into PGH₂. PGH₂ can then be acted upon by various enzymes to produce the various PGs, often in a tissue specific manner (Smith, 1992). The PLA₂ enzymes comprise a family that has been classified into 19 forms, and these divided into four groups in humans. The groups IV PLA₂s, also known as cytosolic (c) PLA₂, appear to be the principal enzyme(s) producing the initial release of AA used for prostanoid biosynthesis (reviewed in Balsinde et al., 1999). The cPLA₂ group itself is comprised of four isoforms, of which cPLA₂α, cPLA₂β, and cPLA₂γ (also referred to group IVA, IVB, and IVC) were the focus of this study. Although the cPLA₂α isoform has long been considered the principal enzyme involved, the participation of the other

cPLA₂ paralogues, cPLA₂β, and cPLA₂γ, must also be considered as potential contributors.

Our studies show that both the MDA-MB 231 and MCF-7 breast cancer cell lines express the cPLA₂α, cPLA₂β, and cPLA₂γ proteins. However, this was not the case in the non-tumorigenic human breast cell line, MCF-10A, where the cPLA₂ isozymes could not be detected. It will be of interest to determine if tumorigenic potential correlates with cPLA₂ expression in additional model systems.

cPLA₂ is expressed in a wide variety of cell types including platelets, macrophages, neutrophils, endothelial cells, vascular smooth muscle cells, alveolar epithelial cells, renal mesangial cells, keratinocytes, and others (Sharp et al., 1991). Several human tumors, including colorectal, small bowel, and lung exhibit elevated expression of cPLA₂α (Heasley et al., 1997; Wendum et al., 2003). The overexpression of cPLA₂α has also been reported in NSCLC and cholangiocarcinomas (Nakanishi and Rosenberg, 2006). RT-PCR and Western blot analyses revealed varying levels of cPLA₂α in several human breast cancer cell lines such as SKBR33, UACC893, HCC38, MDA-MB 231, BT474 and MCF-7. The BT474 and MCF-7 cells exhibited significantly lower expression of cPLA₂α mRNA and protein than the other cell lines tested (Caiazza et al., 2010). Clinical evidence of cPLA₂α expression in human breast tumors has not yet been reported.

As was previously reported (Caiazza et al., 2010), we also found that both the MDA-MB 231 and the MCF-7 cell lines express cPLA₂α. However, to our knowledge this is the first immunohistochemical analysis demonstrating the presence of cPLA₂α protein in these cells. The present studies also revealed that cPLA₂α was predominantly localized in the cytoplasm of both the cancer cell lines. In contrast, the cPLA₂α protein was not detected in

the MCF-10A cells, an observation not previously reported. AACOCF₃ is an arachidonic acid analogue and a potent inhibitor of cPLA₂ α (Riendeau et al., 1994). Pretreatment with AACOCF₃, caused a decrease of cPLA₂ α signal in the MDA-MB 231 and MCF-7 cells, and antigenic adsorption of the cPLA₂ α antibody with its corresponding blocking peptide (also referred to as the pre-incubated cPLA₂ α antibody) prevented detection of the cPLA₂ α protein in either of the cell lines, providing evidence in support of antiserum specificity.

cPLA₂ α remains the most widely studied member of the cPLA₂ family, primarily due to its selectivity for *sn*-2 AA. An approximately 20-fold preference for AA hydrolysis as compared to *sn*-2 PA, has been reported (Clark et al., 1991; Leslie, 2004; Leslie et al., 1988). During basal conditions, cPLA₂ is primarily located in the cytosol (Grewal et al., 2003; Schievella et al., 1995). Stimulation by a variety of agonists causes relocation of cPLA₂ to the intracellular membranes. It has been suggested that the translocation is mediated through the CaLB domain, which upon an elevation in intracellular free Ca²⁺, promotes the binding of cPLA₂ to the phospholipids (Gijon et al., 1999; Song et al., 1999). In the present study, stimulation by the Ca²⁺ ionophore A23187 caused a relocation of the immunohistochemically detected cPLA₂ α to the nuclear membranes in both the MDA-MB 231 and MCF-7 cells. On the other hand, in A23187 negative conditions, cPLA₂ α was predominantly evident in the cytoplasm. Given the observations that the pre-incubated cPLA₂ α antibody and AACOCF₃ treatment caused a loss or decrease, of cPLA₂ α signal, implies that the detected signal is specific to cPLA₂ α . Additionally, A23187 mediated relocation of cPLA₂ α to the nuclear membranes, further suggests that the cPLA₂ α is expressed in the breast cancer cell lines, MDA-MB 231 and MCF-7.

Corresponding studies with whole cell lysates of MDA-MB 231, MCF-7 and MCF-10A cells, were unable to detect cPLA₂α by Western blot analyses. These results seem to present a paradox. Although the actual reason is unknown at this moment, it may be that the cPLA₂α protein is unstable and is degraded during the disruption of the cells, even with the use of protease inhibitors. Alternatively, the enzymes maybe present at levels below the threshold requirement for detection by Western blotting. Several cytokines, such as IL-1β or TNFα and growth factors such as EGF, cause elevated cPLA₂α expression in several cell types (Hoeck et al., 1993; Hulkower et al., 1992; Maxwell et al., 1993). However, we found by Western blot assays that neither TNFα or IL-1β had any apparent effect on the cPLA₂α protein in either of the cancer cell lines, or in the normal breast cell line model MCF-10A.

cPLA₂β mRNA and protein is expressed in various human tissues such as the pancreas, liver, heart, brain (Ghosh et al., 2006a; Song et al., 1999). The cPLA₂β mRNA is predominantly present in an unspliced form, which, after final processing, encodes a protein of 110-114 kDa (Pickard et al., 1999). There is also evidence of a 100kDa cPLA₂β splice variant in the lung, spleen and ovary (Ghosh et al., 2006a). Previous RT-PCR studies done in this laboratory (Maricica Pacurari, 2006) using MCF-7 cell RNA indicated the presence of both the processed cPLA₂β and a pre-processed variant (containing intron I). This was not tested on the MDA-MB 231 and MCF-10A cells.

Due to the presence of cPLA₂β mRNA in the spliced and unspliced forms and because of the possibility of multiple splice variants, antibodies against cPLA₂β were generated against two selected regions. The first antiserum, referred as cPLA₂βI, was against amino acids (aa) 1-57 corresponding to exon I, and the second, cPLA₂βII, was against region

808-829 aa corresponding to exon 6, that is in between catalytic domain A and B (Maricica Pacurari, 2006). In the present study the cPLA₂β protein was detected in both the MDA-MB 231 and MCF-7 cells. In the MDA-MB 231 cells, the cPLA₂β signal was more intense using the cPLA₂βI antibody than the cPLA₂βII antibody. Whether this reflects actual distinctions in the amounts of the cPLA₂β protein cannot be determined based on these results. Antisera characteristics might independently yield unique results. In the MCF-7 cells; cPLA₂β was detected only with the cPLA₂βI antibody. Subsequent studies were conducted using the cPLA₂βI antibody. The cPLA₂β signal could not be detected in the MCF-10A cells assayed with either of the cPLA₂β antibodies. The cPLA₂β antibody blocked with the corresponding blocking peptide did not detect cPLA₂β in either the MDA-MB 231 or MCF-7 cells, suggesting that the signal is specific to cPLA₂β.

It has been proposed that AACOCF₃ can also serve as an inhibitor of cPLA₂β and cPLA₂γ (Meyer et al., 2005), as well as inhibiting cPLA₂α. In the present study AACOCF₃ treatment caused a decrease in the cPLA₂β signal in the MDA-MB 231 and MCF-7 cells in the immunohistochemistry assays. Based on the apparent physical change elicited, these results support Meyer's (2005) proposition that AACOCF₃ may be a cPLA₂β inhibitor. Similar to cPLA₂α, cPLA₂β also harbors a CaLB domain and exhibits calcium dependent phospholipase activity (Song et al., 1999). Therefore, it was speculated that cPLA₂β protein may also be sensitive to A23187 treatment. In the studies described here, we did observe A23187 dependent redistribution of cPLA₂β to the perinuclear region in both the MDA-MB 231 and the MCF-7 cells. In the absence of A23187, cPLA₂β was predominantly located in the cytoplasm.

In parallel studies, the cPLA₂β protein was not immunodetected by Western blotting in total cell protein preparations from any of the cell lines tested. There are no reports from other laboratories on the regulation of cPLA₂β, and whether or not its expression can be upregulated, or by what stimuli. In the present study there was no effect of either TNF-α or IL-1β on the cPLA₂β protein in any of the 3 cell lines when tested by Western blotting. However there are possibilities that the cPLA₂β protein is unstable and was degraded during cell disruption, despite the use of protease inhibitors, or it may present at a level below the sensitivity of Western blotting.

The cPLA₂γ isoform shares 29% homology with cPLA₂α, however, unlike cPLA₂α, it is Ca²⁺ independent. In contrast to the widely expressed cPLA₂α and cPLA₂β, cPLA₂γ is predominantly found in the skeletal and cardiac muscle, and to a lesser extent in the brain (Pickard et al., 1999; Underwood et al., 1998). Human macrophages have also been shown to express cPLA₂γ (Duan et al., 2001). In the present study, immunohistochemical assays did detect the cPLA₂γ protein in the MDA-MB 231 and the MCF-7 cells. It was not detected in the MCF-10A cells. The cPLA₂γ antisera blocked with its corresponding blocking peptide, and in specimens treated with AACOCF₃ the cPLA₂γ protein was not detected.

The cPLA₂γ isoform has been reported to be membrane bound and contains putative acylation and a C-terminal prenylation site that may regulate its membrane association (Underwood et al., 1998). In mammalian cells, cPLA₂γ is farnesylated and acylated, and immunohistochemical analyses have revealed that it co-localizes with the mitochondria (Tucker et al., 2005). Although the data from both the MDA-MB 231 and the MCF-7 cells

strongly suggest the presence of cPLA₂γ, further investigation is necessary to ascertain the precise location of cPLA₂γ in these cells. A corresponding study examining the cPLA₂γ expression by Western blotting was performed using microsomal preparations and whole cell lysates (data not shown) from the MDA-MB 231, MCF-7, and MCF-10A cells. Similar to the other cPLA₂ isoforms, cPLA₂γ was not detected in any of the cell lines. Cytokines induce the release of cPLA₂γ dependent AA in the HEK293 cells (Murakami et al., 2003). Earlier studies in this laboratory indicated that TNF-α upregulates cPLA₂γ mRNA and protein expression in the MCF-7 cells (Maricica Pacurari, 2006). No such effect on the protein was found in this study in the MDA-MB 231, MCF-7 or the MCF-10A cells treated with either TNF-α or IL-1β. The basis of this discrepancy is unclear and additional efforts will be required for clarification.

A subsequent study was performed to assess the cPLA₂ isozyme expression in surgically removed human cancerous breast tissue. Inconclusive results were obtained from the Western blot analyses performed. There are the possibilities of protein degradation or undetectable expression of the cPLA₂ isoforms in light of these results. Future experiments involving immunohistochemical techniques may detect the expression of the cPLA₂ isozymes in surgically removed breast cancer tissue.

The studies described in this thesis, indicate the presence of the cPLA₂ isozymes -α, -β and -γ in the breast cancer cell lines, MDA- MB 231 and MCF-7, but not in the non-transformed breast cell line MCF-10A. Stimulation by Ca²⁺ ionophore A23187 caused relocation of the Ca²⁺ dependent cPLA₂ isoforms, -α and -β, within the MDA-MB 231 and the MCF-7 cells. cPLA₂α, -β and -γ peptide blocked antisera failed to detect the respective cPLA₂ isoforms in the cancer cell lines, suggesting that the detected signal was cPLA₂

isoform specific. This was supported by the results from AACOCF3 pretreatment where the cPLA₂ isoform signals were lost upon treatment with the inhibitor. cPLA₂ α is believed to be the principal enzyme responsible for AA release for eicosanoid biosynthesis. However, cPLA₂ β and cPLA₂ γ , each of which displays ~30% sequence homology to cPLA₂ α , may also be associated with AA release and eicosanoid synthesis in the cell lines tested. AACOCF3, the cPLA₂ α inhibitor, caused a decrease in cPLA₂ β and cPLA₂ γ signals as well. This is an observation that, to our knowledge, has not been reported elsewhere. Whether or not the cPLA₂ isozymes are expressed in breast cancer tissue remains undetermined and further investigations are warranted.

FIGURES

Fig. 2: Characterization of cPLA₂α antisera: A. The schematic location of cPLA₂α peptide selected as potential antigen for antibody generation relative to the cPLA₂α amino acid (aa) sequence (not drawn to scale) (Adapted from Song et al., 1999). B. cPLA₂α antibody titer determination by Western analysis. 5 μg of purified MBP-cPLA₂α peptide was loaded in each lane. cPLA₂α antibody dilutions were used as follows: Lanes 1, 2: 1:50; Lanes 3,4: 1:100; Lanes 5,6:1:500; Lanes 7,8:1:1000. Arrows indicate the selected antibody titers used in subsequent studies (Results are representative of 3 trials). C. cPLA₂α Specificity Analysis. Bacterially expressed and amylose beads purified cPLA₂α specific fragment with maltose binding (MBP) epitope was used for this analysis. Amounts of MBP-cPLA₂α peptide used are indicated in the figure. The antibody dilution used was 1:500. Assays with the purified MBP peptide did not produce any results (Results are representative of 3 trials). D. cPLA₂α Crossreactivity Analysis. Bacterially expressed and amylose beads purified cPLA₂ isozyme specific fragments with maltose binding epitopes were used in this

analysis. α : MBP-cPLA₂ α (411 - 442); β I: MBP-cPLA₂ β (1 - 57) or cPLA₂ β I ; β II: MBP-cPLA₂ β II (808-829) or cPLA₂ β II; γ : MBP-cPLA₂ γ (307 - 327); MBP: MBP alone. 5 μ g peptide was used in the analysis. The antibody dilution used was 1:500. (Results are representative of 3 trials).

Fig. 3: Basal expression of cPLA₂ isoforms in MDA-MB 231, MCF-7 and MCF-10A cells. Total protein was prepared as described in Materials and Methods and 50 µg per lane was used to assay for cPLA₂α, cPLA₂βI and cPLA₂βII isozyme expression. For cPLA₂γ isozyme expression microsomal extracts were prepared as described in Materials and Methods and 50 µg per lane was used. (A) Tested against cPLA₂α antibody (1:50). (B)

Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ β II antibody (1:40). (F) Tested against pre-incubated cPLA₂ β II antibody (1:40). (G) Tested against cPLA₂ γ antibody (1:40). (H) Tested against pre-incubated cPLA₂ γ antibody (1:40) (Results are representative of 3 trials for each isozyme).

Fig. 4: Effects of TNF α and IL-1 β on cPLA₂ isozyme expression in MDA-MB 231 cells: MDA-MB 231 cells were treated with a control for steroid hormone treatment (0.1% ethanol) in lane 1, TNF α (40 nM) in lane 2 and IL-1 β (600 pM) in lane 3. Total protein was prepared as described in Materials and Methods and 50 μ g per lane was used to assay for cPLA₂ α and cPLA₂ β isozyme expression. For cPLA₂ γ isozyme expression, microsomal extracts were prepared as described in Materials and Methods and 50 μ g was used. A) Tested against cPLA₂ α antibody (1:50). (B) Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ γ antibody (1:40). (F) Tested against pre-incubated cPLA₂ γ antibody (1:40) (Results are representative of 3 trails for each isozyme).

Fig. 5: Effects of TNF α and IL-1 β on cPLA₂ isozyme expression in MCF-10A cells:

MCF-10A cells were treated with control for steroid hormone treatment (0.1% ethanol) in lane 1, TNF α (40 nM) in lane 2, or IL-1 β (600 pM) in lane 3. Total protein was prepared as described in Materials and Methods and 50 μ g per lane was used to assay for cPLA₂ α and cPLA₂ β isozyme expression. For cPLA₂ γ isozyme expression, microsomal extracts were prepared as described in Materials and Methods and 50 μ g per lane was used. (A) Tested against cPLA₂ α antibody (1:50). (B) Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ γ antibody (1:40). (F) Tested against pre-incubated cPLA₂ γ antibody (1:40) (Results are representative of 3 trials for each isozyme).

Fig. 6: Immunohistochemical detection of cPLA₂α and cPLA₂βI in MDA-MB 231 cells.

Cells were grown on gelatin coated coverslips, fixed and probed with cPLA₂ isozyme antibody as indicated in Materials and Methods. The secondary antibody used was goat-anti-rabbit IgG coupled with Alexafluor 488. (A) Detection and distribution of cPLA₂α. cPLA₂α antibody

(1:50) was used in this study. (B) cPLA₂ α probed with the cPLA₂ α antibody pre-incubated with the specific blocking peptide (1:50). (C) Detection and distribution of cPLA₂ β . cPLA₂ β I antibody (1:50) was used in this study. (D) cPLA₂ β probed with the cPLA₂ β I antibody pre-incubated with the specific blocking peptide (1:50). (E) Cells probed with Alexafluor 488 secondary antibody. Images were taken using 40X oil immersion objective (Results are representative of 2 trails).

Fig. 7: Immunohistochemical detection of cPLA₂βII and cPLA₂γ in MDA-MB 231 cells. Cells were grown on gelatin coated coverslips, fixed and probed with cPLA₂ isozyme antibody as indicated in Materials and Methods. Secondary antibody was goat-anti-rabbit IgG coupled with Alexafluor 488. (A) Detection and distribution of cPLA₂β. cPLA₂βII antibody (1:40)

was used in this study. (B) cPLA₂β probed with the cPLA₂βII antibody pre-incubated with the specific blocking peptide (1:40). (C) Detection and distribution of cPLA₂γ. cPLA₂γ antibody (1:40) was used in this study. (D) cPLA₂γ probed with the cPLA₂γ antibody pre-incubated with the specific blocking peptide (1:40). (E) Cells probed with Alexafluor 488 secondary antibody. Images were taken using 40X oil immersion objective (Results are representative of 2 trails).

cPLA₂α

cPLA₂α Pre-incubated

cPLA₂βI

cPLA₂βI Pre-incubated

cPLA₂γ

cPLA₂γ Pre-incubated

Alexafluor 488 2° Ab

Fig. 8: Immunohistochemical detection of cPLA₂α, cPLA₂β and cPLA₂γ in MCF-7 cells.

Cells were grown on gelatin coated coverslips, fixed and probed with cPLA₂ isozyme antibody as indicated in Materials and Methods. Secondary antibody used was goat-anti-rabbit IgG coupled with Alexafluor 488. (A) Detection and distribution of cPLA₂α. cPLA₂α antibody (1:50) was used in this study. (B) cPLA₂α probed with the cPLA₂α antibody pre-incubated with the specific blocking peptide (1:50). (C) Detection and distribution of cPLA₂β. cPLA₂βI antibody (1:50) was used in this study. (D) cPLA₂β probed with the cPLA₂βI antibody pre-incubated with the specific blocking peptide (1:50). (E) Detection and distribution of cPLA₂γ. cPLA₂γ antibody (1:40) was used in this study. (F) cPLA₂γ probed with the cPLA₂γ antibody pre-incubated with the specific blocking peptide (1:40). (G) Cells probed with Alexafluor 488 secondary antibody. Images were taken using 40X oil immersion objective (Results are representative of 2 trials).

Fig. 9: Immunohistochemical assay of cPLA₂α, cPLA₂β and cPLA₂γ in MCF-10A cells.

Cells were grown on gelatin coated coverslips, fixed and probed with cPLA₂ isozyme antibody as indicated in Materials and Methods. Secondary antibody used was goat-anti-rabbit IgG coupled with Alexafluor 488. (A) Distribution of cPLA₂α. cPLA₂α antibody (1:50) was used in this study. (B) Distribution of cPLA₂β. cPLA₂βI antibody (1:50) was used in this study. (C) Distribution of cPLA₂γ. cPLA₂γ antibody (1:40) was used in this study. (D) Cells probed with Alexafluor 488 secondary antibody. Images were taken using 40X oil immersion objective (Results are representative of 2 trials).

Fig. 10: Modulation of cPLA₂ isozymes in MDA-MB 231 cells by AACOCF₃.

AACOCF₃, an analogue of arachidonic acid has been reported to be a cPLA₂ α inhibitor. (A) Detection of cPLA₂ α after treatment with 0.05% ethanol (vehicle). The cPLA₂ α antibody dilution used was 1:50. (B) cPLA₂ α after treatment with AACOCF₃ (10 μ M for 15 mins). The cPLA₂ α antibody dilution used was 1:50. (C) Detection of cPLA₂ β after treatment with 0.05% ethanol (vehicle). The cPLA₂ β I antibody dilution used was 1:50. (D) cPLA₂ β after treatment with AACOCF₃ (10 μ M for 15 mins). The cPLA₂ β I antibody dilution used was 1:50. (E) Detection of cPLA₂ γ after treatment with 0.05% ethanol (vehicle). The cPLA₂ γ antibody dilution used was 1:40. (F) cPLA₂ γ after treatment with AACOCF₃ (10 μ M for 15 mins). The cPLA₂ γ antibody dilution used was 1:40. Images were taken using 40X oil immersion objective (Results are representative of 2 trials).

Fig. 11: Modulation of cPLA₂ isozymes in MCF-7 cells by AACOCF₃. (A) Detection of cPLA₂ α after treatment with 0.05% ethanol (vehicle). The cPLA₂ α antibody dilution used was 1:50. (B) cPLA₂ α after treatment with AACOCF₃ (10 μ M for 15 mins). The cPLA₂ α antibody dilution used was 1:50. (C) Detection of cPLA₂ β after treatment with 0.05% ethanol (vehicle). The cPLA₂ β antibody dilution used was 1:50. (D) cPLA₂ β after treatment with AACOCF₃ (10 μ M for 15 mins). The cPLA₂ β antibody dilution used was 1:50. (E) Detection of cPLA₂ γ after treatment with 0.05% ethanol (vehicle). The cPLA₂ γ antibody dilution used was 1:40. (F) cPLA₂ γ after treatment with AACOCF₃ (10 μ M for 15 mins). The cPLA₂ γ antibody dilution used was 1:40. Images were taken using 40X oil immersion objective (Results are representative of 2 trials).

cPLA₂α

cPLA₂βI

cPLA₂α+A23187 (40X)

cPLA₂βI+A23187 (40X)

cPLA₂α+A23187 (60X)

cPLA₂βI+A23187 (60X)

Fig. 12: Modulation of cPLA₂ α and cPLA₂ β isozymes by Ca²⁺ ionophore (A23187) in MDA-MB 231 cells. (A) Distribution of cPLA₂ α after treatment with HEPES buffer with 0.05% ethanol (vehicle). The cPLA₂ α antibody dilution used was 1:50. Images were taken using 40X oil immersion objective. (B) Distribution of cPLA₂ α after treatment with A23187 (5 μ M for 5 mins). The cPLA₂ α antibody dilution used was 1:50. Images were taken using 40X oil immersion objective. (C) Distribution of cPLA₂ α after treatment with A23187 (5 μ M for 5 mins). The cPLA₂ α antibody dilution used was 1:50. Images were taken using 60X oil immersion objective. (D) Distribution of cPLA₂ β after treatment with HEPES buffer with 0.05% ethanol (vehicle). The cPLA₂ β I antibody dilution used was 1:50. Images were taken using 40X oil immersion objective. (E) Distribution of cPLA₂ β after treatment with A23187 (5 μ M for 5 mins). The cPLA₂ β I antibody dilution used was 1:50. Images were taken using 40X oil immersion objective. (F) Distribution of cPLA₂ β after treatment with A23187 (5 μ M for 5 mins). The cPLA₂ β I antibody dilution used was 1:50. Images were taken using 60X oil immersion objective. Arrows indicate distribution of cPLA₂ isozyme within the cell after treatment with A23187 (Results are representative of 2 trials).

Fig. 13: Modulation of cPLA₂α and cPLA₂β isozyme distribution by Ca²⁺ ionophore (A23187) in MCF-7 cells. (A) Distribution of cPLA₂α after treatment with HEPES buffer with 0.05% ethanol (vehicle). The cPLA₂α antibody dilution used was 1:50. (B) Distribution of cPLA₂α after treatment with A23187 (5 μM for 5 mins). The cPLA₂α antibody dilution used was 1:50. (C) Distribution of cPLA₂β after treatment with HEPES buffer with 0.05% ethanol (vehicle). The cPLA₂βI antibody dilution used was 1:50. (D) Distribution of cPLA₂β after treatment with A23187 (5 μM for 5 mins). The cPLA₂βI antibody dilution used was 1:50. Images were taken using 40X oil immersion objective. Arrows indicate distribution of cPLA₂ isozyme within the cell after treatment with A23187 (Results are representative of 2 trials).

Fig. 14: Immunoblotting assays of cPLA₂ isozymes α, β and γ in human breast tumor

sample I: Tumor sample I was diagnosed as an invasive ductal carcinoma. Total protein was prepared as described in Materials and Methods and 20 μg protein was used in each of the above Western blots. In each specimen, tumor preparation was loaded in the left lane and the protein marker in the right lane. (A) Tested against cPLA₂α antibody (1:50). (B) Tested against pre-incubated cPLA₂α antibody (1:50). (C) Tested against cPLA₂βI antibody (1:50). (D) Tested against pre-incubated cPLA₂βI antibody (1:50). (E) Tested against cPLA₂γ antibody (1:40). (F) Tested against pre-incubated cPLA₂γ antibody (1:40). No detection of any cPLA₂ isozyme was evident.

Fig. 15: Immunochemical assays of cPLA₂ isozymes α , β and γ in human breast tumor

sample II: Tumor sample II was diagnosed as an infiltrating ductal carcinoma. Total protein was prepared as described in Materials and Methods and 30 μ g protein was used in each of the above Western blots. In each specimen, tumor preparation was loaded in the left lane and the protein marker in the right lane. (A) Tested against cPLA₂ α antibody (1:50). (B) Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ γ antibody (1:40). (F) Tested against pre-incubated cPLA₂ γ antibody (1:40). No detection of any cPLA₂ isozyme was evident.

Fig. 16: Immunoblotting assays of cPLA₂ isoforms α , β and γ in human breast tumor sample III: Tumor sample III was diagnosed as an infiltrating ductal adenocarcinoma. Total protein was prepared as described in Materials and Methods and 4.8 μ g protein was used in each of the above Western blots. In each specimen, tumor preparation was loaded in the left lane and the protein marker in the right lane. (A) Tested against cPLA₂ α antibody (1:50). (B) Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ γ antibody (1:40). (F) Tested against pre-incubated cPLA₂ γ antibody (1:40). No detection of any cPLA₂ isozyme was evident.

Fig. 17: Immunochemical assays of cPLA₂ isozymes α , β and γ in human breast tumor

sample IV: Tumor sample IV was diagnosed as an invasive ductal carcinoma. Total protein was prepared as described in Materials and Methods and 4 μ g protein was used in each of the above Western blots. In each specimen, tumor preparation was loaded in the left lane and the protein marker in the right lane. (A) Tested against cPLA₂ α antibody (1:50). (B) Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ γ antibody (1:40). (F) Tested against pre-incubated cPLA₂ γ antibody (1:40). No detection of any cPLA₂ isozyme was evident.

Fig. 18: Immunochemical assays of cPLA₂ isozymes α , β and γ protein in human breast tumor sample V: Tumor sample V was diagnosed as an invasive ductal carcinoma. Total protein was prepared as described in Materials and Methods and 8 μ g protein was used in each of the above Western blots. In each specimen, tumor preparation was loaded in the left lane and the protein marker in the right lane. (A) Tested against cPLA₂ α antibody (1:50). (B) Tested against pre-incubated cPLA₂ α antibody (1:50). (C) Tested against cPLA₂ β I antibody (1:50). (D) Tested against pre-incubated cPLA₂ β I antibody (1:50). (E) Tested against cPLA₂ γ antibody (1:40). (F) Tested against pre-incubated cPLA₂ γ antibody (1:40). No detection of any cPLA₂ isozyme was evident.

BIBLIOGRAPHY

- Abe, A., Hiraoka, M., and Shayman, J.A. (2007). A role for lysosomal phospholipase A2 in drug induced phospholipidosis. *Drug Metab Lett* 1, 49-53.
- Adler, D.H., Cogan, J.D., Phillips, J.A., 3rd, Schnetz-Boutaud, N., Milne, G.L., Iverson, T., Stein, J.A., Brenner, D.A., Morrow, J.D., Boutaud, O., *et al.* (2008). Inherited human cPLA(2alpha) deficiency is associated with impaired eicosanoid biosynthesis, small intestinal ulceration, and platelet dysfunction. *J Clin Invest* 118, 2121-2131.
- Andela, V.B., Schwarz, E.M., Puzas, J.E., O'Keefe, R.J., and Rosier, R.N. (2000). Tumor metastasis and the reciprocal regulation of prometastatic and antimetastatic factors by nuclear factor kappaB. *Cancer research* 60, 6557-6562.
- Asai, K., Hirabayashi, T., Houjou, T., Uozumi, N., Taguchi, R., and Shimizu, T. (2003). Human group IVC phospholipase A2 (cPLA2gamma). Roles in the membrane remodeling and activation induced by oxidative stress. *The Journal of biological chemistry* 278, 8809-8814.
- Balkwill, F. (2004). Cancer and the chemokine network. *Nature reviews* 4, 540-550.
- Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *Lancet* 357, 539-545.
- Balsinde, J., Balboa, M.A., and Dennis, E.A. (1998). Functional coupling between secretory phospholipase A2 and cyclooxygenase-2 and its regulation by cytosolic group IV phospholipase A2. *Proceedings of the National Academy of Sciences of the United States of America* 95, 7951-7956.
- Balsinde, J., Balboa, M.A., Insel, P.A., and Dennis, E.A. (1999). Regulation and inhibition of phospholipase A2. *Annual review of pharmacology and toxicology* 39, 175-189.
- Barry, M., Cahill, R.A., Roche-Nagle, G., Neilan, T.G., Treumann, A., Harmey, J.H., and Bouchier-Hayes, D.J. (2009). Neoplasms escape selective COX-2 inhibition in an animal model of breast cancer. *Irish journal of medical science* 178, 201-208.
- Bennett, A., Charlier, E.M., McDonald, A.M., Simpson, J.S., Stamford, I.F., and Zebro, T. (1977). Prostaglandins and breast cancer. *Lancet* 2, 624-626.
- Bidgood, M.J., Jamal, O.S., Cunningham, A.M., Brooks, P.M., and Scott, K.F. (2000). Type IIA secretory phospholipase A2 up-regulates cyclooxygenase-2 and amplifies cytokine-

mediated prostaglandin production in human rheumatoid synoviocytes. *J Immunol* 165, 2790-2797.

Bierie, B., and Moses, H.L. (2006a). TGF-beta and cancer. *Cytokine & growth factor reviews* 17, 29-40.

Bierie, B., and Moses, H.L. (2006b). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nature reviews* 6, 506-520.

Bishop, J.M., and Weinberg, R.A. (1996). *Molecular Oncology*. New York: Scientific American, Inc.

Bond, M., Baker, A.H., and Newby, A.C. (1999). Nuclear factor kappaB activity is essential for matrix metalloproteinase-1 and -3 upregulation in rabbit dermal fibroblasts. *Biochemical and biophysical research communications* 264, 561-567.

Bonventre, J.V., Huang, Z., Taheri, M.R., O'Leary, E., Li, E., Moskowitz, M.A., and Sapirstein, A. (1997). Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 390, 622-625.

Bonventre, J.V., and Sapirstein, A. (2002). Group IV cytosolic phospholipase A2 (PLA2) function: insights from the knockout mouse. *Adv Exp Med Biol* 507, 25-31.

Bos, J.L. (1989). ras oncogenes in human cancer: a review. *Cancer research* 49, 4682-4689.

Brash, A.R. (1999). Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *The Journal of biological chemistry* 274, 23679-23682.

Brash, A.R. (2001). Arachidonic acid as a bioactive molecule. *J Clin Invest* 107, 1339-1345.

Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C., and Darnell, J.E., Jr. (1999). Stat3 as an oncogene. *Cell* 98, 295-303.

Bussolati, B., Biancone, L., Cassoni, P., Russo, S., Rola-Pleszczynski, M., Montrucchio, G., and Camussi, G. (2000). PAF produced by human breast cancer cells promotes migration and proliferation of tumor cells and neo-angiogenesis. *Am J Pathol* 157, 1713-1725.

Caiazza, F., Harvey, B.J., and Thomas, W. (2010). Cytosolic Phospholipase A2 Activation Correlates with HER2 Overexpression and Mediates Estrogen-Dependent Breast Cancer Cell Growth. *Mol Endocrinol*.

Cailleau, R., Olive, M., and Cruciger, Q.V. (1978). Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro* 14, 911-915.

Capper, E.A., and Marshall, L.A. (2001). Mammalian phospholipases A(2): mediators of inflammation, proliferation and apoptosis. *Progress in lipid research* 40, 167-197.

Channon, J.Y., and Leslie, C.C. (1990). A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A2 with membrane in the macrophage cell line RAW 264.7. *The Journal of biological chemistry* 265, 5409-5413.

Chen, C.R., Kang, Y., and Massague, J. (2001). Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proceedings of the National Academy of Sciences of the United States of America* 98, 992-999.

Chen, F.L., Wang, X.Z., Li, J.Y., Yu, J.P., Huang, C.Y., and Chen, Z.X. (2008a). 12-lipoxygenase induces apoptosis of human gastric cancer AGS cells via the ERK1/2 signal pathway. *Dig Dis Sci* 53, 181-187.

Chen, J.K., Chen, J., Imig, J.D., Wei, S., Hachey, D.L., Guthi, J.S., Falck, J.R., Capdevila, J.H., and Harris, R.C. (2008b). Identification of novel endogenous cytochrome p450 arachidonate metabolites with high affinity for cannabinoid receptors. *The Journal of biological chemistry*.

Chen, X., Li, N., Wang, S., Wu, N., Hong, J., Jiao, X., Krasna, M.J., Beer, D.G., and Yang, C.S. (2003). Leukotriene A4 hydrolase in rat and human esophageal adenocarcinomas and inhibitory effects of bestatin. *Journal of the National Cancer Institute* 95, 1053-1061.

Chen, X., Wang, S., Wu, N., Sood, S., Wang, P., Jin, Z., Beer, D.G., Giordano, T.J., Lin, Y., Shih, W.C., *et al.* (2004). Overexpression of 5-lipoxygenase in rat and human esophageal adenocarcinoma and inhibitory effects of zileuton and celecoxib on carcinogenesis. *Clin Cancer Res* 10, 6703-6709.

Choy, H., and Milas, L. (2003). Enhancing radiotherapy with cyclooxygenase-2 enzyme inhibitors: a rational advance? *Journal of the National Cancer Institute* 95, 1440-1452.

Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N., and Knopf, J.L. (1991). A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell* 65, 1043-1051.

Clark, J.D., Schievella, A.R., Nalefski, E.A., and Lin, L.L. (1995). Cytosolic phospholipase A2. *Journal of lipid mediators and cell signalling* 12, 83-117.

Clissold, P.M., and Ponting, C.P. (2001). JmjC: cupin metalloenzyme-like domains in jumonji, hairless and phospholipase A2beta. *Trends in biochemical sciences* 26, 7-9.

Courtois, G., and Gilmore, T.D. (2006). Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene* 25, 6831-6843.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. *Nature* 420, 860-867.

Darnell, J.E., Jr. (2002). Transcription factors as targets for cancer therapy. *Nature reviews* 2, 740-749.

Denkert, C., Winzer, K.J., and Hauptmann, S. (2004). Prognostic impact of cyclooxygenase-2 in breast cancer. *Clin Breast Cancer* 4, 428-433.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nature genetics* 29, 117-129.

Ding, X.Z., Hennig, R., and Adrian, T.E. (2003). Lipoxygenase and cyclooxygenase metabolism: new insights in treatment and chemoprevention of pancreatic cancer. *Mol Cancer* 2, 10.

Dohadwala, M., Batra, R.K., Luo, J., Lin, Y., Krysan, K., Pold, M., Sharma, S., and Dubinett, S.M. (2002). Autocrine/paracrine prostaglandin E2 production by non-small cell lung cancer cells regulates matrix metalloproteinase-2 and CD44 in cyclooxygenase-2-dependent invasion. *The Journal of biological chemistry* 277, 50828-50833.

Dong, B., and Matsumura, F. (2008). Roles of cytosolic phospholipase A2 and Src kinase in the early action of 2,3,7,8-tetrachlorodibenzo-p-dioxin through a nongenomic pathway in MCF10A cells. *Mol Pharmacol* 74, 255-263.

Dong, M., Guda, K., Nambiar, P.R., Rezaie, A., Belinsky, G.S., Lambeau, G., Giardina, C., and Rosenberg, D.W. (2003). Inverse association between phospholipase A2 and COX-2 expression during mouse colon tumorigenesis. *Carcinogenesis* 24, 307-315.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nature reviews* 3, 11-22.

Dreesen, O., and Brivanlou, A.H. (2007). Signaling pathways in cancer and embryonic stem cells. *Stem cell reviews* 3, 7-17.

Duan, L., Gan, H., Arm, J., and Remold, H.G. (2001). Cytosolic phospholipase A2 participates with TNF- α in the induction of apoptosis of human macrophages infected with *Mycobacterium tuberculosis* H37Ra. *J Immunol* 166, 7469-7476.

DuBois, R.N. (2003). Leukotriene A4 signaling, inflammation, and cancer. *Journal of the National Cancer Institute* 95, 1028-1029.

Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England journal of medicine* 315, 1650-1659.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S., and DuBois, R.N. (1994). Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107, 1183-1188.

Ehebauer, M., Hayward, P., and Arias, A.M. (2006). Notch, a universal arbiter of cell fate decisions. *Science (New York, NY)* 314, 1414-1415.

Endsley, M.P., Aggarwal, N., Isbell, M.A., Wheelock, C.E., Hammock, B.D., Falck, J.R., Campbell, W.B., and Nithipatikom, K. (2007). Diverse roles of 2-arachidonoylglycerol in invasion of prostate carcinoma cells: Location, hydrolysis and 12-lipoxygenase metabolism. *International journal of cancer* 121, 984-991.

Ernst, P.B., and Gold, B.D. (2000). The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annual review of microbiology* 54, 615-640.

Fabbro, D., Kung, W., Roos, W., Regazzi, R., and Eppenberger, U. (1986). Epidermal growth factor binding and protein kinase C activities in human breast cancer cell lines: possible quantitative relationship. *Cancer research* 46, 2720-2725.

Fatima, S. (2002). CAM kinase IIa mediates norepinephrine-induced translocation of cytosolic phospholipase A2 to the nuclear envelope. *Journal of cell science* 116, 353-365.

Fedi, P., Tronick, S.R., and Aaronson, S.A. (1997). Growth factors. In *Cancer Medicine*, J.F. Holland, R.C. Bast, D.L. Morton, E. Frei, D.W. Kufe, and R.R. Weichselbaum, eds. Baltimore, MD: Williams and Wilkins, 41-64.

Fegn, L., and Wang, Z. (2009). Topical chemoprevention of skin cancer in mice, using combined inhibitors of 5-lipoxygenase and cyclo-oxygenase-2. *The Journal of laryngology and otology* 123, 880-884.

Ferno, M., Borg, A., Johansson, U., Norgren, A., Olsson, H., Ryden, S., and Sellberg, G. (1990). Estrogen and progesterone receptor analyses in more than 4,000 human breast cancer samples. A study with special reference to age at diagnosis and stability of analyses. Southern Swedish Breast Cancer Study Group. *Acta Oncol* 29, 129-135.

Fiancette, R., Vincent, C., Donnard, M., Bordessoule, D., Turlure, P., Trimoreau, F., and Denizot, Y. (2009). Genes encoding multiple forms of phospholipase A(2) are expressed in immature forms of human leukemic blasts. *Leukemia* 23, 1196-1199.

Fitzpatrick, F.A., and Soberman, R. (2001). Regulated formation of eicosanoids. *J Clin Invest* 107, 1347-1351.

Forman, H.J., and Torres, M. (2001). Redox signaling in macrophages. *Molecular aspects of medicine* 22, 189-216.

Fujishima, H., Sanchez Mejia, R.O., Bingham, C.O., 3rd, Lam, B.K., Sapirstein, A., Bonventre, J.V., Austen, K.F., and Arm, J.P. (1999). Cytosolic phospholipase A2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. *Proceedings of the National Academy of Sciences of the United States of America* 96, 4803-4807.

Funk, C.D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* (New York, NY 294, 1871-1875.

Gao, X., Grignon, D.J., Chbihi, T., Zacharek, A., Chen, Y.Q., Sakr, W., Porter, A.T., Crissman, J.D., Pontes, J.E., Powell, I.J., *et al.* (1995). Elevated 12-lipoxygenase mRNA expression correlates with advanced stage and poor differentiation of human prostate cancer. *Urology* 46, 227-237.

Ghosh, M., Loper, R., Gelb, M.H., and Leslie, C.C. (2006a). Identification of the expressed form of human cytosolic phospholipase A2beta (cPLA2beta): cPLA2beta3 is a novel variant localized to mitochondria and early endosomes. *The Journal of biological chemistry* 281, 16615-16624.

Ghosh, M., Tucker, D.E., Burchett, S.A., and Leslie, C.C. (2006b). Properties of the Group IV phospholipase A2 family. *Progress in lipid research* 45, 487-510.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual review of immunology* 16, 225-260.

Gijon, M.A., Spencer, D.M., Kaiser, A.L., and Leslie, C.C. (1999). Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A2. *The Journal of cell biology* *145*, 1219-1232.

Greenhough, A., Smartt, H.J., Moore, A.E., Roberts, H.R., Williams, A.C., Paraskeva, C., and Kaidi, A. (2009). The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* *30*, 377-386.

Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* *118*, 285-296.

Greten, F.R., and Karin, M. (2004). The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. *Cancer Lett* *206*, 193-199.

Grewal, S., Morrison, E.E., Ponnambalam, S., and Walker, J.H. (2002). Nuclear localisation of cytosolic phospholipase A2-alpha in the EA.hy.926 human endothelial cell line is proliferation dependent and modulated by phosphorylation. *Journal of cell science* *115*, 4533-4543.

Grewal, S., Ponnambalam, S., and Walker, J.H. (2003). Association of cPLA2-alpha and COX-1 with the Golgi apparatus of A549 human lung epithelial cells. *Journal of cell science* *116*, 2303-2310.

Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, inflammation, and cancer. *Cell* *140*, 883-899.

Grossman, E.M., Longo, W.E., Panesar, N., Mazuski, J.E., and Kaminski, D.L. (2000). The role of cyclooxygenase enzymes in the growth of human gall bladder cancer cells. *Carcinogenesis* *21*, 1403-1409.

Guerra, C., Schuhmacher, A.J., Canamero, M., Grippo, P.J., Verdaguer, L., Perez-Gallego, L., Dubus, P., Sandgren, E.P., and Barbacid, M. (2007). Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer cell* *11*, 291-302.

Hall, R.E., Lee, C.S., Alexander, I.E., Shine, J., Clarke, C.L., and Sutherland, R.L. (1990). Steroid hormone receptor gene expression in human breast cancer cells: inverse relationship between oestrogen and glucocorticoid receptor messenger RNA levels. *International journal of cancer* *46*, 1081-1087.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* *100*, 57-70.

Hartmann, C., Johnk, L., Sasaki, H., Jenkins, R.B., and Louis, D.N. (2002). Novel PLA2G4C polymorphism as a molecular diagnostic assay for 19q loss in human gliomas. *Brain Pathol* 12, 178-182.

Hayakawa, M., Ishida, N., Takeuchi, K., Shibamoto, S., Hori, T., Oku, N., Ito, F., and Tsujimoto, M. (1993). Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor. *The Journal of biological chemistry* 268, 11290-11295.

Heasley, L.E., Thaler, S., Nicks, M., Price, B., Skorecki, K., and Nemenoff, R.A. (1997). Induction of cytosolic phospholipase A2 by oncogenic Ras in human non-small cell lung cancer. *The Journal of biological chemistry* 272, 14501-14504.

Hefner, Y., Borsch-Haubold, A.G., Murakami, M., Wilde, J.I., Pasquet, S., Schieltz, D., Ghomashchi, F., Yates, J.R., 3rd, Armstrong, C.G., Paterson, A., *et al.* (2000). Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. *The Journal of biological chemistry* 275, 37542-37551.

Higashi, Y., Kanekura, T., and Kanzaki, T. (2000). Enhanced expression of cyclooxygenase (COX)-2 in human skin epidermal cancer cells: evidence for growth suppression by inhibiting COX-2 expression. *International journal of cancer* 86, 667-671.

Hirabayashi, T., and Shimizu, T. (2000). Localization and regulation of cytosolic phospholipase A(2). *Biochimica et biophysica acta* 1488, 124-138.

Hiraoka, M., Abe, A., and Shayman, J.A. (2002). Cloning and characterization of a lysosomal phospholipase A2, 1-O-acylceramide synthase. *The Journal of biological chemistry* 277, 10090-10099.

Hoeck, W.G., Ramesha, C.S., Chang, D.J., Fan, N., and Heller, R.A. (1993). Cytoplasmic phospholipase A2 activity and gene expression are stimulated by tumor necrosis factor: dexamethasone blocks the induced synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 90, 4475-4479.

Hong, K.H., Bonventre, J.C., O'Leary, E., Bonventre, J.V., and Lander, E.S. (2001). Deletion of cytosolic phospholipase A(2) suppresses Apc(Min)-induced tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 98, 3935-3939.

Honn, K.V., Timar, J., Rozhin, J., Bazaz, R., Sameni, M., Ziegler, G., and Sloane, B.F. (1994). A lipoxygenase metabolite, 12-(S)-HETE, stimulates protein kinase C-mediated release of cathepsin B from malignant cells. *Exp Cell Res* 214, 120-130.

Hulkower, K.I., Hope, W.C., Chen, T., Anderson, C.M., Coffey, J.W., and Morgan, D.W. (1992). Interleukin-1 beta stimulates cytosolic phospholipase A2 in rheumatoid synovial fibroblasts. *Biochemical and biophysical research communications* 184, 712-718.

Irvine, R.F. (1982). How is the level of free arachidonic acid controlled in mammalian cells? *The Biochemical journal* 204, 3-16.

Izaki, S., Yamamoto, T., Goto, Y., Ishimaru, S., Yodate, F., Kitamura, K., and Matsuzaki, M. (1996). Platelet-activating factor and arachidonic acid metabolites in psoriatic inflammation. *Br J Dermatol* 134, 1060-1064.

Jimenez, R.E., Wallis, T., Tabasczka, P., and Visscher, D.W. (2000). Determination of Her-2/Neu status in breast carcinoma: comparative analysis of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol* 13, 37-45.

Johnson, J.P. (1999). Cell adhesion molecules in the development and progression of malignant melanoma. *Cancer metastasis reviews* 18, 345-357.

Jurivich, D.A., Pangas, S., Qiu, L., and Welk, J.F. (1996). Phospholipase A2 triggers the first phase of the thermal stress response and exhibits cell-type specificity. *J Immunol* 157, 1669-1677.

Kallioniemi, O.P., Holli, K., Visakorpi, T., Koivula, T., Helin, H.H., and Isola, J.J. (1991). Association of c-erbB-2 protein over-expression with high rate of cell proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer. *International journal of cancer* 49, 650-655.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nature reviews* 2, 301-310.

Karin, M., Lawrence, T., and Nizet, V. (2006). Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 124, 823-835.

Katz, M., Amit, I., and Yarden, Y. (2007). Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochimica et biophysica acta* 1773, 1161-1176.

Kawamori, T., Uchiya, N., Nakatsugi, S., Watanabe, K., Ohuchida, S., Yamamoto, H., Maruyama, T., Kondo, K., Sugimura, T., and Wakabayashi, K. (2001). Chemopreventive effects of ONO-8711, a selective prostaglandin E receptor EP(1) antagonist, on breast cancer development. *Carcinogenesis* 22, 2001-2004.

Kim, D.K., Kudo, I., and Inoue, K. (1991). Purification and characterization of rabbit platelet cytosolic phospholipase A2. *Biochimica et biophysica acta* 1083, 80-88.

Kim, S., Takahashi, H., Lin, W.W., Descargues, P., Grivennikov, S., Kim, Y., Luo, J.L., and Karin, M. (2009). Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature* 457, 102-106.

Kim, S.W., Rhee, H.J., Ko, J., Kim, Y.J., Kim, H.G., Yang, J.M., Choi, E.C., and Na, D.S. (2001). Inhibition of cytosolic phospholipase A2 by annexin I. Specific interaction model and mapping of the interaction site. *The Journal of biological chemistry* 276, 15712-15719.

Kudo, I., and Murakami, M. (2002). Phospholipase A2 enzymes. Prostaglandins & other lipid mediators 68-69, 3-58.

Kuper, H., Adami, H.O., and Trichopoulos, D. (2000). Infections as a major preventable cause of human cancer. *Journal of internal medicine* 248, 171-183.

Larre, S., Tran, N., Fan, C., Hamadeh, H., Champigneulle, J., Azzouzi, R., Cussenot, O., Mangin, P., and Olivier, J.L. (2008). PGE2 and LTB4 tissue levels in benign and cancerous prostates. *Prostaglandins & other lipid mediators* 87, 14-19.

Leek, R.D., Landers, R., Fox, S.B., Ng, F., Harris, A.L., and Lewis, C.E. (1998). Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. *British journal of cancer* 77, 2246-2251.

Leslie, C.C. (2004). Regulation of the specific release of arachidonic acid by cytosolic phospholipase A2. Prostaglandins, leukotrienes, and essential fatty acids 70, 373-376.

Leslie, C.C., Voelker, D.R., Channon, J.Y., Wall, M.M., and Zelarney, P.T. (1988). Properties and purification of an arachidonoyl-hydrolyzing phospholipase A2 from a macrophage cell line, RAW 264.7. *Biochimica et biophysica acta* 963, 476-492.

Li, X., Massa, P.E., Hanidu, A., Peet, G.W., Aro, P., Savitt, A., Mische, S., Li, J., and Marcu, K.B. (2002). IKKalpha, IKKbeta, and NEMO/IKKgamma are each required for the NF-kappa B-mediated inflammatory response program. *The Journal of biological chemistry* 277, 45129-45140.

Lin, L.L., Lin, A.Y., and DeWitt, D.L. (1992). Interleukin-1 alpha induces the accumulation of cytosolic phospholipase A2 and the release of prostaglandin E2 in human fibroblasts. *The Journal of biological chemistry* 267, 23451-23454.

- Liu, B., Timar, J., Howlett, J., Diglio, C.A., and Honn, K.V. (1991). Lipoxygenase metabolites of arachidonic and linoleic acids modulate the adhesion of tumor cells to endothelium via regulation of protein kinase C. *Cell Regul* 2, 1045-1055.
- Liu, C.H., Chang, S.H., Narko, K., Trifan, O.C., Wu, M.T., Smith, E., Haudenschild, C., Lane, T.F., and Hla, T. (2001). Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *The Journal of biological chemistry* 276, 18563-18569.
- Lupulescu, A. (1978). Enhancement of carcinogenesis by prostaglandins. *Nature* 272, 634-636.
- Maeda, H., and Akaike, T. (1998). Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry* 63, 854-865.
- Mancuso, D.J., Abendschein, D.R., Jenkins, C.M., Han, X., Saffitz, J.E., Schuessler, R.B., and Gross, R.W. (2003). Cardiac ischemia activates calcium-independent phospholipase A2beta, precipitating ventricular tachyarrhythmias in transgenic mice: rescue of the lethal electrophysiologic phenotype by mechanism-based inhibition. *The Journal of biological chemistry* 278, 22231-22236.
- Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. *Nature* 454, 436-444.
- Mantovani, A., Bussolino, F., and Dejana, E. (1992). Cytokine regulation of endothelial cell function. *Faseb J* 6, 2591-2599.
- Markaverich, B.M., Crowley, J., Rodriguez, M., Shoulars, K., and Thompson, T. (2007). Tetrahydrofuran diol stimulation of phospholipase A2, lipoxygenase, and cyclooxygenase gene expression and MCF-7 human breast cancer cell proliferation. *Environ Health Perspect* 115, 1727-1731.
- Massague, J. (1998). TGF-beta signal transduction. *Annual review of biochemistry* 67, 753-791.
- Massague, J. (2008). TGFbeta in Cancer. *Cell* 134, 215-230.
- Matsunaga, K., Yoshimi, N., Yamada, Y., Shimizu, M., Kawabata, K., Ozawa, Y., Hara, A., and Mori, H. (1998). Inhibitory effects of nabumetone, a cyclooxygenase-2 inhibitor, and esculetin, a lipoxygenase inhibitor, on N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats. *Jpn J Cancer Res* 89, 496-501.

- Maxwell, A.P., Goldberg, H.J., Tay, A.H., Li, Z.G., Arbus, G.S., and Skorecki, K.L. (1993). Epidermal growth factor and phorbol myristate acetate increase expression of the mRNA for cytosolic phospholipase A2 in glomerular mesangial cells. *The Biochemical journal* 295 (Pt 3), 763-766.
- Meyer, M.C., Rastogi, P., Beckett, C.S., and McHowat, J. (2005). Phospholipase A2 inhibitors as potential anti-inflammatory agents. *Current pharmaceutical design* 11, 1301-1312.
- Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., *et al.* (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* (New York, NY 266, 66-71.
- Miyashita, A., Crystal, R.G., and Hay, J.G. (1995). Identification of a 27 bp 5'-flanking region element responsible for the low level constitutive expression of the human cytosolic phospholipase A2 gene. *Nucleic acids research* 23, 293-301.
- Moody, T.W., Leyton, J., Martinez, A., Hong, S., Malkinson, A., and Mulshine, J.L. (1998). Lipoxigenase inhibitors prevent lung carcinogenesis and inhibit non-small cell lung cancer growth. *Experimental lung research* 24, 617-628.
- Mounier, C.M., Wendum, D., Greenspan, E., Flejou, J.F., Rosenberg, D.W., and Lambeau, G. (2008). Distinct expression pattern of the full set of secreted phospholipases A2 in human colorectal adenocarcinomas: sPLA2-III as a biomarker candidate. *British journal of cancer* 98, 587-595.
- Muller-Decker, K., and Furstenberger, G. (2007). The cyclooxygenase-2-mediated prostaglandin signaling is causally related to epithelial carcinogenesis. *Mol Carcinog* 46, 705-710.
- Murakami, M., Kambe, T., Shimbara, S., and Kudo, I. (1999). Functional coupling between various phospholipase A2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *The Journal of biological chemistry* 274, 3103-3115.
- Murakami, M., and Kudo, I. (2002). Phospholipase A2. *J Biochem* 131, 285-292.
- Murakami, M., and Kudo, I. (2004). Recent advances in molecular biology and physiology of the prostaglandin E2-biosynthetic pathway. *Progress in lipid research* 43, 3-35.
- Murakami, M., Masuda, S., and Kudo, I. (2003). Arachidonate release and prostaglandin production by group IVC phospholipase A2 (cytosolic phospholipase A2gamma). *The Biochemical journal* 372, 695-702.

- Muthalif, M.M., Hefner, Y., Canaan, S., Harper, J., Zhou, H., Parmentier, J.H., Aebersold, R., Gelb, M.H., and Malik, K.U. (2001). Functional interaction of calcium-/calmodulin-dependent protein kinase II and cytosolic phospholipase A(2). *The Journal of biological chemistry* 276, 39653-39660.
- Nagase, T., Uozumi, N., Ishii, S., Kume, K., Izumi, T., Ouchi, Y., and Shimizu, T. (2000). Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A2. *Nature immunology* 1, 42-46.
- Nakanishi, M., and Rosenberg, D.W. (2006). Roles of cPLA2alpha and arachidonic acid in cancer. *Biochimica et biophysica acta* 1761, 1335-1343.
- Nakatani, Y., Tanioka, T., Sunaga, S., Murakami, M., and Kudo, I. (2000). Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase A(2)alpha. *The Journal of biological chemistry* 275, 1161-1168.
- Nalefski, E.A., McDonagh, T., Somers, W., Seehra, J., Falke, J.J., and Clark, J.D. (1998). Independent folding and ligand specificity of the C2 calcium-dependent lipid binding domain of cytosolic phospholipase A2. *The Journal of biological chemistry* 273, 1365-1372.
- Natarajan, R., Esworthy, R., Bai, W., Gu, J.L., Wilczynski, S., and Nadler, J. (1997). Increased 12-lipoxygenase expression in breast cancer tissues and cells. Regulation by epidermal growth factor. *J Clin Endocrinol Metab* 82, 1790-1798.
- Natarajan, R., and Nadler, J. (1998). Role of lipoxygenases in breast cancer. *Front Biosci* 3, E81-88.
- Nie, D., Che, M., Grignon, D., Tang, K., and Honn, K.V. (2001). Role of eicosanoids in prostate cancer progression. In *Cancer metastasis reviews*, pp. 195-206.
- Ninio, E., Mencia-Huerta, J.M., and Benveniste, J. (1983). Biosynthesis of platelet-activating factor (PAF-acether). V. Enhancement of acetyltransferase activity in murine peritoneal cells by calcium ionophore A23187. *Biochimica et biophysica acta* 751, 298-304.
- Obeid, L.M., and Hannun, Y.A. (1995). Ceramide: a stress signal and mediator of growth suppression and apoptosis. *Journal of cellular biochemistry* 58, 191-198.
- Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. (1996). TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes & development* 10, 2462-2477.

Pan, M.R., Hou, M.F., Chang, H.C., and Hung, W.C. (2008). Cyclooxygenase-2 up-regulates CCR7 via EP2/EP4 receptor signaling pathways to enhance lymphatic invasion of breast cancer cells. *The Journal of biological chemistry* 283, 11155-11163.

Park, J.M., Kanaoka, Y., Eguchi, N., Aritake, K., Grujic, S., Materi, A.M., Buslon, V.S., Tippin, B.L., Kwong, A.M., Salido, E., *et al.* (2007). Hematopoietic prostaglandin D synthase suppresses intestinal adenomas in ApcMin/+ mice. *Cancer research* 67, 881-889.

Patel, M.I., Singh, J., Niknami, M., Kurek, C., Yao, M., Lu, S., Maclean, F., King, N.J., Gelb, M.H., Scott, K.F., *et al.* (2008). Cytosolic phospholipase A2-alpha: a potential therapeutic target for prostate cancer. *Clin Cancer Res* 14, 8070-8079.

Peplow, P.V. (1999). Regulation of platelet-activating factor (PAF) activity in human diseases by phospholipase A2 inhibitors, PAF acetylhydrolases, PAF receptor antagonists and free radical scavengers. *Prostaglandins, leukotrienes, and essential fatty acids* 61, 65-82.

Phillips, R.J., Mestas, J., Gharaee-Kermani, M., Burdick, M.D., Sica, A., Belperio, J.A., Keane, M.P., and Strieter, R.M. (2005). Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *The Journal of biological chemistry* 280, 22473-22481.

Pickard, R.T., Striffler, B.A., Kramer, R.M., and Sharp, J.D. (1999). Molecular cloning of two new human paralogs of 85-kDa cytosolic phospholipase A2. *The Journal of biological chemistry* 274, 8823-8831.

Pierceall, W.E., Woodard, A.S., Morrow, J.S., Rimm, D., and Fearon, E.R. (1995). Frequent alterations in E-cadherin and alpha- and beta-catenin expression in human breast cancer cell lines. *Oncogene* 11, 1319-1326.

Pikarsky, E., Porat, R.M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Gutkovich-Pyest, E., Urieli-Shoval, S., Galun, E., and Ben-Neriah, Y. (2004). NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 431, 461-466.

Rawlings, J.S., Rosler, K.M., and Harrison, D.A. (2004). The JAK/STAT signaling pathway. *Journal of cell science* 117, 1281-1283.

Riendeau, D., Guay, J., Weech, P.K., Laliberte, F., Yergey, J., Li, C., Desmarais, S., Perrier, H., Liu, S., Nicoll-Griffith, D., *et al.* (1994). Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase A2, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. *The Journal of biological chemistry* 269, 15619-15624.

Rioux, N., and Castonguay, A. (1998). Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents. *Carcinogenesis* 19, 1393-1400.

Ristimaki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joensuu, H., and Isola, J. (2002). Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer research* 62, 632-635.

Rolland, P.H., Martin, P.M., Jacquemier, J., Rolland, A.M., and Toga, M. (1980). Prostaglandin in human breast cancer: Evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. *Journal of the National Cancer Institute* 64, 1061-1070.

Roy, M., Pear, W.S., and Aster, J.C. (2007). The multifaceted role of Notch in cancer. *Current opinion in genetics & development* 17, 52-59.

Sapirstein, A., and Bonventre, J.V. (2000). Phospholipases A2 in ischemic and toxic brain injury. *Neurochem Res* 25, 745-753.

Schaloske, R.H., and Dennis, E.A. (2006). The phospholipase A2 superfamily and its group numbering system. *Biochimica et biophysica acta* 1761, 1246-1259.

Schievella, A.R., Regier, M.K., Smith, W.L., and Lin, L.L. (1995). Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *The Journal of biological chemistry* 270, 30749-30754.

Schreinemachers, D.M., and Everson, R.B. (1994). Aspirin use and lung, colon, and breast cancer incidence in a prospective study. *Epidemiology* 5, 138-146.

Schrey, M.P., and Patel, K.V. (1995). Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. *British journal of cancer* 72, 1412-1419.

Serhan, C.N. (2005). Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. Prostaglandins, leukotrienes, and essential fatty acids 73, 141-162.

Shao, J., Lee, S.B., Guo, H., Evers, B.M., and Sheng, H. (2003). Prostaglandin E2 stimulates the growth of colon cancer cells via induction of amphiregulin. *Cancer research* 63, 5218-5223.

Sharp, J.D., White, D.L., Chiou, X.G., Goodson, T., Gamboa, G.C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P.L., Sportsman, J.R., *et al.* (1991). Molecular cloning and expression

of human Ca(2+)-sensitive cytosolic phospholipase A2. *The Journal of biological chemistry* 266, 14850-14853.

Sheridan, A.M., Force, T., Yoon, H.J., O'Leary, E., Choukroun, G., Taheri, M.R., and Bonventre, J.V. (2001). PLIP, a novel splice variant of Tip60, interacts with group IV cytosolic phospholipase A(2), induces apoptosis, and potentiates prostaglandin production. *Molecular and cellular biology* 21, 4470-4481.

Siegel, P.M., and Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature reviews* 3, 807-821.

Six, D.A., and Dennis, E.A. (2000). The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. *Biochimica et biophysica acta* 1488, 1-19.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science (New York, NY)* 235, 177-182.

Smith, W.L. (1992). Prostanoid biosynthesis and mechanisms of action. *The American journal of physiology* 263, F181-191.

Smith, W.L., DeWitt, D.L., and Garavito, R.M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Annual review of biochemistry* 69, 145-182.

Snyder, F. (1995). Platelet-activating factor: the biosynthetic and catabolic enzymes. *The Biochemical journal* 305 (Pt 3), 689-705.

Snyder, F., Lee, T.C., and Blank, M.L. (1992). The role of transacylases in the metabolism of arachidonate and platelet activating factor. *Progress in lipid research* 31, 65-86.

Song, C., Chang, X.J., Bean, K.M., Proia, M.S., Knopf, J.L., and Kriz, R.W. (1999). Molecular characterization of cytosolic phospholipase A2-beta. *The Journal of biological chemistry* 274, 17063-17067.

Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Jr., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., and Brooks, S.C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer research* 50, 6075-6086.

Sparmann, A., and Bar-Sagi, D. (2004). Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer cell* 6, 447-458.

Steele, V.E., Holmes, C.A., Hawk, E.T., Kopelovich, L., Lubet, R.A., Crowell, J.A., Sigman, C.C., and Kelloff, G.J. (1999). Lipoxygenase inhibitors as potential cancer chemopreventives. *Cancer Epidemiol Biomarkers Prev* 8, 467-483.

Stewart, A., Ghosh, M., Spencer, D.M., and Leslie, C.C. (2002). Enzymatic properties of human cytosolic phospholipase A(2)gamma. *The Journal of biological chemistry* 277, 29526-29536.

Sumimoto, H., Imabayashi, F., Iwata, T., and Kawakami, Y. (2006). The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *The Journal of experimental medicine* 203, 1651-1656.

Sun, G.Y., Xu, J., Jensen, M.D., and Simonyi, A. (2004). Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J Lipid Res* 45, 205-213.

Sved, P., Scott, K.F., McLeod, D., King, N.J., Singh, J., Tsatralis, T., Nikolov, B., Boulas, J., Nallan, L., Gelb, M.H., *et al.* (2004). Oncogenic action of secreted phospholipase A2 in prostate cancer. *Cancer research* 64, 6934-6940.

Sveinbjornsson, B., Rasmuson, A., Baryawno, N., Wan, M., Pettersen, I., Ponthan, F., Orrego, A., Haeggstrom, J.Z., Johnsen, J.I., and Kogner, P. (2008). Expression of enzymes and receptors of the leukotriene pathway in human neuroblastoma promotes tumor survival and provides a target for therapy. *Faseb J*.

Tergaonkar, V., Pando, M., Vafa, O., Wahl, G., and Verma, I. (2002). p53 stabilization is decreased upon NFkappaB activation: a role for NFkappaB in acquisition of resistance to chemotherapy. *Cancer cell* 1, 493-503.

Thomas W, C.N., Faherty S, Flatharta CO, Harvey BJ. (2006). Estrogen induces phospholipase A2 activation through ERK1/2 to mobilize intracellular calcium in MCF-7 cells. *Steroids* 71, 256-265.

Thompson, E.W., Paik, S., Brunner, N., Sommers, C.L., Zugmaier, G., Clarke, R., Shima, T.B., Torri, J., Donahue, S., Lippman, M.E., *et al.* (1992). Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J Cell Physiol* 150, 534-544.

Tucker, D.E., Stewart, A., Nallan, L., Bendale, P., Ghomashchi, F., Gelb, M.H., and Leslie, C.C. (2005). Group IVC cytosolic phospholipase A2gamma is farnesylated and palmitoylated in mammalian cells. *J Lipid Res* 46, 2122-2133.

Underwood, K.W., Song, C., Kriz, R.W., Chang, X.J., Knopf, J.L., and Lin, L.L. (1998). A novel calcium-independent phospholipase A2, cPLA2-gamma, that is prenylated and contains homology to cPLA2. *The Journal of biological chemistry* 273, 21926-21932.

Venkitaraman, A.R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108, 171-182.

Vihinen, P., and Kahari, V.M. (2002). Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *International journal of cancer* 99, 157-166.

Vincent, C., Fiancette, R., Donnard, M., Bordessoule, D., Turlure, P., Trimoreau, F., and Denizot, Y. (2008). 5-LOX, 12-LOX and 15-LOX in immature forms of human leukemic blasts. *Leukemia research* 32, 1756-1762.

Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nature reviews* 2, 489-501.

Voelkel-Johnson, C., Thorne, T.E., and Laster, S.M. (1996). Susceptibility to TNF in the presence of inhibitors of transcription or translation is dependent on the activity of cytosolic phospholipase A2 in human melanoma tumor cells. *J Immunol* 156, 201-207.

Wahl, L.M., and Kleinman, H.K. (1998). Tumor-associated macrophages as targets for cancer therapy. *Journal of the National Cancer Institute* 90, 1583-1584.

Wang, D., and Dubois, R.N. (2010). Eicosanoids and cancer. *Nature reviews* 10, 181-193.

Wang, D., Wang, H., Shi, Q., Katkuri, S., Walhi, W., Desvergne, B., Das, S.K., Dey, S.K., and DuBois, R.N. (2004). Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer cell* 6, 285-295.

Ward, E., Halpern, M., Schrag, N., Cokkinides, V., DeSantis, C., Bandi, P., Siegel, R., Stewart, A., and Jemal, A. (2008). Association of insurance with cancer care utilization and outcomes. *CA Cancer J Clin* 58, 9-31.

Warnberg, F., Nordgren, H., Bergkvist, L., and Holmberg, L. (2001). Tumour markers in breast carcinoma correlate with grade rather than with invasiveness. *British journal of cancer* 85, 869-874.

Welsh, P.L., and King, M.C. (2001). BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. *Human molecular genetics* 10, 705-713.

Welsh, P.L., Lee, M.K., Gonzalez-Hernandez, R.M., Black, D.J., Mahadevappa, M., Swisher, E.M., Warrington, J.A., and King, M.C. (2002). BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 99, 7560-7565.

Welling, G.W., Weijer, W.J., van der Zee, R., and Welling-Wester, S. (1985). Prediction of sequential antigenic regions in proteins. *FEBS letters* 188, 215-218.

Wendum, D., Comperat, E., Boelle, P.Y., Parc, R., Masliah, J., Trugnan, G., and Flejou, J.F. (2005). Cytoplasmic phospholipase A2 alpha overexpression in stromal cells is correlated with angiogenesis in human colorectal cancer. *Mod Pathol* 18, 212-220.

Wendum, D., Svrcek, M., Rigau, V., Boelle, P.Y., Sebbagh, N., Parc, R., Masliah, J., Trugnan, G., and Flejou, J.F. (2003). COX-2, inflammatory secreted PLA2, and cytoplasmic PLA2 protein expression in small bowel adenocarcinomas compared with colorectal adenocarcinomas. *Mod Pathol* 16, 130-136.

Winer, I., Normolle, D.P., Shureiqi, I., Sondak, V.K., Johnson, T., Su, L., and Brenner, D.E. (2002). Expression of 12-lipoxygenase as a biomarker for melanoma carcinogenesis. *Melanoma Res* 12, 429-434.

Wong, B.C., Wang, W.P., Cho, C.H., Fan, X.M., Lin, M.C., Kung, H.F., and Lam, S.K. (2001). 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells. *Carcinogenesis* 22, 1349-1354.

Woo, C.H., Lee, Z.W., Kim, B.C., Ha, K.S., and Kim, J.H. (2000). Involvement of cytosolic phospholipase A2, and the subsequent release of arachidonic acid, in signalling by rac for the generation of intracellular reactive oxygen species in rat-2 fibroblasts. *The Biochemical journal* 348 Pt 3, 525-530.

Wu, T., Angus, C.W., Yao, X.L., Logun, C., and Shelhamer, J.H. (1997). P11, a unique member of the S100 family of calcium-binding proteins, interacts with and inhibits the activity of the 85-kDa cytosolic phospholipase A2. *The Journal of biological chemistry* 272, 17145-17153.

Wu, T., Han, C., Lunz, J.G., 3rd, Michalopoulos, G., Shelhamer, J.H., and Demetris, A.J. (2002). Involvement of 85-kd cytosolic phospholipase A(2) and cyclooxygenase-2 in the proliferation of human cholangiocarcinoma cells. *Hepatology (Baltimore, Md)* 36, 363-373.

Xu, R., Feiner, H., Li, P., Yee, H., Inghirami, G., Delgado, Y., and Perle, M.A. (2003). Differential amplification and overexpression of HER-2/neu, p53, MIB1, and estrogen receptor/progesterone receptor among medullary carcinoma, atypical medullary carcinoma, and high-grade invasive ductal carcinoma of breast. *Arch Pathol Lab Med* 127, 1458-1464.

Yarden, Y., and Ullrich, A. (1988). EGF and erbB2 receptor overexpression in human tumors. Growth factor receptor tyrosine kinases. *Annu Rev Biochem* 57, 443-478.

Ye, Y.N., Wu, W.K., Shin, V.Y., Bruce, I.C., Wong, B.C., and Cho, C.H. (2005). Dual inhibition of 5-LOX and COX-2 suppresses colon cancer formation promoted by cigarette smoke. *Carcinogenesis* 26, 827-834.