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THE ROLE OF CYCLIN E IN PKC IOTA-DRIVEN EARLY OVARIAN TUMORIGENESIS

Angela Nanos-Webb

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THE ROLE OF CYCLIN E IN PKC IOTA-DRIVEN EARLY OVARIAN
TUMORIGENESIS

by

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THE ROLE OF CYCLIN E IN PKC IOTA-DRIVEN EARLY OVARIAN
TUMORIGENESIS

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Angela Nanos-Webb
Houston, Texas

Date of Graduation: May, 2011

Abstract

Among the gynecologic malignancies, epithelial ovarian tumors are the leading cause of death. For the past few decades, the only treatment has involved surgical resection of the tumor and/or general chemotherapies. In an attempt to improve treatment options, we have shown that several oncogenes that are overexpressed in ovarian cancer, PI3K, PKC α , and cyclin E, all of which have been shown to lead to a poor prognosis and decreased survival, converge into a single pathway that could potentially be targeted therapeutically.

Because of the ability of either PKC α or cyclin E overexpression to independently induce anchorage-independent growth, a hallmark of cancer, we **hypothesized that targeting PKC α expression in ovarian cancer cells could induce a reversion of the transformed phenotype through down regulation of cyclin E**. To test this hypothesis, we first established a correlation between PKC α and cyclin E in a panel of 20 ovarian cancer cell lines. To show that PKC α is upstream of cyclin E, PKC α was stably knocked down using RNAi in IGROV cells (epithelial ovarian cancer cell line of serous histology). The silencing of PKC α resulted in decreased expression of cell cycle drivers, such as cyclin D1/E and CDK2/4, and an increase in p27. These alteration in the regulators of the cell cycle resulted in a decrease in both proliferation and anchorage-independent growth, which was specifically through cyclin E, as determined by a rescue experiment. We also found that the mechanism of cyclin E regulation by PKC α was at the level of degradation rather than transcription. Using two inhibitors to PI3K, we found that both the active form of PKC α and total cyclin E levels decreased, implying that the PKC α /cyclin E pathway is downstream from PI3K. In conclusion, we have identified a novel pathway in epithelial ovarian tumorigenesis (PI3K \rightarrow PKC α \rightarrow Cyclin E \rightarrow anchorage-independent growth), which could potentially be targeted therapeutically.

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CHAPTER 1

Introduction: The Role of Cyclin E in PKC α -Driven Early Ovarian Tumorigenesis

DISSERTATION OVERVIEW

Briefly, this dissertation will start by focusing on the significance of studying ovarian cancer. Because strides have been made in identifying cancer pathways, more targeted therapeutics have been designed. While ovarian cancer has very few targeted therapies, this dissertation identifies a potential cancer pathway in ovarian cancer that can be targeted therapeutically.

PKC α and cyclin E both have oncogenic characteristics. This dissertation will go into great detail about these oncogenic characteristics. Both PKC α and cyclin E tend to be amplified at the genomic level, thus upstream targets could potentially be inconsequential. This dissertation focuses on PKC α 's regulation of cyclin E. I have found that regulation of cyclin E by PKC α is at the level of protein stability. Inhibiting PKC α expression leads to a decrease in cyclin E levels, both full length and low molecular weight (LMW-E) cyclin E. Importantly, knockdown of PKC α not only leads to a decrease in endogenous cyclin E levels, but also exogenous levels of cyclin E. This is very important because even if cyclin E is amplified at the genetic level, inhibition of PKC α will still cause decreases in the levels of amplified cyclin E.

This dissertation also focuses on the ability of PKC α and cyclin E to be involved in anchorage-independent growth. In this dissertation, I have demonstrated that the role of PKC α in transformation, but not in migration, is through cyclin E.

CANCER OVERVIEW

CANCER

The Centers for Disease Control and Prevention (CDC) states that the leading cause of death in the United States of America is heart disease at 616,067 deaths in 2009. Immediately following heart disease, the second leading cause of death in the US is cancer with 562,875 deaths (CDC, 2010). In 2010, according to the American Cancer Society (ACS), 569,490 people are expected to die from cancer (that is 1500 per day). This number is nearly unchanged from five years ago-570,280. These statistics mean that nearly 1 out of 4 Americans will die from cancer (CF&F, 2005; CF&F, 2010).

The American Cancer Society defines cancer as “a group of diseases characterized by uncontrolled growth and spread of abnormal cells.” Cancer can be due to both intrinsic and extrinsic factors, such as: age, weight, lifestyle, smoking, viral infections, chemical or radiation exposure, and genetic mutations to name a few (CF&F, 2010).

OVARIAN CANCER

Ovarian cancer is the leading cause of death among the gynecologic malignancies. Due to a lack of obvious symptoms and no sufficiently accurate method of screening, early detection is rare, making the prognosis quite poor. Symptoms include pelvic pain and swelling of the abdomen (due to an accumulation of fluid). Other symptoms include a feeling of fullness, abdominal pain or bloating (CF&F, 2010).

Risk factors for ovarian cancer include weight, age, use of post-menopausal hormone supplements (estrogen alone), inherent characteristics such as a family history of breast or ovarian cancer, and genetic mutations such as BRCA1/2 or Lynch syndrome (hereditary nonpolyposis colon cancer). Certain factors can actually decrease ones risk of ovarian cancer, these factors include pregnancy and long-term use oral contraceptives (due to the lack of ovulation). Surgical removal of one's reproductive organs, such as a hysterectomy can also decrease the risk of ovarian cancer. Woman with a high risk of ovarian cancer can have their ovaries removed as a means of prevention (CF&F, 2010).

Treatment of ovarian cancer involves the use of chemotherapy and surgery. Surgery includes the removal of one or both ovaries (one in the case of younger women who still desire to have children), the uterus (hysterectomy), and the fallopian tubes (salpingo-oophorectomy). Chemotherapy can be administered either systemically or localized to the intraperitoneal

cavity. Better prognosis has been observed in patients who have had their surgeries performed by a gynecologic oncologist.

Treatment of ovarian cancer through the use of first-line chemotherapeutics differs between institutions, but likely involves cisplatin, carboplatin, gemcitabine, doxorubicin, or paclitaxel or combinations of these agents (Campos, 2010). As for therapeutics that target specific cancer pathways in ovarian cancer, many clinical trials exist. Some of the more promising clinical trials include targeting VEGF. Because the treatment of ovarian cancer results in chemoresistance, VEGF inhibitors are promising reagents due to cancers' need for angiogenesis in order to promote metastases and tumor growth (Campos, 2010). Currently, Avastin (Bevacizumab) a VEGF (vascular endothelial growth factor) humanized monoclonal antibody that inhibits its activity (through inhibition of angiogenesis) is being tested in clinical trials for the treatment of ovarian cancer (CF&F, 2010; Los, 2007). Bevacizumab was the first VEGF inhibitor. The initial clinical trial testing Bevacizumab was published in 2004 and was performed in colon cancer in combination with irinotecan, fluorouracil, and leucovorin. These combinations between Bevacizumab and first-line metastatic colon cancer chemotherapeutics resulted in an increase in overall survival and progression-free survival (Hurwitz, 2004). Currently, at least twelve clinical trials exist for the Bevacizumab in several cancer types, including ovarian cancer (Campos, 2010). Another targeted therapy in clinical trials for the treatment of ovarian cancer is Recentin (Cediranib or AZD2171) by AstraZeneca which is also a VEGF inhibitor, by inhibiting the kinase activity of vascular endothelial growth factor receptor-2 tyrosine kinase (Wedge, 2005). Several clinical trials involving AZD2171 have been performed in ovarian cancer with a positive response. Matulonis et al published a phase II clinical trial that, although no patients exhibited a complete response, 17% exhibited a partial response and 13% exhibited stable disease (Matulonis, 2009). Also, Garcia et al in a phase II clinical trial, which dichotomized patients according to platinum sensitivity, demonstrated that AZD 2171 treatment led to 41% response rate in patients that were sensitive to platinum therapy while only a 29% response rate was observed in patients who exhibited platinum resistance (Garcia, 2008). Currently, this drug is in phase III clinical trials for the treatment of ovarian cancer (Campos, 2010). Because Avastin and Recentin are potent VEGF (angiogenesis) inhibitors, they were believed to be potentially useful for preventing recurrence and extending survival (Wedge, 2005). However, thus far, these two drugs only extended overall survival by less than six months. Thus, the need for more targeted treatments of ovarian cancer pathways exist. At this time, the 5 year survival rate is only 46%, thus more

research needs to be done to investigate the important pathways to target in ovarian tumorigenesis.

The ovaries are composed of three tissue types: epithelial, germ, and stromal cells. Ovarian cancer can arise from abnormalities in any of these three tissue types and are termed ovarian epithelial tumors, germ cell tumors, and stromal tumors accordingly. In normal ovaries, the epithelial cells form a polarized monolayer and make up the covering of the ovaries. Ovarian cancer that arises from epithelial cells is the predominant form of ovarian cancer and will be the focus of this dissertation. The germ cells make up the eggs, and the stromal cells (or stroma) are the remainder of the ovaries which are composed of hormones and hold the ovaries together (ACS, 2010).

According to the American Cancer Society (ACS), epithelial ovarian tumors can be further divided into three types: benign epithelial tumors, LMP tumors (tumors of low malignant potential- or borderline epithelial ovarian cancer), and malignant epithelial ovarian tumors. Both benign epithelial tumors and LMP tumors are rarely life-threatening. However, the malignant epithelial ovarian tumors, which account for 90% of all ovarian cancers, have a worse prognosis than the other two epithelial tumors, but the survival depends on the stage. Invasive epithelial ovarian cancer diagnosed at stage I has 89% 5-year survival, while stage II has 66%, stage III has 34%, and stage IV has only 18% 5-year survival. LMP tumors have 99% 5-year survival at stage I, 98% at stage II, 96% at stage III, and 77% at stage IV (ACS, 2010). Thus, invasive epithelial ovarian tumors exhibit the need for more targeted therapies.

Based on the histology of epithelial ovarian tumors, these tumors can be further classified into subtypes: serous, mucinous, endometrioid, or clear-cell tumors. While the latter three tumor types are likely low-stage lesions, serous tumors are usually high-stage lesions. Serous epithelial ovarian tumors are the predominant subtype of ovarian epithelial tumors comprising 40% of this type of ovarian cancer and will be the focus of this dissertation. Endometrioid tumors compose 20% of ovarian epithelial tumors, mucinous are only 1%, and clear-cell tumors are 6% (Health-Communities, 2010).

Both germ cell and stromal cell tumors are rarer than epithelial tumors but exhibit a better prognosis. Germ cell tumors, which account for approximately 3% of ovarian cancers, exhibit more than 90% 5-year survival. While stromal tumors, which account for approximately 5% of ovarian cancers, are found in older women (over the age of 50); they can also be found in women of younger age (young girls). Stromal tumors tend to produce hormones and can be either benign or malignant. Malignant stromal tumors still have an overall good prognosis with greater than 70% overall survival (ACS, 2010).

Like most cancer types, ovarian cancer contains hotspots of mutations that occur leading to the onset of ovarian cancer. These mutations include gene amplification or deletions, activating or inactivating mutations, promoter and hypo-methylation, as well as loss of heterozygosity (LOH). Table 1 contains a complete list of the genetic abnormalities that occur in ovarian cancer along with the genes affected by these abnormalities (Bast, 2009). Included in the list of genes with genomic amplification, which result in overexpression of their transcripts, are PKC α and cyclin E. Several known oncogenes exist specifically in the epithelial type of ovarian cancer (Table 2). Both PKC α , which is amplified in 44% and overexpressed in 78% of epithelial ovarian cancers, and cyclin E, which is amplified in 12-36% and overexpressed in 42-63% of epithelial ovarian cancers, are oncogenes in this ovarian cancer subtype and will be discussed below in great detail (Bast, 2009). The discrepancy between the percent of ovarian cancers with genomic amplification and the percent containing overexpression could be due to the ability of several other aberrant genes on these lists to regulate PKC α and cyclin E.

Table 1: Genetic abnormalities in epithelial ovarian cancer
Adapted from (Bast, 2009)

Event	Effect	Chromosome	Gene
Gene amplification	Activation	1q22 3q26 5q31 8q24 19q 20p 20q13.2	RAB25 PRKCI , EVI1 and PIK3CA FGF1 MYC PIK3R1 , CCNE1 , and AKT2 ND AURKA
Gene deletion	Inactivation	4q, 5q, 16q, 17p, 17q, Xp and Xq	ND
Mutation	Activation	NA	KRAS (15%), BRAF (12%), CTNNB1 (12%), CDKN2A (10%), APC (9%), PIK3CA (8%), KIT (7%) and SMAD4 (7%)
Hypomethylation	Activation	NA	IGF2 and SAT2
Loss of heterozygosity	Inactivation	17p13 and 17q21 (in 50% of cases or more) 1p, 3p, 5q, 5q, 6q, 7q and 8q (in fewer than 30% of cases)	ARHI , PEG3 , PLAGL1 , RPS6KA2 , TP53 , BRCA1 , BRCA2 , PTEN , OPCML and WWOX
Mutation	Inactivation	NA	TP53 (62%), BRCA1 (5%), BRCA2 (<5%) and PTEN (3–8%)
Promoter methylation	Inactivation	NA	ARHI , DAPK1 , CDH13 , MLH1 , ICAM1 , PLAGL1 , DNAJC15 , MUC2 , OPCML , PCSK6 , PEF3 , CDKN2A , CDKN1A , RASSF1 , SOCS1 , SOCS2 , PYCARD and SFN

Table 2: Oncogenes associated with epithelial ovarian cancer
Adapted from (Bast, 2009)

Gene	Chromosome	Percentage of cancers in which amplified	Percentage of cancers in which overexpressed	Percentage of cancers in which mutated	Function
<i>RAB25</i>	1q22	54%	80–89%	ND	Cytoplasmic GTPase and apical vesicle trafficking
<i>EVI1</i>	3q26	ND	ND	ND	Transcription factor
<i>EIF5A2</i>	3q26	ND	ND	ND	Elongation factor
<i>PRKCI</i>	3q26	44%	78%	ND	Cytoplasmic serine-threonine protein kinase
<i>PIK3CA</i>	3q26	9–11%	32%	8–12%	Cytoplasmic lipid kinase
<i>FGF1</i>	5q31	ND	51%	ND	Growth factor for cancer and angiogenesis
<i>MYC</i>	8q24	20%	41–66%	ND	Transcription factor
<i>ECFR</i>	7q12	11–20%	9–28%	<1%	Protein tyrosine kinase growth factor receptor
<i>NOTCH3</i>	9p13	20–21%	62%	ND	Cell surface growth factor receptor
<i>KRAS</i>	12P11–12	5%	30–52%	2–24%	Cytoplasmic GTPase
<i>ERBB2</i>	17q12–21	6–11%	4–12%	ND	Protein tyrosine kinase growth factor receptor
<i>PIK3R1</i>	19q	ND	ND	ND	Cytoplasmic lipid kinase
<i>CCNE1</i>	19q12	12–36%	42–63%	ND	Cyclin
<i>AKT2</i>	19q13.2	12–27%	12%	ND	Cytoplasmic serine–threonine protein kinase
<i>AURKA</i>	20q13	10–15%	48%	ND	Nuclear serine–threonine protein kinase

CYCLIN E

EUKARYOTIC CELL CYCLE

As mentioned, the American Cancer Society defines cancer as “a group of diseases characterized by uncontrolled growth and spread of abnormal cells” (ACS, 2010). Proliferation of a eukaryotic cell is controlled by the cell cycle, which is defined as the sequence of events between two cell divisions and is highly regulated. The cell cycle begins with a single cell with one copy of its DNA (N), the cell replicates its DNA (2N), and ends with two genetically identical daughter cells, each with one copy of the DNA (Heichman, 1994). A cell about to divide must exit G_0 (the dormant, quiescent stage) by exposure to a mitogenic stimulus and enter into the first phase of the cell cycle, Gap1 or G1 phase (Pardee, 1989). The G1 phase is important for normal cell division because in the G1 phase, the cell prepares for DNA replication (Synthesis or S phase).

The pause between G1 and S phase is known as the G1/S transition or checkpoint. The G1/S checkpoint is where cells, which have the proper protein expression pattern are able to make the transition into the S phase (Reed, 1997)-review. In S phase, DNA from the mother cell is replicated and chromosomes are duplicated. As with all processes within the cell cycle, DNA replication and chromosome duplication are also highly regulated processes to ensure that the DNA is only replicated once (Heichman, 1994). Also in S phase, the centrosomes, which are involved in the division of the DNA between the two daughter cells, are duplicated (Lacey, 1999). Upon completion of proper chromosome and centrosome duplication, the cycling cell enters the second gap phase, G2.

Like G1 phase, G2 phase is time that the cell spends examining its ability to enter the next phase of the cell cycle without error (Johnson, 1999). In the case of G2, the next phase of the cell cycle is mitosis or M phase. Mitosis is the phase of the cell cycle in which the cells physically divides into two genetically identical daughter cells. See Table 3 for a detailed view of the phases of the cell cycle.

Table 3: Regulators of the different stages of the eukaryotic cell cycle

	Phase	Function	Predominant CDK	Predominant Cyclin	CDK Inhibitor
Interphase	G1 Gap1	Receive Mitogenic Stimuli Cell Growth Protein Synthesis Preparation for S Phase	CDK4 CDK6 CDK2	Cyclin D1/D2/ D3	p16 ^{INK4A} p15 ^{INK4B} p18 ^{INK4C} p19 ^{INK4D}
	S Synthesis	DNA Replication Chromosome Duplication Centrosome Duplication	CDK2	Cyclin E1/E2 Cyclin A1/A2	(cyclin E) p21 ^{CIP1} p27 ^{KIP1} p57 ^{KIP2}
	G2 Gap2	Preparation for M Phase Centrosome Maturation Chromosome Condensation	CDK2 CDK1	Cyclin A1/A2	-
Mitosis	M Mitosis	Chromosome Separation Cell Division	CDK1	Cyclin B1/B2/ B3 Cyclin F	-

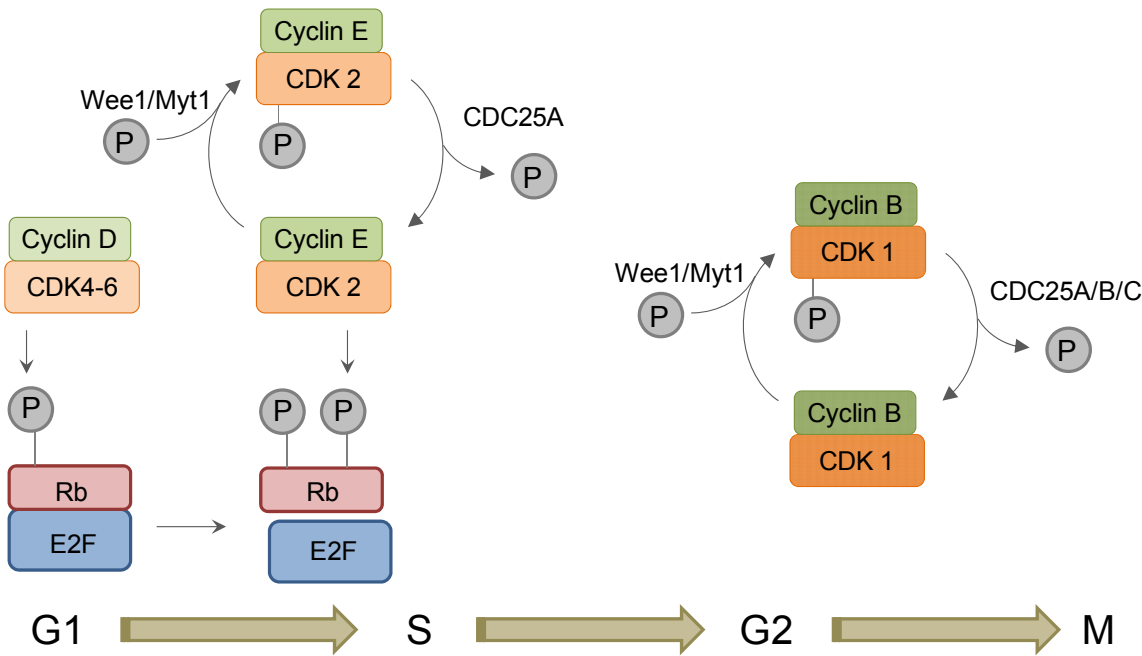
Mitosis can be further divided into stages: prophase, prometaphase, metaphase, anaphase, and telophase. In prophase, the nuclear envelope is broken down and the condensed chromosomes start to align for cell division. In prometaphase, the spindle begins to form. In metaphase, the chromosomes align at the metaphase plate. Then, during anaphase, the sister chromatids migrate to the poles, and the cleavage furrow begins to form, marking the beginning of cytokinesis. At telophase, the final phase of mitosis, the spindles disassemble, the nuclear envelopes of each new daughter cell form, and the chromosomes decondense. At that point, one round of the cell cycle has completed, resulting in two genetically identical daughter cells that are equivalent to the initial mother cell. See Table 4 for detailed view of the phases of mitosis.

Table 4: Functions of the different stages of mitosis

Mitosis	Phase	Function
	Prophase	Nuclear Envelope Break Down Chromosome Alignment
	Prometaphase	Spindle Formation
	Metaphase	Sister chromatids migrate to the poles Chromosomes align on metaphase plate
	Anaphase	Cytokinesis
	Telophase	Spindle Disassembly Chromosome Decondensation Nuclear Envelope Reformation

Each of these cell cycle phases contains protein regulators (See Table 3). These regulators are the accelerators and breaks for the cell cycle. The accelerators of the cell cycle are complexes made of two subunits, a catalytic subunit (CDK) and a regulatory subunit (cyclin). The catalytic subunit of the cell cycle is a kinase, whose activity drives the cycle forward and is ubiquitously expressed throughout the cell cycle. The catalytic subunits are known as cyclin dependent kinases (or CDKs) because the CDKs depend on binding of the regulatory subunits (cyclins) for enzymatic activation. The expression patterns of cyclins are temporal throughout the cell cycle (see Table 3). The cyclins are up-regulated by transcription and down-regulated by ubiquitin-mediated proteasomal degradation in a highly organized fashion. When a cyclin is transcriptionally up-regulated and binds to a CDK, this cyclin-CDK complex is then active and drives the cell cycle forward. Activation occurs through a series of phosphorylation events, both activating and inactivating (see Figure 1). An inhibitory kinase is able to phosphorylate the CDK-subunit until the cell is adequately prepared to go into the next phase. At which point, the cyclin/ CDK complex is de-phosphorylated by the phosphatase, CDC25. Another phosphorylation event necessary to progress the cell cycle is the phosphorylation of the E2F inhibitor, RB. Phosphorylation of Rb results in release of the transcription factor, E2F and transcriptional activation of cell cycle genes (see Figure 1). In the case of the G1 and S cyclin-CDK complexes, CDK-inhibitors are able to bind the cyclin-CDK complexes and render these complexes inactive, thereby halting the cell cycle. These CDK-inhibitors are specific to cyclin-CDK complexes (see Table 3). The G1/S CDK/cyclin complexes are activated by phosphorylation of the CDK subunit by CAK, CDK-activating complex, which is composed of cyclin H and CDK7 (Fisher, 1994). The cyclin that this dissertation will focus on will be cyclin E, the cyclin involved in the G1-S phase transition by binding CDK2 due to the importance of cyclin E in driving tumorigenesis.

Figure 1: Schematic of the Phosphorylation Events that Lead to Cell Cycle Progression

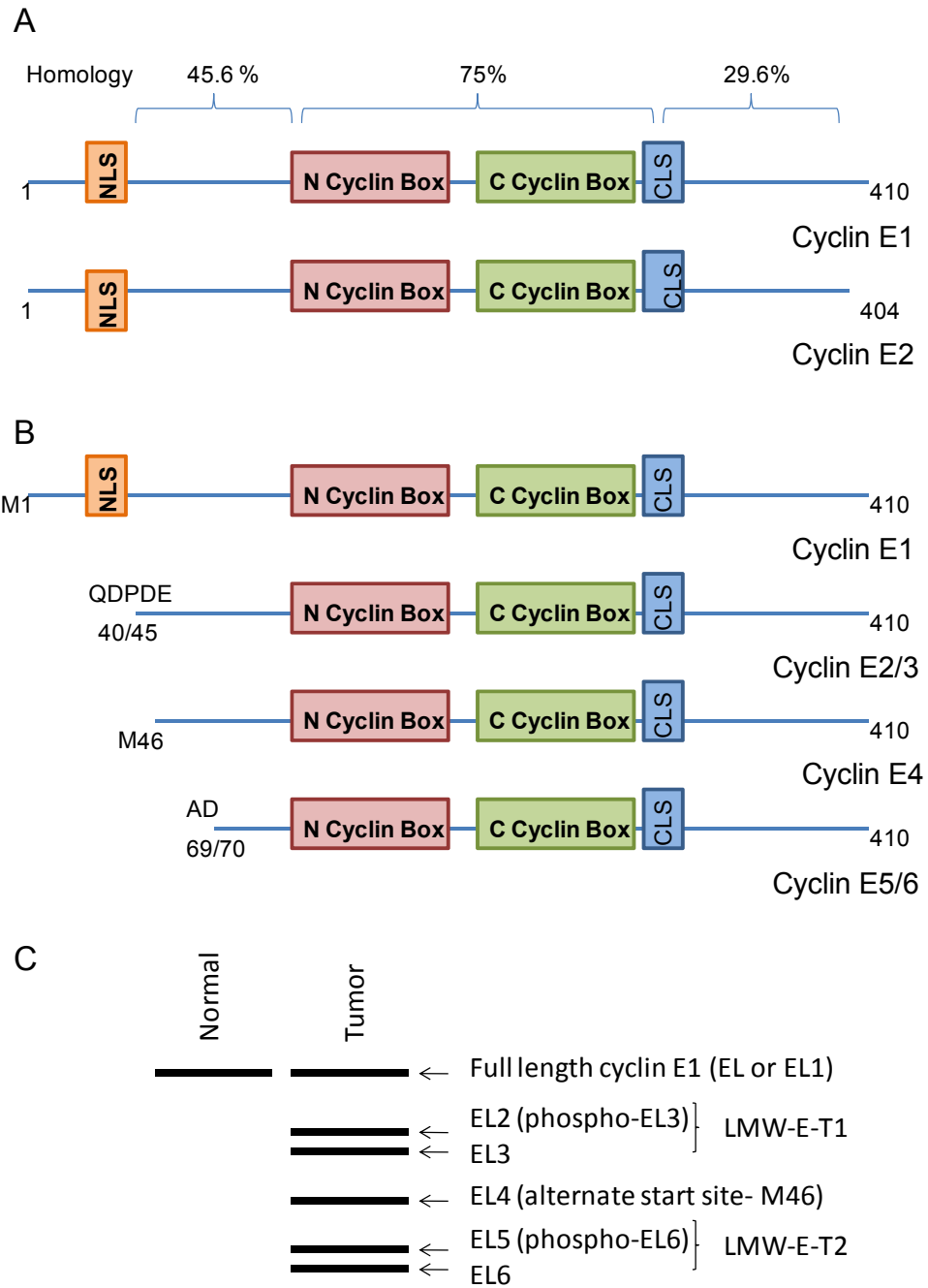


CYCLIN E

The cyclin E family consists of two genes, *CCNE1* (cyclin E1) and *CCNE2* (cyclin E2). Cyclin E1 was originally identified in two separate labs using a yeast complementation assay. Both laboratories, using budding yeast (*Saccharomyces cerevisiae*), performed this assay to rescue the deficiency of the G1 cyclin function using a human cDNA library prepared from the human glioblastoma cell line, U118 (Koff, 1991; Lew, 1991). However, the cyclin E1 mRNA that was originally identified was not the full length form, but a spliced form, which is 15 aa shorter than the endogenous cyclin E and not translated under physiological conditions in human cell lines. Both the long and short forms of cyclin E1 that were identified in this study bound CDK2 and led to its activation, as determined by rapid G1 progression and increased cyclin E-associated kinase activity. Additionally, the short form of cyclin E1 used in its original discovery was an artifact of NotI restriction enzyme digestion, and does not exist *in vivo* (Ohtsubo, 1995). Both mouse and human cyclin E2 were identified using a yeast two-hybrid assay with p27 (Lauper, 1998) and Cdk2 (Zariwala, 1998) as bait. Cyclin E2 was found to bind CDK2, phosphorylate histone H1, and isolate (increase in G1 phase and get degraded in S) throughout the cell cycle like cyclin E1 (Lauper, 1998). However, the cyclin E2 protein appeared to be regulated similar to cyclin E1 in regards to Rb but not p53, as determined by response to the viral E6 and E7 proteins (Zariwala, 1998). Essentially, both cyclin E1 and cyclin E2 were upregulated in response to the E7 oncoprotein (which suppresses Rb), but only cyclin E2 was upregulated by the suppression of p53 via viral E6.

Cyclin E1 and cyclin E2 have overall sequence homology at 48 percent (Lauper, 1998; Zariwala, 1998; Gudas, 1999). However, some regions of cyclin E1 and cyclin E2 are more conserved than others. For example, the cyclin box domain of cyclin E1 and E2 shares over 75 percent homology (Lauper, 1998; Zariwala, 1998; Gudas, 1999). The centrosome localization sequence (CLS) is also conserved in both cyclin E1 and E2 (Matsumoto, 2004). The NLS of cyclin E1 appears to be dispensable for nuclear localization (Porter, 2001; Geisen, 2002; Kelly, 1998). Most likely, cyclin E1 and E2 are able to translocate into the nucleus through interactions with other proteins containing NLSs. Although cyclins E1 and E2 are similar but not conserved in their substrate binding motifs (MRAIL and VDCLE), cyclins E1/CDK2 and E2/CDK2 are both able to bind the substrates, p21, p27, pRb, and histone H1 (Lauper, 1998; Zariwala, 1998; Gudas, 1999). See Table 5 for a complete list of CDK2 substrates and the sites phosphorylated by CDK2.

Figure 2: Cyclin E Protein Domains



NLS = nuclear localization sequence

CLS = centrosome localization sequence

Table 5: CDK2 Substrates

Substrate	Residue (Site)
Cyclin E1	T (380), S (399)
Myogenic differentiation antigen 1	S (200)
Nucleophosmin 1	T (199)
Parathyroid hormone related protein	T (108)
Retinoblastoma 1	S (230), S (567), S (608), S (612), S (807), S (811), T (821)
Nuclear transcription factor Y, alpha	S (320), S (326)
p53	S (315), S(392)
Ubiquitin conjugating enzyme E2A	S (120)
HIRA	T (555), S (687)
Coilin	S (184)
Inhibitor of DNA binding 3	S (5)
Inhibitor of DNA binding 2	S (5)
Upstream binding factor	S (389), S (484)
Cyclin dependent kinase inhibitor 1B	T (187)
B Myb	T (266), S (393), T (405), T (440), T (444), S (452), T (487), T (494), T (515), T (518), T (520), S (577)
ORC1	S (258), S (273), T (375)
p73	T (86)
Forkhead box protein M1	T (649)
CDC6	S (106), S (54), S (74)
Cell division autoantigen 1	S (20), T (340)
Protein tyrosine phosphatase, non receptor type 2	S (304)
Cell division cycle 7	T (376)
CP110 protein	S (170), T (194), S (366), S (372), S (400), S (45), S (516), T (566)
Cyclin dependent kinase 7	S (164), T (170)
Protein regulator of cytokinesis 1	T (470), T(481)
CDX2	S (283), S (287), S (291), S (295)
SMAD3	T (179), S (204), S (208), S (213), T (8)
Protein kinase, cAMP dependent, regulatory, type I, alpha	T (209), S (83)
DNA replication licensing factor MCM4	T (110), T (19), S (3), S (32), S (54), T (7)

* Adapted from the Human Protein Research Database- CDK2

Cyclins E1 and E2 also likely have similar methods of degradation as they both contain a PEST motif, which is involved in the E3 ubiquitin ligase, FBW7, binding (Moroy, 2004)-review, (Koepp, 2001; Gudas, 1999; Won, 1996; Strohmaier, 2001). Both the phosphorylation sites that target cyclin E for degradation and the prolyl bonds that allow PIN1 to aid in their isomerization, which also targets cyclin E for degradation, are highly conserved. The studies that have been performed to date comparing the two cyclin Es, suggest that cyclin E1 is the dominant one and also the cyclin that is most likely to be the driver of oncogenesis. Thus, from this point forward, all references to cyclin E are for cyclin E1.

Cyclin E is important in cell cycle regulation, as cyclin E is involved in the checkpoint at the G1 to S phase transition (Johnson, 1999)-review. In G1, upon phosphorylation of the Rb-E2F complex, E2F is released to transcriptionally upregulate G1 to S phase transition genes, including cyclin E (Cobrinik, 2005)-review. The cyclin E promoter is transcriptionally activated by E2F, as discovered using a cyclin E reporter gene with mutations at two potential E2F binding sites (Botz, 1996; Ohtani, 1995). Cyclin E, when bound to CDK2, leads to hyperphosphorylation of Rb, creating a positive feedback loop (Moroy, 2004). When Rb is phosphorylated by cyclin E-CDK2, Rb undergoes a conformational change releasing E2F, a transcription factor which drives the cell cycle forward by transcriptionally regulating genes involved in S phase (Sherr, 1996)- review.

As cells move through S phase, cyclin E gets down-regulated by ubiquitin-mediated proteasomal degradation (Singer, 1999; Nishitani, 2006). Cyclin E, which is unbound to CDK2, gets degraded by the E3 ubiquitin ligases, Cul3 (shown using Cul3^{-/-} mice) and Cul4 (shown using RNAi) (Singer, 1999) and (Nishitani, 2006), respectively. The E3 ubiquitin ligase responsible for the degradation of CDK2-bound cyclin E is the SCF complex (Skp/Cullin/F-box, with the F-box protein that binds cyclin E being FBW7) (Nishitani, 2006; Koepp, 2001; Strohmaier, 2001; Moberg, 2001). Using FBW7 knockout mice and FBW7 RNAi in HeLa cells, Fbw7 loss was shown to lead to cyclin E protein accumulation (Tetzlaff, 2004; Yada, 2004). Similarly, mutations in FBW7 can lead to tumorigenesis, most likely through elevated cyclin E protein levels (Calhoun, 2003; Ekholm-Reed, 2004; Gu, 2007).

FBW7 (4q32) is located at a frequently deleted region of the genome (Spruck, 2002; Knuutila, 1999). Overall, mutations in *FBW7* exist in 6% of all tumors. However, some tumor types have higher levels of *FBW7* mutations than others. The highest level of *FBW7* mutations exist in T-cell acute lymphoblastic leukemias (T-ALL) and cholangiocarcinomas (nearly 30%) followed by pancreatic, gastric, colon, prostate, and endometrial cancers (approximately 10%)

(Maser, 2007; Lee, 2006; Kemp, 2005; Hubalek, 2004; Calhoun, 2003; Akhoondi, 2007). However, some tumor types rarely exhibit mutations in *FBW7*, these include bladder, bone, breast, leukemia, liver, lung, and ovarian (Nowak, 2006; Lee, 2006; Kwak, 2005; Sgambato, 2007; Yan, 2006). The majority of *FBW7* mutations are point mutations (approximately 75%), which can result in the inability of FBW7 to either form homodimers or bind its substrate (such as cyclin E). Other mutations that can occur in *FBW7* are nonsense mutations (result in a truncated form of the FBW7 protein by insertion of a premature stop codon), these truncated forms of FBW7 can either be rendered inactive or dominant-negative depending on the location of the mutation (Welcker, 2008). Because inactivating mutations in *FBW7* can result in chromosomal instability (Rajagopalan, 2004), a known effect of cyclin E overexpression (see below), one could infer that the effect of inactivation of FBW7 on tumorigenesis is through the inability to degrade cyclin E, resulting in increased cyclin E/ CDK2 activity, increased proliferation and chromosomal instability, thus increased tumorigenesis.

During the cell cycle, cyclin E is not only required for G₀ exit and the G1 to S transition, but it is also involved in DNA replication and centrosome duplication (Hwang, 2005). Centrosome duplication occurs at the G1/S transition (Lacey, 1999). In *Xenopus* oocytes, inhibition of Cyclin E-CDK2 activity followed by phenotypic reversion with cyclin E-CDK2 showed that cyclin E-CDK2 is required for centrosome duplication (Hinchcliffe, 1999). Also, using confocal microscopy, Hinchcliffe et al was able to show that cyclin E localized to the centrosome (Hinchcliffe, 1999). In 1999, Hinchcliffe et al stated, "The abnormally high number of centrosomes found in many human tumor cells can lead directly to aneuploidy and genomic instability through the formation of multipolar mitotic spindles." Under normal circumstances, the cyclin E/CDK2 complex is able to phosphorylate CDC25C, resulting in its activation. However, in a study by Bagheri-Yarmand et al in which I was involved, in a breast cancer model, we found that when bound to CDK2, tumor-specific LMW-E (but not full length cyclin E) could lead to an increase in chromosomal instability through the premature inactivation of CDC25C. This LMW-E-induced aberrant inactivation of CDC25C in turn resulted in an early exit from mitosis, complete with centrosome amplification, chromosome missegregation, and anaphase bridges as determined by time-lapsed confocal microscopy. In a rescue experiment, by silencing CDC25C in the presence of LMW-E, we found that CDC25C was necessary for this effect of LMW-E on chromosomal instability (Bagheri-Yarmand, 2010; Bagheri-Yarmand, 2010). Thus, cyclin E is very important in its role in centrosome duplication and if altered can lead to genomic instability.

Some of the functions of cyclin E require CDK2 enzymatic activity; however some functions are CDK2-independent. For example, cyclin E, independent of CDK2, was determined to be necessary for *endoreduplication* (DNA replication in the absence of cell division) in drosophila (Sauer, 1995; Knoblich, 1994). This same group found that cyclin E (through activation of CDK2) was necessary for drosophila *embryogenesis*. Specifically, Knoblich et al found that mutant drosophila cyclin E (Dmcyce) embryos lacked the ability to exit the sixteenth round of cell division in early embryogenesis, which was through drosophila CDK2 (Dmcdc2c) activity (Knoblich, 1994). Another function of cyclin E, which is dependent on CDK2 activity to induce Cdc7 accumulation (which phosphorylates Mcm2), is MCM loading onto chromatin at the pre-RC (pre-replication complex) during *DNA replication*, as determined by overexpression of Ad-cyclin E (Ekholm-Reed, 2004; Chuang, 2009). Another event, which is necessary for S-phase entry, that is controlled by cyclin E, through CDK2, is *histone biosynthesis*. Specifically, the cyclin E/CDK2 complex can phosphorylate NPAT, resulting in increased gene expression of histones, as determined by Histone H4 expression (Zhao, 2000). As mentioned above, cyclin E through CDK2 activation is also involved in cell growth through phosphorylation of Rb resulting in release of the transcription factor, E2F (Cobrinik, 2005)-review. Defects in these processes due to aberrant cyclin E expression and/or activity can result in cancer.

CYCLIN E AND CANCER

Many proteins are involved in cell cycle control; defects in these regulators can result in tumorigenesis (Sherr, 1996; Pardee, 1989). Using a breast cancer model system (both tissue samples and breast cancer cell lines), cyclins A, B, E, and D1/D3 and CDKs 1 and 2 were shown to be overexpressed compared to control (either normal adjacent tissue or non-tumorigenic cell lines) as determined by Northern and Western blot analysis, indicating their important role in driving tumorigenesis (Keyomarsi, 1993). Specifically, cyclin E was found to be overexpressed in all 10 breast cancer cell lines, compared to the 3 control cell lines, indicating cyclin E is of particular relevance in cancer (Keyomarsi, 1993). Improper regulation of cyclin E can result in increased proliferation and genomic instability, which can lead to cancer (Ohtsubo, 1993; Ohtsubo, 1995; Resnitzky, 1994). Specifically, cyclin E deregulation through overexpression or increased activity can result in breast, ovarian, prostate, and colon cancers (Bedrosian, 2004; Keyomarsi, 2002; Wingate, 2005; Sherr, 1996; Keyomarsi, 1994; Keyomarsi, 1995; Keyomarsi, 1993). Cyclin E1, cyclin E2, and the cyclin E catalytic subunit CDK2 knockout mice are all viable. However, double knockout of cyclin E1/E2 resulted in an

embryonic lethal phenotype at day 11.5 with defects in extra embryonic tissues most likely due to defects in endoreduplication (see above) (Geng, 2003; Parisi, 2003). In fact, cyclin E1^{-/-}E2^{-/-} MEFs (mouse embryo fibroblasts) derived from these knockouts were able to proliferate normally in culture (Mendez, 2003). Cyclin E1 knockout mice are viable and fertile with no observed abnormalities, possibly due to compensation by cyclin E2 (Parisi, 2003). Similarly, cyclin E2 knockout mice are viable with no observed abnormalities. However, unlike cyclin E1 knockout mice, cyclin E2 knockout mice exhibit reduced male fertility (Geng, 2003). Similarly, the CDK2 knockout mice were also viable exhibiting a reduced body size and sterility (Berthet, 2003; Ortega, 2003). Perhaps the explanation of why the cyclin E and CDK2 knockout mice are viable with very little observed defects results from functional redundancies between cyclins and CDKs, allowing for other cyclins or CDKs to compensate for loss of these essential drivers of the cell cycle. Which is why individual knockout of either cyclin E1 or E2 have no phenotypic abnormalities, but double knockouts of cyclin E1 and E2 are embryonic lethal. While individual cyclin E knockout mice were viable and exhibited no observed phenotypic abnormalities, knockout of both cyclin E1 and cyclin E2 (E1^{-/-}E2^{-/-}) in MEFs (mouse embryo fibroblasts) showed resistance to oncogenic transformation. More specifically, in response to v-HA-Ras (plus either DNp53, myc, or E1A) or myc alone, cyclin E1^{-/-}E2^{-/-} MEF exhibit significant reduced foci formation when compared to the cyclin E1^{+/+}E2^{+/+} MEFs (Geng, 2003), thus implying the role of cyclin E in tumorigenesis is more likely due to transformation than changes in proliferation. Aberrant cyclin E, or down-regulation of cyclin E inhibitors, such as p21, p27, or FBW7, can result in alterations in the G1 to S transition, endoreduplication, centrosomal amplification, and even mitotic catastrophe: all of which could lead to genomic instability and thus cancer (see above).

Cyclin E is involved in several stages of tumorigenesis from initial transformation to metastasis. For example, Geng et al had shown that cyclin E1^{-/-}E2^{-/-} MEFs were resistant to Ras or myc-induced transformation (Geng, 2003). Also, Geisen et al found that cyclin E-binding to CDK2 was not necessary for Ras-induced transformation, indicating this function of cyclin E is CDK2-independent (Geisen, 2002). Similarly, through a mutation on cyclin E resulting in decreased degradation (increased stability- T393A), stabilized cyclin E led to increased Ras-induced transformation *in vitro* and lung cancer *in vivo* (Loeb, 2005). In testicular germ cell tumors, Datta et al found that a significant correlation existed between cyclin E and clinical stage and metastasis (Datta, 2000). In a similar correlative study but in non- small cell lung cancer (NSCLC), cyclin E was found to correlate with survival in early stage NSCLC and metastasis formation (Muller-Tidow, 2001). Through examination of

colorectal cancer patient samples, Li et al found that cyclin E significantly with metastasis formation (Li, 2001). Also, in breast cancer, cyclin E correlates with tumor stage and a poor prognosis, implicating the role of cyclin E as a biomarker in tumorigenesis (Keyomarsi, 1994; Datta, 2000; Keyomarsi, 2002). Post-translational cleavage of cyclin E (LMW-E) is also implicated in the tumorigenic phenotype. Cyclin E in the steps of tumorigenesis will be covered in detail below.

LOW MOLECULAR WEIGHT CYCLIN E (LMW-E)

In a PNAS paper published in 1993, Keyomarsi and Pardee reported the existence of low molecular weight isoforms of cyclin E in breast cancer, which were tumor specific and generated post-translationally as indicated by lack of multiple mRNA transcripts in a Northern blot assay (Keyomarsi, 1993). These LMW-E isoforms, which are also present in several cancer types including ovarian cancer (Bedrosian, 2004; Davidson, 2007), are cleaved post-translationally by the serine protease, elastase (Porter, 2001). Use of elastase *in vitro* with both endogenous and exogenous cyclin E revealed that incubation with elastase was able to cleave cyclin E into low molecular weight isoforms that mimic those observed *in vivo*. Elastase has an endogenous inhibitor, Elafin (Yokota, 2007), which if overexpressed can lead to growth arrest as well as apoptosis of breast cancer cells in an Rb-dependent fashion (Caruso, 2010). LMW-E is generated by elastase cleavage of full length cyclin E (E1) at two sites, between N40/N45 (E3) and between A69/D70 (E6) (Porter, 2001) (see figure 2b and c). LMW-EL2 and LMW-EL5 are phosphorylated forms of EL3 and EL6 respectively (Mull, 2009), while EL4 is expressed due to an alternate M46 start site (Porter, 2001). The EL2/EL3 doublet has been termed cyclin E-T1, LMW-E-T1, or cyclin E-truncation 1, while the EL5/EL6 doublet has been termed cyclin E-T2, LMW-E-T2, or cyclin E-truncation 2. Another group showed that cyclin E could be cleaved by calpains in response to exogenous Ca^{2+} and that inhibition of calpain by calpeptin could result in a decrease in the cleavage of cyclin E (Wang, 2003). However, while calpain activity was increased in breast cancer cells (HTB-126, HTB-123, HTB-124, and ZR75) compared to the control HMECs, the LMW forms of cyclin E generated in response to calpain differed from the elastase cleavage pattern, which more closely resembles the pattern of cyclin E cleavage observed in cancer.

LMW-E is predominantly expressed in the cytoplasm, as compared with full length cyclin E, which is predominantly nuclear. In immortalized breast (MCF10A and 76NE6), breast cancer (MCF7-Her18 and MDA-MB-157), and ovarian cancer (IGROV, FUOV1, and OVCAR-3) cell lines, fractionation experiments revealed that LMW-E expression was predominantly

cytoplasmic, while full length cyclin EL was found in both the nucleus and the cytoplasm (Delk, 2009). Using a protein complementation assay, cytoplasmic LMW-E was determined to bind CDK2 in the cytoplasm. This cytoplasmic LMW-E/CDK2 complex was also determined to display kinase activity, as determined by an *in vitro* kinase assay. Because FBW7, the E3 ubiquitin ligase for cyclin E, is nuclear, this can account for the increased stability of LMW-E compared with cyclin EL (Delk, 2009). The unique localization of LMW-E to the cytoplasm as compared to the nuclear localization of cyclin EL potentially allows for the screening of cancer patients expressing LMW-E via IHC in order to provide a more efficient regiment of treatment for this category of breast and ovarian cancer patients.

The LMW-E isoforms are not merely the effect of tumorigenesis, but are contributors to the process. Studies have been performed on LMW-E using normal breast epithelial cells, as well as breast, ovarian, melanoma, and colorectal cancer cells (Porter, 2001; Bedrosian, 2004; Bales, 2005; Corin, 2006). In these different model systems, our lab and others have found LMW-E to be involved in an accelerated G1/S transition (most likely due to increased cyclin E-associated kinase activity- presumably through CDK2), lengthened period of S phase, shortened M phase and increased genomic instability. LMW-E was demonstrated to be hyperactive as indicated by increased phosphorylation of both histone H1 and GST-Rb, using an *in vitro* kinase assay (Porter, 2001). Specifically, in mortal (70N and 81N), immortalized (MCF10A and 76NE6), and tumor (MDA-MB-436 and MDA-MB-157) breast cell lines, overexpression of exogenous, flag-tagged LMW-E exhibited increased cyclin E-associated kinase activity compared to the full length form of cyclin E. This increase in cyclin E-associated kinase activity translated into an increase in the capability of normal cells (81N) as well as immortalized cells (76NE6) to enter S and subsequently G2/M phases of the cell cycle, indicating LMW-E is hyperactive in its ability to activate CDK2 in a breast model system (Porter, 2001; Wingate, 2003). In spite of the increased cyclin E-associated kinase activity observed with LMW-E overexpressing cells, the LMW-E/CDK2 complex binds p21 and p27 more efficiently than the cyclin EL/CDK2 complex, as determined by a series of immunoprecipitation (IP): Western blot analyses in MCF7 breast cancer cells (Akli, 2004). However, the LMW-E/CDK2 complex is more resistant to these CKIs, as determined by an IP (with p21 or p27) followed by an H1 kinase assay. These results were validated using an *in vitro* kinase assay with purified p21 from SF9 insect cells using GST-Rb as the substrate (Akli, 2004). Thus, even in the presence of increased binding to the CKIs, p21 and p27, LMW-E/CDK2 has increased kinase activity compared to cyclin EL/CDK2.

LMW-E also causes an increase in chromosomal instability as compared to full length cyclin E. Akli et al determined that in MCF7 cells with stably expressed exogenous cyclin E, LMW-E caused an increase in the percentage of metaphases with aberrations as compared to the parental, vector control, of cyclin EL overexpressing MCF7 clones (Akli, 2004). This change in the percentage of aberrations was due to an increase in the number of chromosome breaks, which resulted in an increase in the percent polyploidy in the MCF7-LMW-E clones (Akli, 2004). Using an MCF7-Tet-on cyclin E inducible model system, we found that in addition to chromosome breaks, fragments and dicentric chromosomes were observed in the LMW-E inducible clones compared to the cyclin EL induced MCF7-Tet-on cells (Nanos-Webb in revision). Exploring the mechanism of chromosomal instability induced by LMW-E, we found that LMW-E (but not cyclin EL), through its binding CDK2, led to increased chromosomal instability through its premature inactivation of CDC25C, leading to centrosome amplification, an early exit from mitosis, chromosome missegregation, and anaphase bridges, resulting in chromosomal instability (Bagheri-Yarmand, 2010; Bagheri-Yarmand, 2010). Thus, at least one of the roles of LMW-E in tumorigenesis is through induction of chromosomal instability.

CYCLIN E AND TRANSFORMATION

Several steps are necessary to transform a normal cell into a malignant cell (Hanahan, 2000). These steps include: (1) evading apoptosis, (2) self-sufficiency in growth signals, (3) insensitivity to anti-growth signals, (4) sustained angiogenesis, (5) limitless replicative potential, as well as (6) tissue invasion and metastasis.

(1) *Evading Apoptosis*: Surprisingly, cyclin E overexpression has been shown to sensitize cells to the induction of apoptosis, which is the opposite effect needed for transformation. This phenomenon could be an evolutionarily adaptive mechanism cells have acquired to counteract a deregulation in the expression of cyclin E. In a breast cancer model system (MCF7 and T47D cell lines), exogenous cyclin E overexpression resulted in an anti-apoptotic protein (Bcl-2) and an increase in two pro-apoptotic proteins (Bad and Bax), as determined by Western blot analysis (Dhillon, 2003). This change in mediators of apoptosis observed upon over expression of cyclin E translated to an increase in cytokine-induced (Fas, TNFalpha, and TRAIL) apoptosis, as determined by TUNEL analysis (Dhillon, 2003). Overall, most studies of cyclin E in apoptosis have been performed in the presence of ionizing radiation (IR). For example, in hematopoietic cell lines (Multiple-myeloma IM-9, U-266, and lymphoma Molt-4) exposed to IR (4-20 Gy), cyclin E is cleaved into a non-CDK2 binding truncation, referred to as p18 (due to its size), as observed by Western blot analysis (Mazumder, 2002).

Exogenous Bcl-2 expression can prevent this truncation and p18 expression is caspase 3-dependent, indicating p18-cyclin E could be involved in apoptosis. In fact, exogenous overexpression of the truncated p18-cyclin E induced apoptosis, as determined by annexin V-FITC analysis (Mazumder, 2002). Thus, contrary to the first hallmark of cancer, evasion of apoptosis, cyclin E seems to exhibit a pro-apoptotic function. However, because cyclin E is oncogenic, this role of cyclin E is likely suppressed by the tumorigenic characteristics of cyclin E.

(2) *Self-Sufficiency in Growth Signals*: This particular hallmark of cancer has not been extensively studied in regards to cyclin E. However, transformed cells that, by another mechanism unrelated to cyclin E, have induced an autocrine response (self-sufficiency in growth signals) require cyclin E activity to perform proliferation. An example of this being in endometrial carcinoma cells, where estrogen-induction caused an autocrine response via upregulation of IGR1 (insulin growth factor 1), resulting in an increase in cyclin E expression followed by increased proliferation (Kashima, 2009). Thus, although cyclin E may not be responsible for the induction of the autocrine response, cyclin E is still necessary to accomplish the effect of this response.

(3) *Insensitivity to Anti-Growth Signals*: The majority of studies of cyclin E in the transformation process have been on this particular hallmark of cancer. Previously, I have mentioned that in breast cancer, LMW-E is able to bind the CKIs p21 and p27. However, LMW-E is resistant to their inhibitory effects (Akli, 2004). Other studies have also shown that cyclin E is able to evade growth arrest signals. For example, in MCF7 (ER+) breast cancer cells, addition of the antiestrogen (tamoxifen) causes growth arrest. However, exogenous expression of cyclin E is able to delay this growth arrest (Dhillon, 2002). Similarly, in a breast cancer model system, MCF7 cells expressing ectopic LMW-E show a reduction in growth arrest in response to the antiestrogen, ICI 182,780 (fulvestrant), when compared to the MCF7 vector control and cyclin EL overexpressing cells, as determined by flow cytometry cell cycle analysis (Akli, 2004). Along the same lines, also in a breast cancer model (MCF-7/Ac1- MCF7 cells which have been transfected with aromatase, the enzyme that converts androgens to estrogens), MCF7/Ac1 cells infected with LMW-E (but not the LacZ control of cyclin EL) exhibit resistance to the G1-growth arrest induced by addition of the aromatase inhibitor (AI), letrozole (Akli, 2010). This bypass of growth arrest was due to an increase in cyclin E-associated CDK2 kinase activity, and could therefore be blocked by addition of the CDK-inhibitor, Roscovitine. Thus, several studies have indicated that cyclin E and LMW-E can exhibit insensitivity to anti-growth signals, the third hallmark of cancer.

(4) *Sustained Angiogenesis*: Minimal research has been performed regarding cyclin E's role in angiogenesis. However, in a melanoma model system, LMW-E was demonstrated to cause an increase in human melanoma cell xenograft tumors with sustained angiogenesis, the fourth hallmark of cancer, compared to vector control or cyclin EL overexpressing melanoma cells (Bales, 2005). This model system was used due to the differential expression of LMW-E in melanoma cells and metastatic melanoma as compared to normal melanocytes, which only express cyclin EL (Bales, 2005). Thus, although the mechanism of LMW-E induced angiogenesis has not been resolved, LMW-E can lead to an increase in angiogenesis.

(5) *Limitless Replicative Potential*: By examining IMR-90 cells (human fetal lung fibroblasts) that lack limitless replicative potential (senescent cells), Dulic et al found that cyclin E protein (as determined by Western blot analysis) and mRNA (as determined by Northern blot analysis) levels were 10 to 15 fold higher in quiescent cells (high confluency) versus senescent cells (high passage), indicating cyclin E could be down regulated in senescence, which could account for their inability to enter S-phase (Dulic, 1993). Similarly, upon induction of both senescent and quiescent cells with growth factors, cyclin E protein levels (as measured by Western blot analysis) and cyclin E-associated kinase activity (as measured by an H1 kinase assay) only increased in the quiescent cells, while the senescent cells remained unaffected. Also, upon treatment of these cells under these two conditions with CDC25, cyclin E-associated kinase activity only increased in the quiescent cells, while the senescent cells exhibited no change in cyclin E activity (Dulic, 1993). While this mechanism of downregulation of cyclin E levels and activity in this study of senescence was not addressed, this implies that downregulation of cyclin E can result in a decrease in limitless replicative potential, the fifth hallmark of cancer, indicating the necessity of cyclin E for limitless replicative potential. In an effort to devise the mechanism of cyclin E regulation in senescent versus proliferating melanocytes, Bandyopadhyay et al found that p300/CBP (a histone acetyltransferase- HAT) was recruited to the cyclin E promoter during proliferation of melanocytes versus in senescent cells that recruit histone deacetylases (HDACs), as determined by ChIP analysis (Bandyopadhyay, 2002). Thus, cyclin E through its transcriptional regulation, at least in part, can control the ability of cells to senesce versus undergo limitless replicative proliferation.

(6) *Tissue Invasion and Metastasis*: Very little research on invasion has been performed in regards to cyclin E expression. The only study, to my knowledge, involved a negative correlation (increased cyclin E correlated with a decrease in invasion) between cyclin E overexpression and an infiltrative phenotype as determined by IHC (immuno-histochemistry) in 985 breast cancer patient primary tumor samples (Berglund, 2006). However, no direct link

or mechanism was established between cyclin E overexpression and invasion, nor were any invasion assays performed. Cyclin E has however been studied more extensively in its effect on metastasis.

Cyclin E expression has been shown to be correlated with metastasis in several model systems. For example, in gastric carcinoma, by examination of cyclin E levels in tissue samples by IHC, Sakaguchi et al was able to show that cyclin E levels correlated with metastasis: 39% lymph node metastasis positive with strong cyclin E staining versus only 12% with low cyclin E staining ($p < 0.05$) (Sakaguchi, 1998). However, no direct studies of the effect of cyclin E expression on metastasis were investigated in this study. Another group showed the correlation between cyclin E and metastasis in colorectal carcinoma. Using tissue samples stained for cyclin E expression using IHC, Li et al found that cyclin E levels significantly correlated with both lymph nodal metastasis and peritoneal metastasis in a colorectal carcinoma model (Li, 2001). However, like the Sakaguchi study, no direct investigation of the effect of cyclin E expression on metastasis was reported. However, in these two studies, cyclin E expression, either cyclin EL or LMW-E was not differentiated.

Several studies have been performed which directly link LMW-E expression with metastasis. In a transgenic mouse model system, Akli et al found that LMW-E could contribute to metastatic mammary carcinomas and that this effect is through the interruption of the ARF-p53 pathway (Akli, 2007). More specifically, generation of transgenic mouse models exogenously expressing cyclin E (either full length alone, cyclin EL- M46A, which is unable to generate the alternatively translated LMW form of cyclin E, EL4, or LMW-E-T1) under control of the MMTV promoter, showed an increase in the number of lung metastases formed. Transgenic mice expressing LMW-E-T1 exhibited 25% (with wt p53) and 35.2% (with p53^{-/+}) metastatic tumors ($n = 53$ and 26 , respectively) as compared to cyclin EL-M46A transgenic mice which exhibited 14.3% (with wt p53) and 0% (with p53^{-/+}) metastatic tumors ($n = 67$ and 17 , respectively) (Akli, 2007). Thus, LMW-E was demonstrated to be a direct initiator of lung tumor metastases in the breast cancer model system. Also, melanoma xenograft models demonstrated that LMW-E led to increasing lung metastasis (Bales, 2005). More specifically, in a mouse xenograft model, SB-2 melanoma cells expressing exogenous cyclin E (either vector alone, cyclin EL, LMW-E-T1 or LMW-E-T2) were injected into mice. The vector alone group did not form lung metastases, the cyclin EL group formed metastases in 40% of the mice, while both the LMW-E-T1 and LMW-E-T2 groups formed metastases in 100% of the mice ($n = 10$ for each group) (Bales, 2005). Thus, LMW-E, more so than cyclin EL, is able to

lead to an increase in metastasis in both the breast cancer and melanoma model systems. However, this dissertation will be focusing on the role of cyclin E in the initial stages of tumorigenesis: transformation and anchorage-independent growth.

CYCLIN E AND ANCHORAGE-INDEPENDENT GROWTH

One of the factors that sets apart a transformed cell from a non-transformed cell is anchorage-independent growth (Kang, 1996). The loss of anchorage-dependence correlates with the tumorigenic phenotype *in vivo* (Shin, 1975). Adherent cells that have undergone transformation are able to grow in suspension (anchorage-independent growth).

Cells that lack substrate adhesion (adherent cells cultured in suspension) tend to arrest in late G1 (Otsuka, 1975; Campisi, 1983; Guadagno, 1991; Han, 1993). Three G1 events occur in adherent cells which have been cultured in suspension that cause these cells to arrest in G1 (fail to grow in suspension): (1) decrease in cyclin E-CDK2 kinase activity, (2) decrease in Rb phosphorylation, and (3) a decrease in cyclin A expression (Fang, 1996; Kang, 1996). (1) Predictably, changes in cyclin E expression would alter the activity of the cyclin E/CDK2 complex. (2) Similarly, because cyclin E, when bound to CDK2, leads to hyper-phosphorylation of Rb, creating a positive feedback loop (Moroy, 2004), alterations in cyclin E could affect the phosphorylation of Rb, which could in turn affect anchorage-independent growth. (3) Likewise, cyclin E-CDK2 is upstream in the cell cycle, thus a decrease in cyclin A expression is likely a result of loss of cyclin E-CDK2 kinase activity (Fang, 1996). Therefore, according to these three necessary steps for non-transformed cells to be able to grow in suspension independently of anchorage, cyclin E can be linked to all three steps.

Cyclin E exhibits a role in Ras-induced transformation. Kang et al, in ER-1-2 (somatic cell mutant rat fibroblasts, which are defective in oncogene-mediated, anchorage-independent growth), PKC3-F4 (non-transformed fibroblasts that only grow in suspension upon oncogene-induction, i.e. RasV12), and NIH3T3 (mouse embryonic fibroblasts) all non-transformed fibroblasts, showed that Ras induced-transformation induced cyclin E kinase activity. When ER-1-2, PKC3-F4, and NIH3T3 cells were grown in suspension, these cells arrested in G1 phase, had undetectable phospho-Rb levels, and showed no cyclin E-CDK2 kinase activity as demonstrated using a kinase assay with histone H1 as the substrate. However, upon exogenous Ras expression, PKC3-F4 and NIH3T3 cells not only grew in the suspended state but induced cyclin E-CDK2 kinase activity and Rb phosphorylation (Kang, 1996). Similarly, Haas et al found that exogenous expression of the CDK-inhibitors, p21 and p27, abrogated

cyclin E/HA-Ras-induced transformation as determined by loss of foci formation (Haas, 1997). Also, cyclin E1^{-/-}E2^{-/-} MEFs showed resistance to oncogenic transformation (as mentioned above) (Geng, 2003). Thus, an adherent characteristic, such as cyclin E-CDK2 kinase activity is implicated in transformation.

Both CDK2- dependent and CDK2-independent cyclin E-associated activity has been implicated in transformation. In a Science paper, Fang et al showed that the decreased cyclin E-CDK2 activity that was observed in cells that were arrested by being grown in suspension was due to increases in the CDK-inhibitors, p21 and p27. In KD untransformed human diploid fibroblasts, Fang et al synchronized these cells in G₀ by serum starvation and placed the cells in suspension culture. Upon re-addition of growth factors, the arrested, suspended KD cells failed to undergo DNA synthesis, implying that arrest of suspended cells occurs in G1 phase. In both KD and IMR90 untransformed human diploid fibroblasts, the protein levels of cyclin E and CDK2 were unchanged between the adherent and suspended cells. However, the cyclin E/CDK2 kinase activities were lower in the suspended cells than the attached cells. Similarly, HUT12 cells, an anchorage-independent variant of KD cells, showed similar levels of cyclin E and CDK2 kinase activity when attached or adherent. Although CAK is responsible for phosphorylation and activation of cyclin E-CDK2, no changes in CAK levels or activity were observed. The suspended KD and IMR90 cells did however reveal a four-fold increase in the CDK-inhibitors p21 and p27, but not p57 (Fang, 1996), indicating the inhibition of cyclin E/CDK2 activity was responsible for the arrest observed in the G1 phase of the cell cycle. This goes along with the finding by Haas et al that p21 and p27 expression abrogated cyclin E/HA-Ras-induced transformation (Haas, 1997). Thus, the decrease in cyclin E-CDK2 activity was most likely due to this increase in p21 and p27. However, the transformed HUT12 cells exhibited similar elevated levels of p21 and p27, but the higher levels of cyclin E and CDK2 were able to overcome this inhibition (Fang, 1996).

Since the inhibitory roles of p21 and p27 are highly implicated in cyclin E's role in transformation, it seems likely that LMW-E would exhibit increased anchorage-independent growth. As previously stated, research in our laboratory has shown that LMW-E binds p21 and p27 but is resistant to the inhibitory effects of these CDK inhibitors. In fact, the IC₅₀ value of p21 necessary to decrease the cyclin E-associated kinase activity, as determined by a GST-Rb kinase assay, for the full length form of cyclin E was 25 nM, while the IC₅₀ values for LMW-E-T1 and LMW-E-T2 were 125 nM and 105 nM, respectively. While similar results were observed with p27, the exact IC₅₀ values for p27 were not published. Thus, 5-fold more p21 and p27 is necessary to inhibit CDK2/LMW-E activity as compared to CDK2/cyclin EL (Akli,

2004; Wingate, 2005). As discussed previously, Akli et al in our lab has shown that LMW-E transgenic mice exhibit increased incidence of breast cancer development as compared to the full length cyclin E transgenic mice (Akli, 2007). Thus LMW-E could potentially lead to transformation and anchorage-independent growth by evading the inhibitory effects of p21 and p27 up-regulation. While up to this point, active CDK2 has been shown to be necessary for induction of anchorage-independent growth, cyclin E is able to play a role in transformation that is not dependent on CDK2.

Cyclin E could also have a role in transformation that is CDK2-independent. Cyclin E is necessary for Ras-induced transformation. However, kinase-dead cyclin E expressing REFs (rat embryo fibroblasts) still exhibited Ras-induced transformation (Geisen, 2002), thus implying that Ras induction of transformation is dependent on cyclin E, but independent of cyclin E's binding to CDK2. In fact, using a series of truncated forms of cyclin E, Geisen et al showed that mutant cyclin E (which was unable to bind CDK2) was (in cooperation with HA-Ras) able to induce transformation of rat embryo fibroblasts, as measured by foci formation (Geisen, 2002). Thus, in REFs, cyclin E (independently of CDK2 binding) was able to lead to foci formation, an indication of cyclin E's potential to induce transformation. However, in the Geisen study, no *in vivo* analysis of transformation was investigated. Thus, it is highly probable that cyclin E can be involved in transformation and anchorage-independent growth independently of CDK2 binding and activity.

Thus cyclin E plays a critical role in oncogenic transformation, and the effect of cyclin E on anchorage-independent growth could be CDK2-dependent or CDK2-independent. Also, Ras mediated transformation seems to be at least partially through an increase in cyclin E-CDK2 activity and Rb phosphorylation.

PKC ι

NOTE

Due to the high homology between the two atypical PKC family members, PKC ι and PKC ζ , many early papers studying PKC ι and PKC ζ do not differentiate between the two isoforms. Thus, when these two isoforms are not distinguished, they will collectively be referred to as aPKC. Also, the mouse homolog for PKC ι is PKC λ . Thus, in mouse studies, PKC ι will be referred to as PKC ι /lambda.

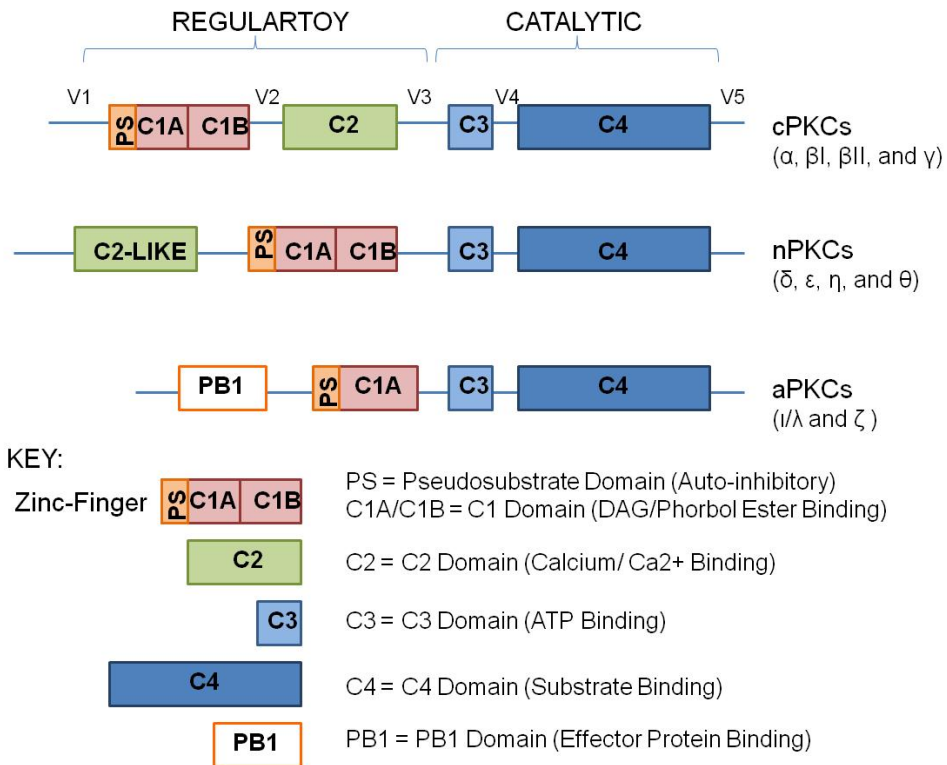
Species	Alternative Names
Humans	PKC ι (or aPKC if not differentiated from PKC ζ)
Mice/Rats	PKC λ
Flies	DaPKC

PKC FAMILY OF SERINE/THREONINE KINASES

The PKC (protein kinase C) family of serine/threonine kinases is involved in various signal transduction cascades and is currently comprised of ten isoforms (Martelli, 2003). To date, the PKC isoforms reside in the cytosol and upon activation translocate to the plasma membrane (Martelli, 2003). These ten isoforms are clustered into three categories: the conventional, novel, and atypical isoforms (see Figure 4). The conventional PKCs (cPKCs) are comprised of α , β I, β II, and γ , and are activated by calcium (Ca^{2+}), diacylglycerol (DAG), and the membrane lipid phosphatidylserine (PS). The novel PKCs (nPKCs) are comprised of δ , ϵ , η , and θ , and are activated by DAG and PS. The third class of the PKC family, the atypical PKCs (aPKCs) will be the focus of this dissertation. The atypical PKCs are comprised of ι/λ and ζ (Newton, 1997).

As the name implies, the atypical PKCs are regulated very differently than the conventional or novel PKCs. The atypical PKCs are not directly regulated by Ca^{2+} , DAG, or phorbol esters (Nishizuka, 1995). Specifically, because of PKC ι 's (PKC ι/λ) homology to PKC ζ (PKC ζ), the PKC ι isoform was found to not be regulated by diacylglycerol (DAG) (Selbie, 1993). While other members of the PKC family bind to phorbol esters (excluding the other atypical PKC family member, PKC ζ), PKC ι /lambda, as determined using a phorbol ester binding assay, did not bind to phorbol esters (Akimoto, 1994). Also, both aPKC isoforms lacked the N-terminal C2 domain and part of the C1 domain, which accounted for aPKC's lack of activation by DAG and Ca^{2+} (see Figure 4). Interestingly, in place of the C1/C2 domains, the aPKCs contain a PB1 domain, allowing these specific isoforms to form protein:protein (PB1:PB1) interactions with effector proteins (Hirano, 2004). See "PKC ι -Binding Partners" section for great detail.

Figure 4: PKC Family of Serine/Threonine Kinases



Class	PKC isoform	Activated By:
Conventional (cPKC)	α(Alpha) βI(Beta I) βII(Beta II) γ(Gamma)	Calcium (Ca ²⁺) Diacylglycerol (DAG) The membrane lipid phosphatidylserine (PS)
Novel (nPKC)	δ(Delta) ε(Epsilon) η(Eta) θ(Theta)	DAG PS
Atypical (aPKC)	ι/λ(Iota/Lambda) ζ(Zeta)	Effector Molecules ie p62, PAR-6:PAR-3, MEK5, CDC42

INITIAL DISCOVERY OF PKC IOTA/LAMBDA AND CHARACTERISTICS

PKC ι is a serine/threonine kinase in the PKC family. PKC ι was first identified by PCR using loose primers against the highly conserved catalytic domain of other PKC family members (Selbie, 1993). Specifically, the PKC ι gene encodes for a 587-amino acid coding region which gives rise to a 65-kDa band (western blot analysis) first identified using an antibody to the C-terminus of PKC ζ (this region is conserved in PKC ι) (Selbie, 1993). A year later, the mouse homolog (PKC lambda) was identified using a mouse P19 embryonal carcinoma cells and mouse brain (Akimoto, 1994). The PKC ι / lambda gene gave rise to a 74-kDa protein, identified using an antiserum raised against a portion of PKC ι / lambda (Akimoto, 1994). Akimoto et al determined that PKC ι / lambda was autophosphorylated, thus possessing protein kinase activity (Akimoto, 1994). In PKC ι / lambda kinase assays, myelin basic protein (MBP) is used for the substrate due to the ability of PKC ι / lambda to phosphorylate MBP (Kazanietz, 1993). As mentioned, this dissertation will refer to PKC ι as such unless the study was conducted in mice, in which case, PKC ι will be referred to as PKC ι /lambda.

The structure of the aPKCs compared to the nPKCs and cPKCs is quite different (see Figure 4). Like the nPKCs and cPKCs, the aPKCs contain a catalytic subunit comprised of the C3 ATP-binding site and the C4 substrate binding site (Tan, 2003). The crystal structure of the catalytic domain of PKC ι revealed that the PKC ι ATP-binding site resembled that of PKB/AKT but not PKA (Messerschmidt, 2005). However, unlike the nPKCs and cPKCs, the aPKCs contain a distinct PB1 domain and a truncated C1 domain (Hirano, 2004). The PB1 domain allows the aPKCs to bind effector molecules.

PKC ι is able to traffic to and from the nucleus. For the most part, PKC ι is localized to either the nucleus or cytosol and upon activation is translocated to the plasma membrane (in polarized cells). However, PKC ι behaves differently in 2D culture versus polarized tissues. In 2D culture, PKC ι is located in the nucleus, but upon activation moves to the cytosol. For example, PKC ι /lambda translocates from the nucleus to the cytoplasm upon PDGF or EGF stimulation (Akimoto, 1996). PKC ι was found to contain canonical NLS (nuclear localization sequence) and NES (nuclear export sequence) sequences allowing the trafficking between the two parts of the cell. Specifically, in HeLa and HEK293 cells, PKC ι /lambda was shown to traffic to and from the nucleus. Localization was visualized by using GFP-fused PKC ι / lambda constructs (and mutant PKC ι / lambda constructs). While wild-type PKC ι / lambda localized in the cytoplasm, the kinase-dead and activation loop

mutant both localized in the nucleus. A canonical NLS and CRM1-dependent leucine-rich NES were identified using truncated forms of PKC ι /lambda. The NLS was mapped to the N-terminal portion of the zinc-finger domain, and the NES was mapped to the linker region, which is located between the catalytic and the zinc-finger domains. Experiments using Leptomycin B, which blocks nuclear export by inhibiting the exportin protein CRM1/exportin1, revealed that PKC ι /lambda possessed the ability to traffic to and from the nucleus (Perander, 2001).

THE ATYPICAL PKCS: PKC IOTA/LAMBDA VERSUS PKC ZETA

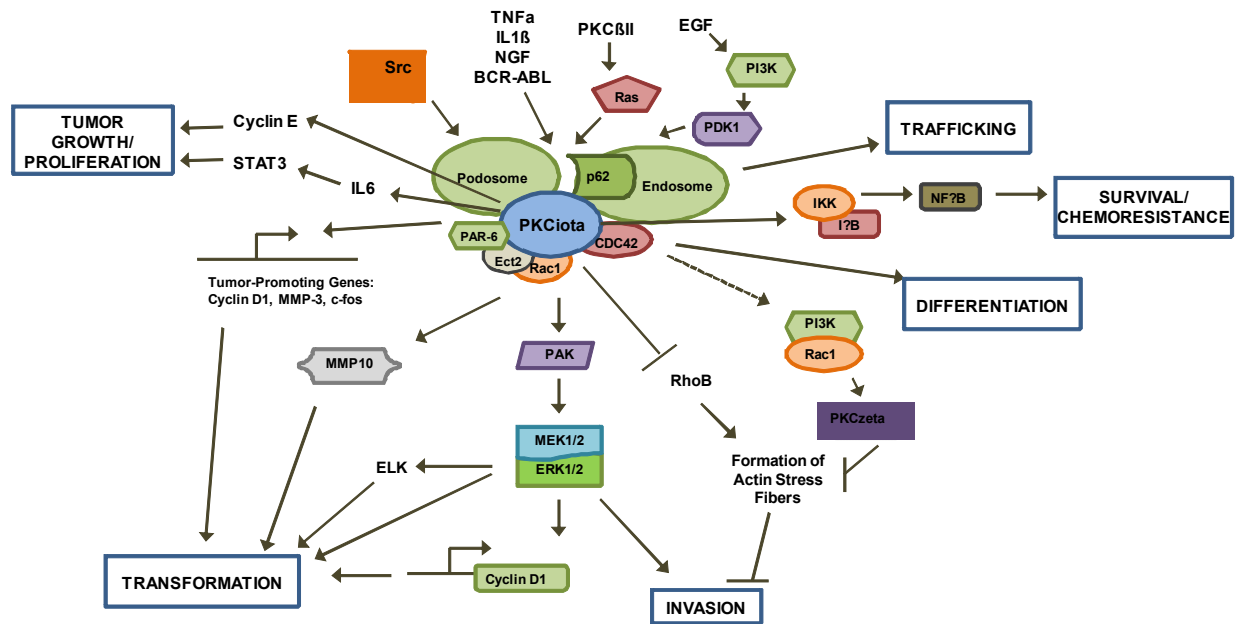
PKC ι /lambda most closely resembled PKC's lone atypical member, PKCzeta, with overall homology of 72% and catalytic domain homology of 84% (Selbie, 1993; Akimoto, 1994). After PKCzeta, the closest that PKC ι has homology to other PKC family members is less than 53% (Selbie, 1993). PKC ι shares similarities with PKCzeta, having a highly conserved pseudosubstrate domain and only a single cysteine-rich, zinc finger-like region (C1) (Selbie, 1993). Like PKCzeta, PKC ι lacks an apparent Ca²⁺-binding region (C2) (Selbie, 1993). As expected, PKC ι and PKCzeta, since they are so homologous, can have functional redundancies. For example, PKC ι and PKCzeta show functional redundancy during vertebrate (zebrafish) retinogenesis in several aspects, including "mitotic division location and orientation, cell-type positioning, and retinal pigment epithelial (RPE) and photoreceptor cell morphogenesis" (Cui, 2007). However, PKC ι and PKCzeta also have distinct roles, either through PB1 protein-binding or through distinguishing localization.

PKC ι and PKCzeta can also have the same role but differential expression patterns leading to a loss of functional redundancy. For example, PKC ι /lambda and PKCzeta exhibit extremely different gene expression patterns during mouse embryogenesis (Kovac, 2007). Additionally, each has alternate cellular localization is demonstrated in the mouse epidermis. By examination of the mouse epidermis, which exhibits apical-basal polarity, Helfrich et al found that both PKC ι /lambda and PKCzeta are expressed in these cells, but only PKC ι /lambda is present at the tight junctions (Helfrich, 2007). Thus, in these particular epithelial cells, PKC ι /lambda and PKCzeta do not exhibit functional redundancy due to unique localization.

PKC ι and PKCzeta can activate the same effector molecule but through different pathways. Thus is the case in Ras-induced c-fos expression. Ras through PKC ι is able to transcriptionally up-regulate c-fos, a tumor-promoting transcription factor. The PKC ι -dependent pathway seems to be Ras \rightarrow PKC ι /lambda \rightarrow c-fos transcriptional activation.

However, the Raf/PKCzeta pathway appears to be Ras → Raf-1 → MEK → PKCzeta (but not PKCdelta/ lambda) → c-fos transcriptional activation (Kampfer, 1998). See figure OPP (Overall PKCdelta Pathway) for a complete overview of the upstream and downstream proteins involved in PKCdelta signaling.

Figure OPP: Overall PKC α Pathway



PKC ι and PKC ζ can also function in the same pathway but at different locations in that pathway. Thus is the case of Ras-induced cyclin D1 transcriptional activation and actin remodeling, both of which lead to transformation. Both of these pathways indicate that PKC ι is upstream of PKC ζ : Ras \rightarrow PKC ι \rightarrow Rac1 \rightarrow PKC ζ \rightarrow cyclin D1 induction (Kampfer, 2001) and Ras \rightarrow PKC ι \rightarrow Rac1 \rightarrow PKC ζ \rightarrow actin remodeling (Uberall, 1999) (see OPP figure).

PKC ι and PKC ζ can also exhibit isoforms-specific functions. For example, in order to determine the roles of PKC ι versus PKC ζ in the innate immune response, Soloff et al generated a PKC ι /lambda knockout mouse. The PKC ι / lambda knockout mouse was embryonic lethal at day 9, however, they were able to generate a chimera composed of the PKC ι /lambda^{-/-} embryonic stem cells and blastocysts. In the PKC ι /lambda^{-/-} chimera, Soloff et al found that these cells responded normally to the innate immune response, i.e. TNF α serum, EGF, IL-1, or LPS stimulation. Thus, Soloff et al concluded that PKC ι had more of a role in embryogenesis and polarity than the innate immune response (Soloff, 2004). Unfortunately, the authors never did these studies in a PKC ζ knockout mouse model (or PKC ι /lambda^{-/-}PKC ζ ^{-/-} mouse), which would better rule out the possibility of redundancy among the atypical PKCs. In another study, PKC ι and PKC ζ were found to have different characteristics. While caspase-3 cleaved PKC ζ upon UV-irradiation in HeLa cells, PKC ι / lambda was not cleaved in this manner. Mutating the caspase-3 cleavage site in PKC ζ resulted in reduce apoptosis (Frutos, 1999).

An example of PKC ι and PKC ζ having the same role but under different conditions is in the case of Na⁺/K⁺ATPase activity, where PKC ι and PKC ζ have opposing roles. Dominant negative PKC ζ inhibited Na⁺/K⁺ATPase activity in serum-starved MCF7 cells stimulated with AngII. However, using MCF7 cells cultured in 10% serum, PKC ζ activity was not changed upon stimulation with AngII; nor did the Na⁺/K⁺ATPase activity change upon expression of a dominant negative PKC ζ . However, PKC ι antisense was able to block AngII-induced Na⁺/K⁺ATPase activity in MCF7 cells cultures in 10% serum. Thus, PKC ι is involved in Na⁺/K⁺ATPase activity under high growth factor conditions, while PKC ζ is involved in Na⁺/K⁺ATPase activity under quiescent conditions (Muscella, 2005).

PKC ι and PKC ζ are not ubiquitously expressed. Thus, the activation of these two PKC isoforms can be dependent on their expression. In DU-145 prostate cancer cells, PKC ι was able to phosphorylate IKK β , a protein that upon phosphorylation gets degraded

allowing NF κ B to translocate to the nucleus. However, in RWPE-1 prostate cancer cells, the kinase responsible for IKK β phosphorylation is PKCzeta (Win, 2008).

Thus, PKC ι and PKCzeta can show similar effects but their roles may be distinct because of unique localization, different conditions of activation or expression, or through alternative binding (activating) partners (see Table 7).

PKC IOTA PHOSPHORYLATION/ ACTIVATION

The analysis of crystal structure of PKC ι revealed that it had two important phosphorylation sites. Phosphorylation of Thr403 occurred at the activation loop, while phosphorylation of Thr555 (an autophosphorylation site) was in the turn motif (Messerschmidt, 2005).

PKC ι is also activated by tyrosine phosphorylation. Src tyrosine phosphorylation of PKC ι is necessary for beta-COP recruitment to the pre-golgi intermediates (VTCs) (Tisdale, 2006) and to the endosome, where it binds p62 (Samuels, 2001). In PC12 cells that have been stimulated with NGF, PKC ι is phosphorylated by Src at three sites (tyrosines 256, 271, 325) which was inhibited in a dose-dependent manner by the Src inhibitors, PP2 and K252a. In response to NGF, Src phosphorylates PKC ι at tyrosines 256, 271, 325. However, site directed mutagenesis revealed that only Y325 phosphorylation by Src was required for PKC ι activation and subsequently NF κ B activation (Wooten, 2001).

By liquid chromatography-high accuracy mass spectrometry analysis, Macek et al observed several other potentially important phosphorylation sites, S217 and S237/S238 (possibly linked to improved stability/solubility) and S35/S37 (which is located in the PB1 domain thus implying the possibility of substrate specificity) (Macek, 2008). However, to date, the exact purpose of the phosphorylation of these sites has not been published.

Overall, PKC ι regulation occurs at the level of localization, where PKC ι is inactive in the cytosol and upon activation translocates to the plasma membrane (Martelli, 2003). PKC ι degradation is mediated by the E3 ubiquitin ligase, VCB-Cul2, which is a complex composed of pVHL (von Hippel-Lindau), a hypoxic induced tumor suppressor gene, elongins B and C, Cul-2, and Rbx1. Overexpression of pVHL in HEK293 cells resulted in an increase in PKC ι degradation, and an *in vitro* ubiquitination assay revealed the components of the VCB-Cul-2 complex were sufficient for PKC ι ubiquitination (Okuda, 2001).

PKC IOTA HAS DIVERSE FUNCTIONS.

PKC ι /lambda is a highly conserved polarity protein involved in development of multicellular organisms (Suzuki, 2003). PKC ι /lambda has also been shown to be evolutionarily conserved, being expressed in three strains of baker's yeast extracts, wheat germ, *C. elegans*, goldfish, mice, rats, chickens, rabbits, guinea pigs, and monkeys, just to name a few (Kuo, 1995; Izumi, 1998; McCord, 1996).

PKC ι is involved from initial stages of meiosis and embryogenesis to epithelial cell polarity and differentiation. PKC ι is expressed in various tissues throughout the body. In chickens, PKC ι was found to be expressed in mesenchymal connective tissue (the embryonic tissue that becomes connective tissue, bone, cartilage, the lymphatic system, or the circulatory system) of embryonic chick limb buds (Yang, 1998). In the initial discovery of PKC ι /lambda, it was identified by northern blot analysis to be expressed in brain, kidney, testis, and ovarian tissues (Akimoto, 1994). The retinas of several different species (rabbit, rat, guinea pig and goldfish) exhibited positive immunoreactivity for PKC ι /lambda (McCord, 1996). Also, PKC ι was expressed in rat testis (gonocytes) (Li, 1997), porcine ASM (airway smooth muscle) (Togashi, 1997), and in bovine parathyroid (and is translocated to the cell periphery among high Ca^{2+} levels- but not in low levels) (Onyango, 1999). In humans, PKC ι was expressed in kidney, heart, aorta, and vein samples in both the cytosolic (except the heart) and membrane fractions. In the latter study, PKC ζ expression patterns were the same as PKC ι , except that PKC ζ was only found in the cytosol of the kidney (but not the aorta or vein samples) (Erdbrugger, 1997). Oddly, PKC ι /lambda was found in the cytosolic fraction of the heart in rats (but not in humans) (Erdbrugger, 1997). Thus, PKC ι is expressed in several tissue types.

PKC ι is expressed in various cell types as well. However, PKC ι levels are highest in epithelial cell lines compared to fibroblasts, lymphoid, or monocytic cells (Bonizzi, 1999). PKC ι was also expressed in gonocytes (rat testis) (Li, 1997), in dermal papilla cells (the cells that make up the ridges of the skin) (Eicheler, 1997), adipocytes (make up adipose tissue), (Kotani, 1998), erythrocytes (the "most abundant cells in blood and carry out the vital function of oxygen transport") (Govekar, 2001), and in beta pancreas cells (important in glucose-stimulated insulin secretion) (Hashimoto, 2005; Yang, 2005).

Polarity (During Embryogenesis/ Development and Apical-Basal Polarity of Tissues)

PKC ι through binding to PAR-6 and PAR-3, which together make up the "Par" or "aPKC" polarity complex, is involved in asymmetric polarization of cells, at embryogenesis,

development, and epithelial sheet formation. This complex is conserved throughout species: from xenopus and zebrafish to mice and humans. PKCιota has been shown to localize near the polar bodies during oocytes maturation. Atypical PKC also plays a role in animal-vegetal polarity of oocytes of *Xenopus laevis*. Upon maturation of oocytes, either naturally or through addition of exogenous hormones, XaPKC (xenopus ortholog to aPKC) localizes to the animal region (near the nucleus, where the polar bodies are formed), where XaPKC plays a role in maturation, which is dependent on XaPKC kinase function (Nakaya, 2000).

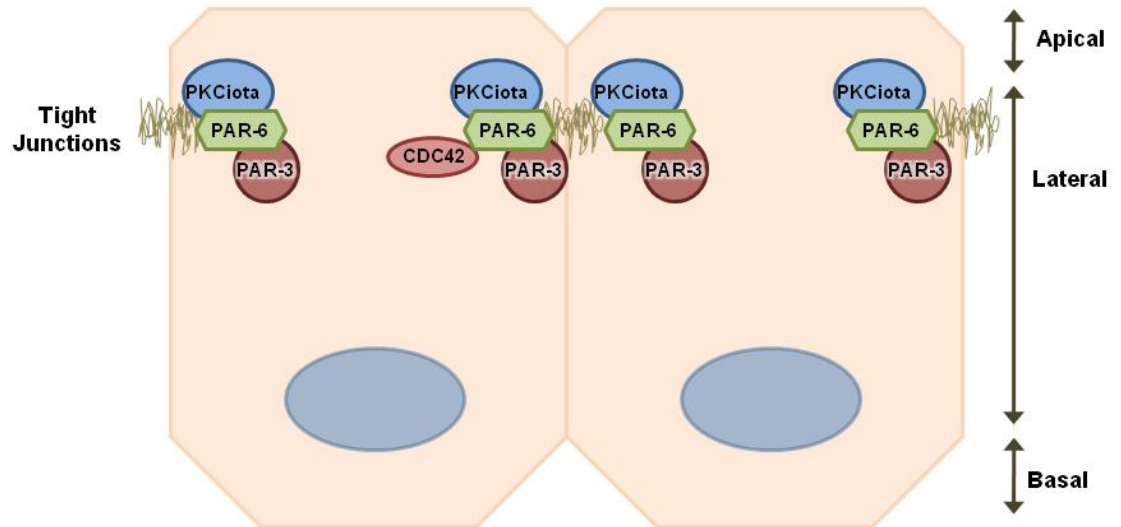
In zebrafish organogenesis, PKCιota/ λ is necessary for both the formation and the maintenance of tight junctions (zonula adherens) in early vertebrate epithelial development (Horne-Badovinac, 2001). Mutations in PKCιota/ λ in cardiogenic cells results in disruption of epithelial cell-cell interactions and cause the heart field to fuse improperly (Peterson, 2001).

PKCιota also plays a role in the polarity of the eye. For example, PKCιota overexpression in the drosophila eye can lead to loss of apical-basal polarity (Eder, 2005). Also, in a conditional knockout of PKCιota in differentiating photoreceptors in mice, severe laminar disorganization was exhibited in both the photoreceptor layer and the entire retina (Koike, 2005).

The intestinal epithelium is also dependent on PKCιota to maintain polarity and microvilli formation. In CACO-2 (human intestinal epithelial) cells and *in vivo*, PKCιota is able to directly phosphorylate Ezrin at T567. Ezrin is, like PKCιota, localized to the apical membrane upon activation. When PKCιota was silenced by RNAi, levels of Ezrin T567 phosphorylation decreased, as did the ability to form microvilli structures, a known role of active Ezrin. Thus, PKCιota also plays a role in microvilli formation in polarized intestinal epithelial cells (Wald, 2008).

PKCιota is involved in apical-basal polarity of asymmetrically polarized epithelial cells through its localization at tight junctions (along with PAR-3/ASIP and PAR-6) and interactions with the cytoskeleton. PKCιota levels are the highest in epithelial cell lines compared to fibroblasts, lymphoid, or monocytic cells (Bonizzi, 1999). Both PAR-3 (*C. elegans*) and ASIP (rats) contain PDZ domains, which were originally believed to allow these proteins to bind the atypical PKC members. However, later PAR-6 was determined to be the linker protein that allowed PKCιota and PAR-3 to co-localize (Noda, 2001). When PKCιota/ λ and ASIP are bound in rat intestinal epithelial cells, the complex localizes to the tight junctions, as determined by immunoelectron microscopy (Izumi, 1998) (see “PKCιota- Binding Partners: PB1 Domain” for details).

Figure 5: Apical-Basal Polarized Epithelial Cells



PKC ϵ is localized to the tight junctions of epithelial cells in several species (see Figure 5). For example, PKC ϵ / λ (XaPKC) and PAR-3/ASIP (XASIP) colocalize in *Xenopus laevis* epithelial cells at the *Xenopus* equivalent of tight junctions (Nakaya, 2000). Similarly, in zebrafish organogenesis, PKC ϵ / λ is necessary for both the establishment and maintenance of tight junctions (zonula adherens) (Horne-Badovinac, 2001). In canine MDCK cells (an excellent model system for apical-basal polarity), mutations in the PKC ϵ / λ binding site on PAR-3/ASIP blocked cell-cell contact-induced formation of tight junctions (Hirose, 2002). Lastly, in MTD1-A mouse mammary epithelial cells, dominant-negative PKC ϵ / λ was found to block maturation of spot-like junctions into belt-like adherens and tight junctions (Suzuki, 2002). Thus, regardless of species, PKC ϵ is localized to the tight junctions, where it plays a role in the establishment and maintenance of epithelial cell polarity.

Although PKC ϵ is one of the last proteins to be recruited to the tight junctions, PKC ϵ binding to the PAR-3/ASIP complex is necessary for the establishment of polarity in epithelial cells (Suzuki, 2002; Hirose, 2002). PKC ϵ is localized to the apical edge of tight junctions. In MDCK cells, mutations in the PKC ϵ / λ binding site on PAR-3/ASIP blocked cell-cell contact-induced formation of tight junctions. Also, PAR-3/ASIP is phosphorylated by PKC ϵ / λ at S827. As expected by PKC ϵ / λ localization, phospho-ser827 is localized to the apical edge during the formation of tight junctions (Hirose, 2002). Similarly, PAR-3/ASIP expression decreases the cytosolic PKC ϵ levels, recruiting PKC ϵ to tight junctions. Specifically, in 3T3-L1 adipocytes, overexpression of PAR-3/ASIP resulted in an increase in the PKC ϵ / λ levels in the LDM and PM fractions, but a decrease in the cytosolic fraction. Therefore, PAR-3/ASIP overexpression results in altered localization of PKC ϵ / λ (Kotani, 2000).

PKC ϵ has also been implicated in the maturation of tight junctions upon polarization. In MTD1-A mouse mammary epithelial cells, PKC ϵ / λ was examined upon repolarization by immunofluorescence (IF). Repolarization occurred when a monolayer of MTD1-A cells was scratched with a sterile needle and allowed to repolarize and wound heal. Upon repolarization as determined by IF, the spot-like adherens junctions (AJs) and tight junctions (TJs) form. PKC ϵ / λ is one of the last proteins to localize to the tight junctions, preceded by E-cadherin and ZO-1 at the adherens junctions and JAM, occludin, and claudin-1 at the tight junctions. Exogenous expression of a dominant-negative mutant PKC ϵ / λ construct revealed that PKC ϵ / λ was not necessary for the initial

recruitment of polarity proteins to the tight junction, but rather blocked cortical bundle formation which was required for maturation of spot-like junctions into belt-like adherens and tight junctions (Suzuki, 2002). Thus, PKC ι / λ plays a critical role in the formation of tight junctions in epithelial cells.

One of the roles of PKC ι at the tight junction involves regulation of zonula occludens 2 (ZO-2 or tight junction protein 2). PKC ι regulates ZO-2 through phosphorylation of ZO-2 at the tight junctions of polarized cells. ZO-2 is involved with the maintenance of cell polarity in epithelial cells through interactions between the plasma membrane and the actin cytoskeleton (Avila-Flores, 2001).

Apical-Basal Polarity and transformation- In cancer, amplification or mislocalization of PKC ι can occur and result in poor prognosis. Over-expression of oncogenic Ras or oxidants can lead to changes in the cytoskeleton. Many of these cytoskeletal alterations occur through PKC ι .

Using intestinal epithelial cells (Caco-2), which inducibly express either wild-type (wt) or dominant negative (dn) PKC ι / λ , Banan et al was able to show that PKC ι / λ was the key player in oxidant-induced disruption of the epithelial barrier. While dn PKC ι / λ expression blocked the effect of H₂O₂ (an oxidant) on the disruption of the epithelial barrier, wt PKC ι / λ exacerbated the effect of H₂O₂ on the epithelial barrier. Similarly, wt PKC ι / λ expression was independently able to cause effects that mimicked H₂O₂ treatment, i.e. cytoskeletal/ barrier hyperpermeability. Thus either oxidant exposure (through PKC ι / λ) or direct activation of PKC ι can lead to disassembly of the cytoskeleton through disruption of the integrity of the epithelial barrier, cytoskeletal disruption, and discord in the permeability of the monolayer barrier (Banan, 2005).

Ras, Rac, CDC42, PKC ι / λ , and PKC ζ have all individually been implicated in stress fiber loss and actin remodeling (Zhao, 1998; Uberall, 1999). Cells, which have undergone transformation, have alterations in their actin cytoskeleton, one of these alterations is a loss of stress fibers (Mackay, 1998). While Uberall et al linked Ras with PKC ι / λ and PKC ζ , Coghlan et al linked Ras-CDC42-PKC ι / λ in stress fiber loss. While Rac expression is able to cause stress fiber loss, this phenotype is through a different pathway than Ras-CDC42-PKC ι / λ pathway (Coghlan, 2000), a study that was contradicted by Uberall et al (Uberall, 1999) (see "PKC ι -Signaling: Ras" for details).

PKC ι is involved in stress fiber loss by its interaction with CDC42. In NIH3T3 cells, ectopic expression of constitutively active CDC42 (V12 CDC42) led to a loss in stress fibers

(Kozma, 1995). Coghlan et al was able to show that transient exogenous expression of PKC ι / λ (but not PKC ζ) was able to lead to the same loss of stress fibers observed in response to CDC42 overexpression. To show that PKC ι / λ was essential for CDC42-induced loss of stress fibers, kinase dead mutants of PKC ι / λ (KD PKC ι / λ) and PKC ζ (KD PKC ζ) were used to block CDC42-induced loss of stress fibers. The KD PKC ι mutant blocked CDC42- induced loss of stress fibers, but had no effect on CDC42- induced membrane ruffling or filopodia formation, indicating that while PKC ι / λ was essential for CDC42-induced loss of stress fibers, CDC42 induced other pathways independently of the PKC ι / λ or PKC ζ pathways. Because Rac-1 had also been implicated in loss of stress fibers, the KD PKC mutants were used to block loss of stress fibers in response to Rac. However, the KD PKC mutants were unable to block Rac-induced loss of stress fibers. Uberall et al had published that Ras-induced loss of stress fibers, was not only dependent on PKC ι / λ and PKC ζ , but also dependent on Rac, contrary to the finding of Coghlan et al. Coghlan et al was also able to link PKC ι / λ and PKC ζ to Ras-induced loss of stress fibers by use of the KD PKC mutants. However, contrary to the findings of Uberall, upon induction of NIH 3T3 cells with constitutively active Ras and either dominant negative CDC42 (N17 CDC42) or dominant negative Rac, only N17 CDC42, but not dominant negative Rac, was able to block Ras-induced loss of stress fibers. Thus, according to Coghlan et al at least two pathways exist for loss of stress fibers in transformation, one pathway involving Ras- CDC42- PKC ι / λ and one involving Rac (independently of Ras or the atypical PKCs). It is important to note that Coghlan et al did not directly test the KD PKC mutants on transformation just on indicators of transformation (loss of stress fibers).

Similarly, in NIH3T3 and COS fibroblasts, PKC ι / λ is essential for transforming RAS-mediated disassembly of actin fibers. PI3K had already been shown to be necessary for Ras-induced reorganization of the actin cytoskeleton (Rodriguez-Viciano, 1994). Upon addition of transforming Ha-RAS L61, re-organization of the cytoskeleton occurs. RAS activates PKC ι / λ , which in turn, activates PI3K/Rac-1, leading to disassembly of actin fibers. This role of PKC ι was demonstrated using both dominant negative PKC ι / λ (in the presence of transforming Ha-RAS L61) and constitutively active PKC ι / λ to mimic the effect of Ha-RAS L61 on cytoskeleton remodeling. Likewise, PKC ζ is involved in RAS-mediated cytoskeleton re-modeling, but unlike PKC ι / λ , PKC ζ acts downstream of PI3K/Rac-1 (Uberall, 1999). Thus, Ras signaling is involved in the remodeling of actin in a PKC ι -dependent (Ha-Ras L61 \rightarrow PKC ι / λ \rightarrow PI3K/Rac-1

→actin remodeling) and PKC ζ -independent (Ha-Ras L61 → PI3K/Rac-1 → PKC ζ → actin remodeling) manner.

Overexpression of PKC ζ results in alterations in polarity through cytoskeletal changes, from disruption of the integrity of the epithelial barrier to loss of actin stress fibers. Many model systems have been used to show these changes, and many other factors are involved (Ras, CDC42, Rac-1, and PI3K). Cytoskeletal changes are essential for the transformation process, thus demonstrating PKC ζ 's vital role in polarity and transformation. The role of PKC ζ in Ras-induced transformation, which is a large majority of the transformation studies, will be discussed in detail in a later section.

Membrane Trafficking- PKC ζ is also involved in membrane trafficking and microtubule dynamics in the early phase of the secretory pathway (which carries molecules between the endoplasmic reticulum (ER) and the Golgi). Upon stimulation with Rab2, a protein involved in protein sorting and recycling, PKC ζ is recruited to the pre-golgi intermediates (VTCs-vesicular tubular clusters), where it is involved in recruitment of other secretory proteins, such as β -COP. The phosphorylation of GAPDH, in the Src-PKC ζ -Rab2-GAPDH complex by PKC ζ is necessary for the early phase of the secretory pathway. The role of PKC ζ in this process is also to link these secretory proteins to the cytoskeleton, by recruitment of dynein (a microtubule motor protein), to the Rab2-GAPDH-PKC ζ complex. However, PKC ζ has also been implicated in other secretory pathways. For example, PKC ζ binds ProF, which has WD (tryptophan-aspartic acid dipeptide)-repeats and a propeller-FYVE domain (named for its identification in Fab1p, YOTB, Vac1p and EEA1- early endosome antigen 1) (Fritzius, 2006).

In NRK (normal rat kidney) cells, in response to stimulation by Rab2, both PKC ζ / λ and β -COP translocate to the membrane of the pre-golgi intermediates or VTCs in a dose-dependent manner. This association of PKC ζ / λ and β -COP at the VTCs was visualized by immunofluorescence. Antibodies against PKC ζ / λ but not against PKC α or γ (the only other PKC isoforms expressed in NRK microsomes) were able to reduce β -COP association with the VTCs. Also, VTC-associated β -COP levels are increased with PKC ζ / λ overexpression, indicating PKC ζ / λ lies upstream of β -COP recruitment to the VTCs in response to Rab2 (Tisdale, 2000).

Using a kinase dead mutant of PKC ζ / λ (His6-PKC ζ / λ K275W) in HeLa cells, Tisdale was able to show that the kinase activity of PKC ζ / λ , although not activated by Rab2, was necessary for Rab2 induced β -COP VTC association (Tisdale, 2000).

Thus Rab2, causes PKC ι / λ and β -COP recruitment to the VTCs, which is necessary for vesicle budding (Tisdale, 2000).

Rab2 recruits both PKC ι and GAPDH to the VTCs. Both PKC ι and GAPDH bind directly to Rab2 allowing PKC ι to interact with GAPDH. Initially, Tisdale et al found that upon stimulation by Rab2, PKC ι phosphorylates GAPDH at the VTCs, which is involved in microtubule dynamics in the early phase of the secretory pathway (Tisdale, 2002). A year later, Tisdale found that the phosphorylation of GAPDH by PKC ι in endoplasmic reticulum to golgi transport, is inhibited by the binding of the small GTPase, Rab2, at the vesicular tubular clusters (VTCs) (Tisdale, 2003).

Src is also involved in this role of PKC ι in VTCs. Tisdale et al found through a quantitative membrane binding assay that while PKC ι and GAPDH both interact with Src upon Rab2 stimulation, Src does not interact directly with Rab2. More importantly, Src tyrosine phosphorylation of PKC ι is necessary for β -COP recruitment to the pre-golgi intermediates (VTCs) (Tisdale, 2006). Thus Rab2, GAPDH, PKC ι , Src form a complex. Particularly, Rab2 binds directly to PKC ι and GAPDH, but Src binds only to PKC ι and GAPDH (Tisdale, 2006).

One of the roles of PKC ι in VTCs is recruitment of the microtubule motor protein, dynein to the Rab2-GAPDH-PKC ι complex (Tisdale, 2009). Thus, PKC ι is involved with protein trafficking through interaction with the VTC components coupling them to the re-organization of the cytoskeleton.

PKC ι recruitment to the endosome can also occur in response to NGF (nerve growth factor) stimulation. PKC ι must be phosphorylated by Src before it can be recruited to the endosome, where it binds p62 (Samuels, 2001) (Figure 15).

Inflammation- PKC ι plays a role in the inflammatory response through activation of NF κ B and thus activation of interleukins. For example, PKC ι in prostate cancer can induce IL-6 through NF κ B leading to both survival and growth in an autocrine manner (Ishiguro, 2009). While some of the complexes formed with PKC ι induce NF κ B activation, others inhibit it. The ability of PKC ι to activate NF κ B is through its interactions with the PKC ι binding partners LIP (lambda interacting protein) (Diaz-Meco, 1996) and p62 (Sanz, 1999). However, TNF α -induced NF κ B activation is blocked by the binding of PKC ι to PAR-4 (Diaz-Meco, 1999).

PKC ι has been shown to be induced in response to inflammation. In canine colonic circular muscle cells, PKC ι was up-regulated in response to inflammation induced by

mucosal exposure to both ethanol and acetic acid (Ali, 2002). Similarly, PKC ζ was found to be in a signalsome with TRAF6, p62, and IKK through a series of immunoprecipitations in response to Thymosin α 1, a treatment of cancer in clinical trials. This induction of the signalsome led to IL-6 expression through the Thymosin α 1 (Ta1) \rightarrow TRAF6/p62/PKC ζ /zeta/ IKK \rightarrow NF κ B \rightarrow IL-6 pathway (Zhang, 2005). Also, PKC ζ has been shown to be upregulated in response to the inflammatory cytokines TNF α and IL-1 β (Diaz-Meco, 1999; Anthonsen, 2001) and NGF (nerve growth factor) (Wooten, 2000; Wooten, 2001).

In Jurkat T-cells, PKC ζ binds the cytoplasmic portion of L-selectin. L-selectin, a leukocyte adhesion molecule, is involved in the inflammatory response by aiding in the leukocyte extravasation step (Vestweber, 1999). A PKC ζ pseudosubstrate (dominant negative) mutant blocked L-selectin phosphorylation thus implicating PKC ζ as an activator of L-selectin-mediated signal transduction (Kilian, 2004). Thus, PKC ζ interacts with L-selectin, a leukocyte receptor involved in extravasation, and leads to the phosphorylation of its cytoplasmic domain.

Loss of PKC ζ can result in impaired inflammatory response. For example, in PKC ζ knockout of activated T cells, Yang et al found that cytokine production in Th2 (T-helper) cells as well as NF κ B activation is blocked (Yang, 2009). Additionally, most studies used to identify the role of PKC ζ in NF κ B activation did so by using dominant negative, kinase dead, or knockouts of PKC ζ , which led to a decreased immune response.

Proliferation- While PKC ζ plays an established role in transformed growth, its role in proliferation is not as straight forward. It seems that PKC ζ can affect growth in a three-dimensional model, but is not as likely to influence two-dimensional adherent growth.

While PKC ζ is not cell cycle regulated, PKC ζ is able to regulate members of the cell cycle, including cyclin E, cyclin D1, AP-1 (c-fos and c-jun), and GSK3 β . Alteration in the cell cycle do not change PKC ζ levels. In C6 glioma cells, upon contact inhibition (induction of the quiescent state, Go) in the presence of serum, PKC ζ protein expression, unlike several other PKC family members, remained unchanged (Moreton, 1995).

PKC ζ , in glioma cells, was shown to phosphorylate and co-localize with CAK (or least CDK7). However, these two papers did not show convincing data, in regard to the co-localization experiments. The co-localization, which was extremely modest, was only upon stimulation with DMSO, so these papers lack physiological relevance (Acevedo-Duncan, 2002; Bicaku, 2005).

There are several studies which conclude that PKC ζ is not involved in cell proliferation: 1) Zhang et al examined the ability of PKC ζ to alter proliferation in three ovarian cancer cell lines (Zhang, 2006). In A1847 ovarian cancer cells, CMV-PKC ζ was transiently overexpressed, and changes in cell number as determined by growth curves were documented. No change was observed in proliferation between control and PKC ζ -overexpressing A1847 cells. Similarly, in 2008 and OVCAR10 ovarian cancer cell lines, PKC ζ was transiently silenced using RNAi, but no changes were observed in doubling time, as measured by growth curves (Zhang, 2006). Thus, in three ovarian cancer cell lines, A1847 and OVCAR10 (PKC ζ normal copy number) and 2008 (PKC ζ is genomically amplified), changes in PKC ζ levels alone did not exhibit an effect on cell proliferation in a two-dimensional model system.

2) In another study using a non-small cell lung cancer cells (NSCLC), PKC ζ (but not PKC η) was highly expressed. By use of wild type (wt) or kinase dead (kd) PKC ζ exogenous expression in A549 lung cancer cells, PKC ζ was determined to be expendable in proliferation of adherent cells in culture. However, these PKC ζ expressing stable cells revealed that PKC ζ was necessary for transformed growth, as determined by soft agar colony formation, and for tumor growth, as determined by measurement of tumor volume (Regala, 2005). Similarly, in H1299 and ChaGo lung cancer cells, expression of kinase dead PKC ζ resulted in decreased transformed growth, but no change in adherent cell proliferation (Regala, 2005). Lastly, both aurothioglucose-ATG (an aPKC-specific inhibitor) and kinase dead PKC ζ did not affect the rate of proliferation of adherent cells. This decrease in transformation of NSCLC cells in the presence of ATG was due to a disruption of the PKC ζ /PAR-6 complex, resulting in decrease in the level of active Rac1 (GTP versus GDP-bound) (Stallings-Mann, 2006). Thus, in NSCLC, PKC ζ is not likely involved in proliferation of adherent cells.

3) In human chronic myeloid leukemia cells (H562), while PKC ζ did not play a role in proliferation, PKC ζ protected these cells from apoptosis induced by OA (okadaic acid) and taxol. Inhibition of PKC ζ resulted in a re-sensitization of these cells to the apoptotic stimuli; thus indicating PKC ζ could play a role in survival rather than proliferation in leukemia (Murray, 1997).

Contrary to the studies described above which show lack of involvement of PKC ζ in cell proliferation, the following studies show that PKC ζ does play a role in proliferation.

1) In the first ovarian cancer study, by use of IHC in tissue samples of ovarian cystadenomas, borderline ovarian tumors, primary tumors, and recurrent invasive ovarian cancer that had been formalin-fixed and paraffin-embedded, Weichert et al found that PKCιota was expressed in half of the primary and recurrent tumors, but not in the cystadenomas or borderline tumors, thus implicating the role of PKCιota in the transformation stage of tumorigenesis. PKCιota showed a positive correlation with histopathological grading, FIGO stage, and the proliferation index. Specifically, in primary ovarian tumors, the expression of PKCιota exhibited a significant correlation with a reduced median survival time (Weichert, 2003).

2) In the second ovarian cancer study, PKCιota was genomically amplified leading to its over expression in ovarian cancer. In serous ovarian carcinomas, an increase in PKCιota DNA copy number correlated with a decreased progression free survival. In a drosophila eye, which was used as a model of epithelial cell apical-basal polarity and normal PKCιota localization, overexpression of constitutively active PKCιota (DaPKM and rPKCζ) resulted in a disruption in polarity, an increase in proliferation, and an increase in cyclin E levels (Eder, 2005).

3) In HL-60 (human promyelocytic leukemia) cells, upon stimulation with granulocyte colony-stimulating factor (G-CSF), a cytokine and growth factor involved with proliferation, differentiation, and survival, PKCιota translocated from the nucleus to the plasma membrane. Using PKCιota RNAi, Kanayasu-Toyoda et al was able to block proliferation in response to G-CSF and thus blocked differentiation (Kanayasu-Toyoda, 2007). Thus, PKCιota plays an important role in G-CSF-induced proliferation.

4) In the brain, PKCιota was differentially expressed in normal versus tumor tissues. Using western blot analysis of patient samples, Patel et al found that while PKCιota was not expressed in normal brain tissue, it was expressed in a majority of benign meningiomas and expressed in all malignant meningiomas and gliomas. Using T98G and U-138MG glioma cells, PKCιota RNAi resulted in a decrease in proliferation of these cells in culture (Patel, 2008). Thus, PKCιota is involved in the proliferation of malignant meningiomas and gliomas.

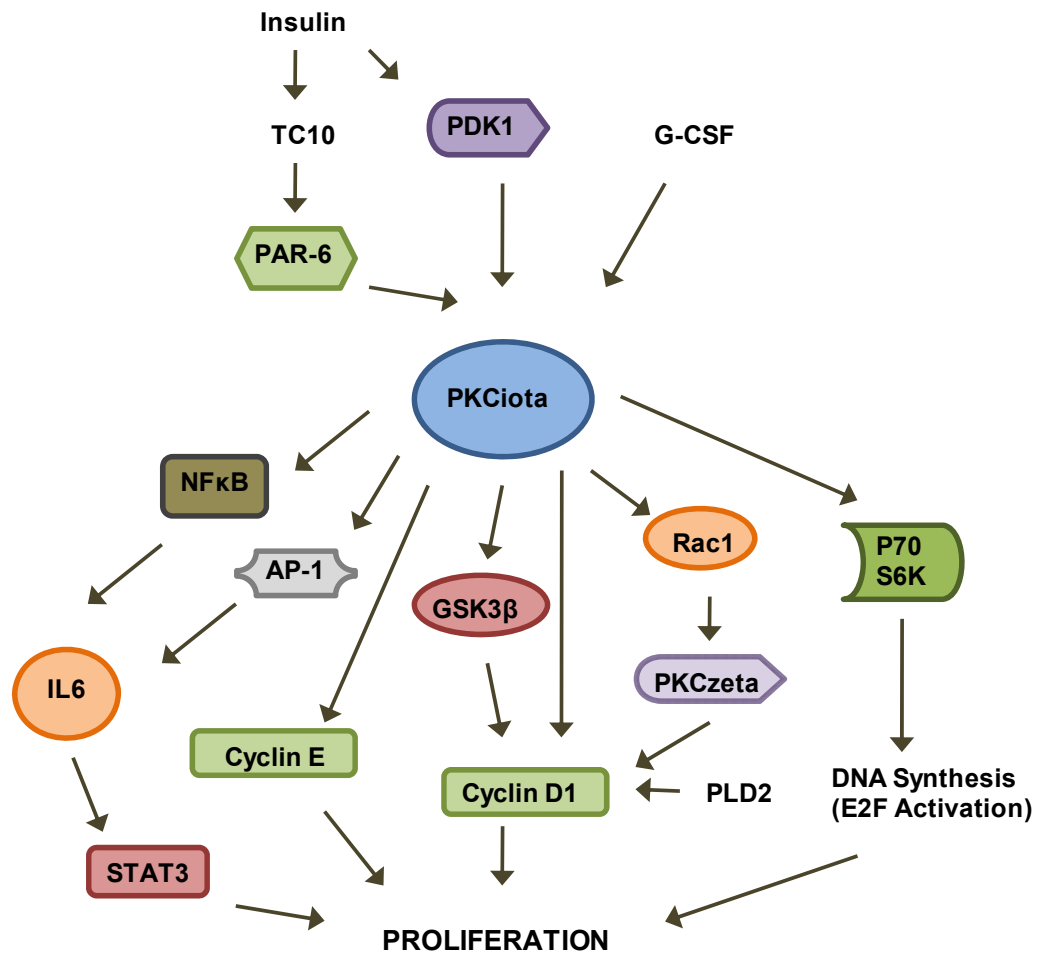
5) PKCιota has also been shown to correlate with the recurrence of hormone refractory prostate cancer (HRPC). Inhibition of PKCιota by RNAi resulted in decreased prostate cancer cell growth *in vitro* and decreased tumor volume *in vivo*. Ishiguro et al found that PKCιota was able to induce an autocrine loop of prostate cancer cell proliferation through the increased expression of IL-6. In prostate cancer cells, inhibition of PKCιota by RNAi suppressed STAT3

phosphorylation and IL-6 expression. Specifically, PKC α increased IL-6 expression through activation of the two transcription factors, AP-1 and NF κ B (Ishiguro, 2009).

While there is no doubt that PKC α can play an essential role in proliferation, the exact mechanism of this effect had not been investigated in two-dimensional versus three-dimensional culture conditions. All of the studies mentioned above, in which PKC α had no role in proliferation were *in vitro* studies, examining PKC α in a two-dimensional model on plastic culture dishes (growth curves), while many of the studies indicating PKC α is vital for proliferation were examined either *in vivo* or on IHC of patient samples examining BrdU incorporation or Ki67 staining. PKC α plays an essential role in three-dimensional polarity, as mentioned previously, and the absence of three-dimensional polarity observed in two-dimensional model systems may cause some of the functions of PKC α to be hindered, thus changes in PKC α in a two-dimensional model system would not change the rate of proliferation. While the polarity predicament may account for some, if not most, of the reasoning behind the discrepancies in the proliferation studies with PKC α , this does not account for all the discrepancies. In chapters 2 and 3 of this dissertation, I will show that PKC α causes alterations in proliferation using IGROV ovarian cancer cells in an *in vitro* two-dimensional model system, which would not display apical-basal polarity. When PKC α is knocked down in IGROV cells, the result is a decrease in the expression levels of several cell cycle regulator proteins, including cyclin E, which translates to a decrease in proliferation, even in two-dimensional culture. However, in another ovarian cancer cell line, OVCAR3, knockdown of PKC α does not change cyclin E levels, and inhibition of PKC α in this cell line does not lead to changes in proliferation. Thus, some of the discrepancies in the study of PKC α in proliferation could be due to downstream effector molecules, such as cyclin E, that may or may not be downstream of PKC α in every system investigated.

Another link between PKC α and cell proliferation has come about from a cell cycle regulatory prospective where several studies have found that PKC α to be upstream of modulators and effectors of the cell cycle (see Figure 6). For example, PKC α is involved in the regulation of **cyclin E**. The link between cyclin E and PKC α which is the topic of this dissertation was originally described by Eder et al as summarized on page (124-126).

Figure 6: Mechanism of PKC ι -Induced Proliferation



In another study using a hepatocellular carcinoma model, the expression of PKC ζ correlated with the expression of cyclin E (Wang, 2009). However, this correlation between PKC ζ and cyclin E was observed but no mechanism was determined.

PKC ζ has also been implicated in the Ras-Rac1-**cyclin D1** pathway and described in the section on Ras-Transformation. In HC11 mouse mammary epithelial cells, dominant negative mutants of PKC ζ / λ (or PKC ζ) suppress Ras-induced cyclin D1 expression, indicating that both PKC ζ / λ and PKC ζ are essential for cyclin D1 induction by oncogenic Ras. The induction of cyclin D1 was measured using a cyclin D1 luciferase reporter assay (Hellbert, 2000).

In a separate study, in HC11 mouse mammary epithelial cells, through the use of a cyclin D1 luciferase reporter gene, Mwanjewe et al was able to show that PLD2 and PKC ζ / λ cooperate in the transcriptional activation of cyclin D1 (Mwanjewe, 2001).

The transcriptional regulation of cyclin D1 by transforming Ras required two pathways, one PKC ζ -dependent and one PKC ζ -independent. By using constitutively active Ras, Raf, MEK, and a cyclin D1 reporter gene, in HC11 mouse mammary epithelial cells, Kampfer et al found that PKC ζ was not involved in Ras induction of cyclin D1 through the MEK-ERK pathway (however PKC ζ was required). By using constitutively active Ras, Rac, and a cyclin D1 reporter gene, PKC ζ was found to be downstream of Ras but upstream of Rac. Interestingly, PKC ζ was found to be involved downstream of Rac (Kampfer, 2001). Thus, Ras transcriptionally activates cyclin D1 in a PKC ζ -independent (HA-Ras \rightarrow MEK-1 \rightarrow ERKs \rightarrow cyclin D1 transcription) pathway and a PKC ζ -dependent (HA-Ras \rightarrow PKC ζ \rightarrow Rac \rightarrow cyclin D1 transcription) pathway.

PKC ζ is also able to activate **AP-1**, which transcriptionally regulates cyclin D1. While PKC ζ induction of cyclin D1 is downstream of Raf-1, as in the case of c-fos induction, PKC ζ / λ does not seem to be in the Raf-Mek pathway for cyclin D1 induction but rather in a pathway involving Rac1 (Hellbert, 2000). This is not surprising that the two pathways work separately in cyclin D1 induction. The cyclin D1 promoter had already been shown to contain an Ets2 binding site (activated by the Raf/MEK/ERK pathway) in addition to an AP-1 site.

In ovarian cancer, **GSK3 β** (glycogen synthase kinase 3 beta) leads to proliferation by regulating cyclin D1 (Cao, 2006). PKC ζ leads to activation of GSK3 β . In 3T3-L1 adipocytes, while PKC ζ was shown to be phosphorylated and activated by insulin induction via PI3K, only TC10 was able to cause PKC ζ to associate with the plasma membrane lipid rafts.

TC10 is a Ras-like small GTPase that in response to insulin causes PKC η to associate with PAR-3 and PAR-6 at the plasma membrane. Also, the ability of PKC η to phosphorylate GSK3 β required TC10 rather than PI3K, which led to GSK3 β phosphorylation via AKT (Kanzaki, 2004). Thus, AKT through the Insulin \rightarrow PI3K \rightarrow AKT \rightarrow P-GSK3 β pathway and PKC η through the Insulin \rightarrow TC10 \rightarrow PAR-6/PKC η \rightarrow P-GSK3 β pathway are able to lead to activation of GSK3 β (Kanzaki, 2004). Interestingly, Etienne-Manneville found that both PKC η and PAR-6 bind directly to GSK3, leading to reorganization of the cell, while PAR-3 was not necessary for the activation of GSK3 (Etienne-Manneville, 2001; Etienne-Manneville, 2003).

PKC η is involved in **DNA synthesis** in fibroblasts (Berra, 1993; Bjorkoy, 1997) and in COS and 293 cells (Akimoto, 1998). p70 S6K, a kinase involved in the PI3K pathway, is involved in cell cycle progress through DNA synthesis (Lane, 1993; Reinhard, 1994; Pullen, 1997; Alessi, 1998). p70 S6K binds to PKC η and PKC ζ via both the regulatory and kinase domains, although S6K is not a PKC η substrate. PKC η is required but not on its own sufficient to activate serum-induced DNA synthesis via S6K. In both COS and 293 cells, two dominant negative mutants (kinase dead and a truncated regulatory domain) of PKC η /lambda were able to suppress the ability of p70 S6K to lead to DNA synthesis in response to serum stimuli, determined by BrdU incorporation. However, a constitutively active form of PKC η was unable to stimulate p70 S6K-induced DNA synthesis, indicating that PKC η is required but not on its own sufficient for the activation of p70 S6K. Specifically, PKC η 's role in DNA synthesis appears to be through the Insulin \rightarrow PI3K \rightarrow PDK1 \rightarrow PKC η \rightarrow p70 S6K \rightarrow G1 to S phase progression (DNA synthesis and E2F activation) pathway (Akimoto, 1998).

While PKC η plays a role in proliferation, other downstream factors need to be examined. As previously stated, we have seen that IGROV cells through the PKC η /cyclin E pathway exhibit decreased proliferation in response to PKC η inhibition, while OVCAR3 cells, which do not display the PKC η /cyclin E pathway do not exhibit a decrease in proliferation in response to PKC η inhibition. Thus, it is likely that the effect of PKC η on proliferation is a consequence of modifications in the expression pattern of cyclin E, a well documented regulator of proliferation.

Differentiation- PKC η also plays a role in differentiation. The majority of research performed on PKC η in differentiation has been through NGF stimulation, which leads to both survival and differentiation through PKC η . However, other groups have also examined PKC η 's role in differentiation without NGF stimulation.

In HL-60 (human promyelocytic leukemia) cells, upon stimulation with granulocyte colony-stimulating factor (G-CSF), a cytokine and growth factor involved with proliferation, differentiation, and survival, PKCιota translocated from the nucleus to the plasma membrane. At 5 minutes post G-CSF-stimulation, PKCιota translocation occurred, while at 15 minutes post G-CSF-stimulation, PKCιota activation occurred. Using PKCιota RNAi, Kanayasu-Toyoda et al was able to block proliferation in response to G-CSF and thus blocked differentiation (Kanayasu-Toyoda, 2007). Thus, PKCιota plays an important role in G-CSF-induced proliferation and differentiation.

Conversely, a decrease in PKCιota has also been shown to correlate with differentiation. In undifferentiated mouse P19 embryonal carcinoma cells, PKCιota/ λ was the most abundantly expressed PKC family member; while the differentiation of these cells resulted in a decrease in PKCιota/ λ expression (Akimoto, 1994). Thus, in P19 embryonal carcinoma cells, PKCιota has the opposite effect on differentiation than in HL-60 human promyelocytic leukemia cells. This contradiction between PKCιota's role in differentiation is similar to the variance in the role of PKCιota in proliferation, and is likely due to similar reasoning.

Diseases- Diabetes, Alzheimers, Cancer- Alterations in PKCιota levels, localization, or activity can also lead to a number of other diseases. Particularly, aberrant PKCιota can lead to Alzheimer's disease, diabetes and insulin-resistance, inflammatory disease, and cancer. Diabetes and insulin-resistance are covered in full detail in the "PKCιota- Signaling: Insulin/ GLUT4 transport" section.

PKCιota has a potential role in Alzheimer's disease. In response to amyloid beta-peptide (ABP), a peptide linked to neuronal degeneration in Alzheimer's disease, PC12 neural cells undergo apoptosis. However, if PKCιota is overexpressed, PC12 cells display resistance to apoptosis. Thus, in Alzheimer's disease, PKCιota activation could be seen as a potential therapy for prevention of this age-related disease (Xie, 2000).

PKCιota has also been implicated in inflammatory disease. In human chondrocytes, PKCιota contributes to inflammatory joint disease through the pathway: IL1 → PKCιota → ERK/STAT3 phosphorylation → c-fos expression → collagenase gene induction → MMP1 (Litherland, 2010). PKCιota also has other implication in inflammation (see "PKCιota- Functions: Inflammation" and "PKCιota-Signaling: NFκB" for details).

PKCιota has briefly been studied in other diseases. For example, PKCιota is implicated in cardiac hypertrophy through the Npr1-PKC-MAPK-GATA1 pathway (Ellmers, 2007). Also, in

a PKC α /lambda mouse model, Sajan et al found that PKC α / lambda through activation of SREBP-1 (sterol receptor element binding protein-1c) and NF κ B is involved in hepatic lipogenesis, hyperlipidemia, as well as systemic insulin resistance (Sajan, 2009).

The role of PKC α in **cancer** is the one that has been studied the most- PKC α is located on chromosome 3q26, which is an amplicon in several types of cancer and they are described in detail in this section:

NSCLC (Non-Small Cell Lung Cancer)- PKC α is a *bona fide* oncogene in NSCLC. Regala et al found that PKC α (but not PKC ζ) was overexpressed in lung cancer patient primary tumor samples when compared to the normal adjacent lung epithelium. PKC α DNA copy number was amplified in a large majority of squamous cell carcinomas but not in lung adenocarcinoma. This increase in PKC α DNA copy number correlated with PKC α expression levels, which were also amplified in lung cancer patient primary tumor samples when compared to the normal adjacent lung epithelium. Importantly, overexpression of PKC α correlated with poor survival, which did not depend on the stage of the tumor. In H1299 and ChaGo lung cancer cells, expression of kinase dead PKC α resulted in decreased transformed growth, but no change in adherent cell proliferation. Thus, PKC α is an oncogene in NSCLC (Regala, 2005).

ESCC (Esophageal Squamous Cell Carcinoma)- PKC α is genomically amplified in several types of squamous cell carcinomas (SCCs). Using fluorescence in situ hybridization (FISH), Yang et al found that PKC α was genomically amplified in esophageal squamous cell carcinomas (ESCCs)- both patient samples (n =108) and ESCC cells lines (n = 9). In ESCCs examined, PKC α amplification was positively correlated (statistically significant) with tumor size, lymph node metastasis, and clinical stage. Also, PKC α genomic amplification was correlated with elevated PKC α protein expression. Using a tissue array, elevated PKC α protein expression was statistically correlated with lymph node metastasis and clinical stage (Yang, 2008).

Hepatic Metastasis of Colorectal Carcinoma- Examination of the expression levels of PKC α in patients with hepatic metastasis of colorectal carcinoma by qRT-PCR revealed that as compared to the paratumor mucosa, both the primary foci and hepatic metastatic foci exhibit elevated levels of PKC α . Also, the expression level of PKC α in the hepatic metastatic foci

was higher than in the primary foci (Li, 2004). Thus, PKC ζ mRNA expression levels correlated with aggressiveness of the tumor in colorectal carcinoma patients.

Malignant Meningioma and Glioma- In the brain, PKC ζ was differentially expressed in normal versus tumor tissues. Using western blot analysis of patient samples, Patel et al found that while PKC ζ was not expressed in normal brain tissue, it was expressed in a majority of benign meningiomas and expressed in all malignant meningiomas and gliomas. Using T98G and U-138MG glioma cells, PKC ζ RNAi resulted in a decrease in proliferation of these cells in culture (Patel, 2008).

Breast Cancer- Upon examination of 110 breast cancer patient samples by IHC, 80% showed elevated levels of PKC ζ . Specifically, PKC ζ expression was low in non-invasive DCIS (ductal carcinoma in situ) but high in invasive ductal carcinomas. In cancer patient samples, PKC ζ no longer localized to the apical membrane, but was present throughout the cytoplasm (Kojima, 2008). Thus, in breast cancer, PKC ζ expression correlates with the aggressiveness of the disease.

Colon Cancer- Upon induction of colon cancer in rats using azoxymethane, rats had higher levels of membrane-bound PKC ζ / λ and lower cytosolic levels. These levels were exacerbated by feeding the rats a high sugar diet and decreased when rats were given fish oil. Levels in PKC ζ / λ mRNA did not change; only the protein localization changed (Jiang, 1997).

HCC (Hepatocellular Carcinoma)- PKC ζ correlated with invasion potential in hepatocellular carcinoma. The expression level of PKC ζ in hepatocellular carcinoma is significantly higher than in the surrounding normal tissue, as determined by both qRT-PCR and IHC. Also, the expression levels of PKC ζ positively correlated with the invasive potential (Wang, 2009). Also, in paraffin-embedded hepatocellular carcinoma tumor tissues, PKC ζ was mislocalized to the cytoplasm. Cytoplasmic PKC ζ in hepatocellular carcinoma correlated with a reduction in cell-cell contact, through inhibition of adherens and tight junction formation, as well as a reduction in E-cadheren expression and an increase in cytoplasmic beta-catenin. Thus, the decrease in cell-cell contacts is likely responsible for the increase in the invasion and metastatic potential of HCCs (Du, 2009).

Gastric Cancer- PKC η expression was detected in over 70% of the 177 gastric cancer samples examined. The expression of PKC η , while it did not correlate with a decrease in E-cadherin, PKC η was determined to be a strong prognostic factor for recurrence along with presence of nodal metastases (Takagawa, 2010).

PDAC (Pancreatic Ductal Adenocarcinoma)- Ras is mutated in more than 90% of PDACs (Pancreatic Ductal Adeno-carcinomas). Predictably, examination of PKC η status in PDACs revealed that PKC η was also highly expressed, and its expression correlated with poor survival. *In vitro*, using PDAC cells, Scotti et al found that PKC η inhibition resulted in decreased transformation. Similarly, in a mouse model, Scotti found that PKC η inhibition resulted in decreased tumorigenesis (as well as a decrease in both angiogenesis and metastasis). The role of PKC η in pancreatic cancer transformation is also through the Rac-Pak-Mek-Erk pathway, indicating inhibition of PKC η -Par-6 binding may be a feasible target in pancreatic cancer treatment (Scotti, 2010).

Cholangiocarcinoma (Cancer of the Bile Ducts)- In cholangiocarcinoma (cancer of the bile ducts), PKC η , which was examined by immunohistochemistry, was expressed in tumors but absent in benign bile duct tissues. Similarly, PKC η correlated with the invasion potential of cholangiocarcinomas and negatively correlated with the expression of E-cadherin, whose loss of function/expression is involved in cancer progression and metastasis (Li, 2007; Li, 2008).

Glioblastoma Multiforme- PKC η plays a large role in invasion in glioblastoma multiforme, which is a primary brain tumor characterized by its highly invasive phenotype. Glioblastomas often have overactive PI3K signaling due to mutations in PTEN (tumor suppressor) or in EGFR (epidermal growth factor receptor). In U87MG cells, an increase in actin stress fibers as well as a decrease in both cell motility and invasion were observed when PKC η was silenced (RNAi). Through a gene expression microarray, RhoB was identified as being down-regulated by PKC η (Baldwin, 2008). Importantly, RhoB is essential for formation of actin stress fibers (Nobes, 1995). This inverse relationship between PKC η and RhoB was validated by both depletion and inhibition of PKC η , which resulted in increased RhoB expression. Interestingly, constitutively active RhoB expression repressed PKC η activation, thereby implying a switch between PKC η overexpression via PI3K (invasive) and RhoB (non-invasive). Thus, PKC η activation leads to an invasive phenotype in glioblastoma through downregulation of RhoB (Baldwin, 2008).

HRPC (Hormone Refractory Prostate Cancer)- PKC ζ has also been shown to correlate with the recurrence of hormone refractory prostate cancer (HRPC). Inhibition of PKC ζ by RNAi resulted in decreased prostate cancer cell growth *in vitro* and decreased tumor volume *in vivo*. In a PNAS paper, Ishiguro et al found that PKC ζ was able to induce an autocrine loop of prostate cancer cell proliferation through the increased expression of IL-6. In prostate cancer cells, inhibition of PKC ζ by RNAi suppressed STAT3 phosphorylation and IL-6 expression. Specifically, PKC ζ increased IL-6 expression through activation of the two transcription factors, AP-1 and NF κ B (Ishiguro, 2009). Also, fatty acids are involved in prostate cancer, and PKC ζ is involved in fatty acid induced proliferation in LNCaP prostate cancer cells (Pandian, 2001). Also in a human prostate cancer cell line, PC3U, PKC ζ was found to induce apoptosis in response to PKC ζ inhibition (ATM-aurothiomalate) in cancer cells, but not in human primary epithelial prostate cells, PrEC. ATM-induced apoptosis was determined to be through ERK, as determined by a rescue experiment using ERK RNAi with change in apoptosis as the readout (Trani, 2009). Therefore, PKC ζ plays a significant role in prostate cancer growth and provides a potential target to induce apoptosis in a tumor-specific manner.

Melanoma- Out of all eleven PKC isoforms examined, PKC ζ was the only isoform that was expressed in tumor but not normal lysates. Specifically, PKC ζ , while not expressed in normal melanocytes, was up-regulated in spontaneously-transformed melanoma cells, melanoma cell lines, tumor lysates, melanoma lymph node metastases, and cell lines established from these metastases. Thus, PKC ζ is implicated in melanocyte transformation (Selzer, 2002).

Leukemia (CML)- In K562 leukemia cells (CML), BCR-ABL is involved in chemo-resistance to apoptotic drugs, such as taxol. Upon treatment of K562 cells with taxol, PKC ζ became activated. Using tyrphostin AG957, which inhibits BCR-ABL, Jamieson et al was able to block taxol-induced PKC ζ activation and re-sensitize these cells to the apoptotic properties of taxol. Also, constitutively active PKC ζ was able to revert the chemo-sensitive phenotype of K562 cells treated with taxol and tyrphostin AG957 back to the chemo-resistant phenotype. Similarly, in a BCR-ABL negative and taxol-sensitive leukemia cell line (HL60), taxol was unable to induce PKC ζ activation, suggesting that PKC ζ would be downstream of BCR-ABL in response to taxol (Jamieson, 1999).

Table 6: Table of Types of Cancers in which PKC α was Examined

Tumor Type	Number of Patients	Number of Cell Lines	Method of Detection of PKC α	Metastasis	Outcome	References
NSCLC	74 NSCLC (37 LAC & 37 SCC)	4 NSCLC & 1 Non-Transformed Lung Epithelium	qRT-PCR (DNA & mRNA) IHC and Western Blot (protein)	ND	PKC α frequently amplified in SCC not in normal tissue PKC α mRNA/protein increased in tumor samples/ cell lines PKC α expression correlates with poor survival Ectopic PKC α did not alter proliferation <i>in vitro</i>	Regala, 2005
ESCC	108 ESCCs 180 ESCCs	9 ESCC Cell Lines	FISH (DNA copy number) Tissue Microarray and IHC	Significant Correlation	Significant correlation between PRKCI gene copy number and tumor size, lymph node metastasis, and clinical stage PKC α expression correlates with lymph node metastasis and higher stage	Yang, 2008
Colorectal Carcinoma	58 colorectal cancer patients (25 of them had hepatic metastasis)	N/A	qRT-PCR (mRNA)	Significant Correlation	PKC α levels were significantly increased in metastasis versus primary tumor	Li, 2004
Malignant Meningioma and Glioma	12 normal brain biopsies, 15 benign meningiomas, 3 malignant meningiomas and 3 gliomas	2 Glioma Cell Lines	Western Blot Analysis	ND	PKC α was expressed in malignant meningiomas and gliomas but not in normal brain tissue PKC α was involved in proliferation <i>in vitro</i>	Patel, 2008
Breast Cancer	110 Breast Cancer Patient Samples	N/A	IHC	ND	PKC α was elevated/mislocalized in IDC compared to DCIS	Kojima, 2008
HCC	43	N/A	qRT-PCR, Western Blot, IHC	ND	PKC α correlated with invasive potential Both PKC α and cyclin E significantly correlated with increased grade and lack of differentiation Significant correlation between PKC α expression and cyclin E expression in HCC	Wang, 2009
	43	N/A	PCR, Western Blot, IHC	Significant Correlation	PKC α levels are significantly higher in tumor samples compared with normal PKC α levels significantly correlated with increased tumor size, invasion, metastasis, and stage Metastasis: PKC α was elevated in 93% of lymph node metastasis (12 out of 13)	Du, 2009
Gastric Cancer	177	N/A	IHC	No Correlation	PKC α was elevated in 70% of gastric cancer patient samples PKC α expression significantly correlated with recurrence, a decrease in DSS and RFS Metastasis: PKC α expression was NOT correlated with metastasis	Takagawa, 2010
PDAC	28 Pancreatic Tumor Samples	10 Pancreatic Cancer Cell Lines Xenograft Model	qRT-PCR, IHC	PKC α RNAi significantly reduced metastasis	PKC α mRNA levels were higher than adjacent normal tissue in all 28 samples PKC α protein levels were elevated in tumor samples and significantly correlated with reduced survival <i>In vitro</i> , PKC α mRNA/protein was overexpressed in all cancer cell lines <i>In vitro</i> , PKC α RNAi did not alter rate of proliferation but did cause decrease in transformed growth <i>In vivo</i> , PKC α RNAi blocked pancreatic cancer proliferation and angiogenesis, but did not affect apoptosis Metastasis: PKC α RNAi significantly reduced metastasis to the kidney, liver, diaphragm, and mesentery	Scotti, 2010
Cholangiocarcinoma	9 Benign Bile Duct Tissues 35 Cholangiocarcinoma Samples	N/A	IHC	ND	PKC α expression was significantly higher in cholangiocarcinomas than in benign bile duct tissues PKC α expression was significantly negatively correlated with E-cadherin expression PKC α was significantly correlated with differentiation and invasion potential in cholangiocarcinoma samples <i>In vitro</i> , PKC α RNAi decreased cell migration and invasion via RhoB upregulation <i>In vitro</i> , PKC α 's role in migration/invasion is via the PI3K pathway	Li, 2007; Li 2008
Glioblastoma Multiforme	N/A	2 Glioblastoma Cell Lines	Western Blot Analysis	ND	Significant increase of PKC α mRNA levels in prostate cancer than adjacent normal tissue PKC α mRNA levels significantly correlated with PSA failure time (survival) <i>In vitro</i> , PKC α RNAi led to decreased proliferation <i>In vitro</i> , PKC α affected proliferation in an IL-6/STAT3 autocrine feedback loop <i>In vivo</i> , PKC α RNAi led to a significant decrease in tumor volume	Baldwin, 2008
Prostate Cancer	29 Prostate Cancer Tissue Samples	3 Prostate Cancer 1 Normal Prostate Epithelium Xenograft Model	qRT-PCR, IHC	ND	PKC α protein was overexpressed in all tumor samples and all cell lines analyzed	Ishiguro, 2009
Melanoma	8 (from various sites)	4 Melanoma Cell Lines	Western	ND	PKC α activity upregulated in response to Taxol leading to chemoresistance Bcr-Abl inhibitor inhibits PKC α kinase activity	Selzer, 2002
Leukemia	N/A	1 CML 1 PML	Western Blot Analysis Kinase Assay	ND	PKC α DNA copy number correlated significantly with poor survival	Jamieson, 1999
Ovarian Cancer	235 Grade 3 or Stage III or IV- Serous Epithelial Ovarian Cancer Specimens		High-Density Array Comparative Genomic Hybridization (DNA) qRT-PCR, IHC, Western Blot	ND	PKC α mRNA was highest in serous histology PKC α protein is mislocalized and overexpressed in ovarian cancer PKC α protein levels correlated with LMW-E expression	Eder, 2005
	89 Late-stage Primary Ovarian Cancer Specimens	18 Ovarian Cancer Cell Lines 6 HOSEs	Array Comparative Genomic Hybridization (DNA) qRT-PCR, IHC, Western Blot	ND	PKC α DNA copy number was increased in 43.8% of ovarian cancer patient samples PKC α expression is significantly increased in ovarian cancer compared to normal ovary PKC α mRNA was significantly higher in the ovarian cancer cell lines than the HOSEs PKC α mRNA was significantly higher in late stage tumor samples than early stage PKC α protein levels were significantly higher in the ovarian cancer cells than the normal tissue and HOSEs <i>In vitro</i> , PKC α RNAi did not affect proliferation in 3 cell lines <i>In vitro</i> , PKC α expression resulted in transformation of MOSEs PKC α levels did not correlate with drug resistance	Zhang, 2006

N/A	Non-Applicable
ND	Not Determined
NSCLC	Non-Small Cell Lung Cancer
LAC	Lung Adenocarcinoma
SCC	Squamous Cell Carcinoma
ESCC	Esophageal Squamous Cell Carcinoma
IDC	Invasive Ductal Carcinoma
DCIS	Ductal Carcinoma <i>in situ</i>
HCC	Hepatocellular Carcinoma
DSS	Disease-Specific Survival
RFS	Relapse-Free Survival
PDAC	Pancreatic Ductal Adenocarcinoma
Cholangiocarcinoma	Cancer of the Bile Ducts
CML	Chronic Myelogenous Leukemia
PML	Promyelocytic Leukemia
HOSE	Human Ovarian Surface Epithelium
MOSE	Mouse Ovarian Surface Epithelium

Mechanism of PKC α -induced Transformation- The presence of overexpressed, mutated, amplified, or mislocalized PKC α in the different tumor types underscores the importance of this protein in the transformation process. PKC α was found to be upregulated in many spontaneously-transformed cells, and PKC α is implicated in transformation of several types of cells, including melanocytes and lung and ovary epithelium (Selzer, 2002; Regala, 2005; Zhang, 2006). Because the details of the transformation experiments were discussed in detail for each individual type of cancer above, here I will briefly summarize the mechanism of PKC α -induced transformation.

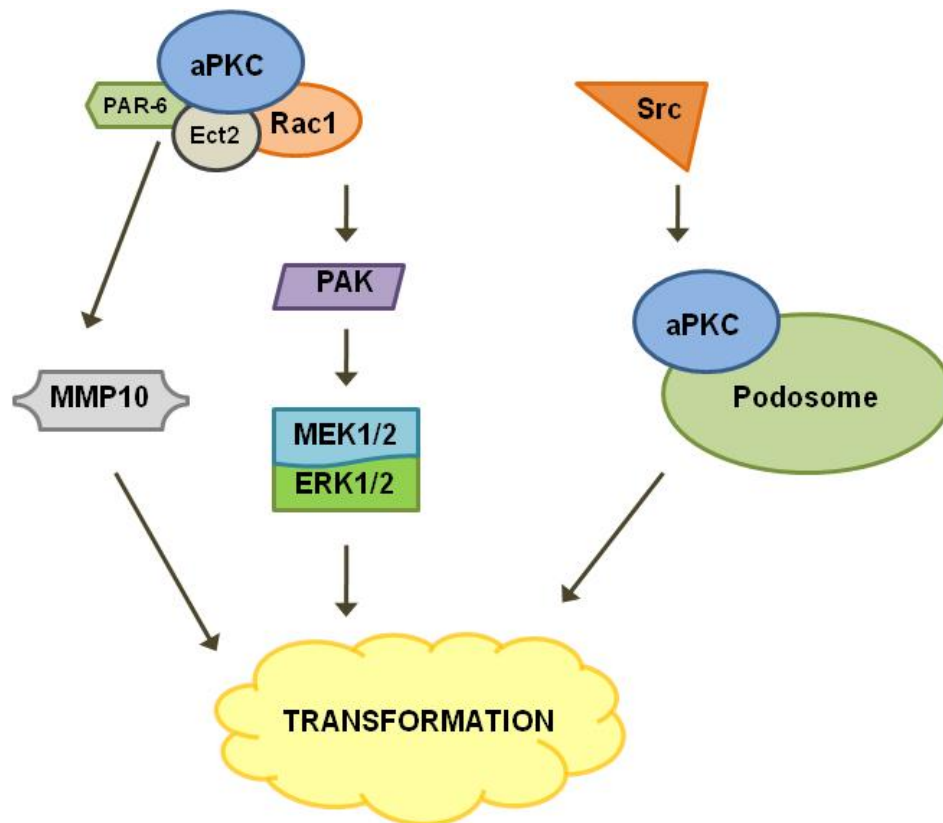
The consensus mechanism of PKC α -induced transformation involves the interaction of PKC α with PAR-6 and Rac1 (see Figure 7). Several studies in various cancer types have been performed to identify PKC α 's role in transformation. (1) In the A549 NSCLC cell line, ectopic wt PKC α but not dn PKC α (negative control) expression revealed that PKC α was necessary for transformation (soft-agar colony formation) and for tumor growth (measurement of tumor volume). Transformation via PKC α involved Rac1, and kd PKC α decreased P-MEK and P-ERK (western blot analysis). Similarly, the dn PKC α phenotype was rescued by constitutively active Rac1 (RacV12) (Regala, 2005). Thus, the pathway for PKC α -mediated transformed and tumor growth in NSCLC is most likely PKC α \rightarrow Rac1 \rightarrow Pak \rightarrow MEK1/2 \rightarrow ERK1/2. (2) PKC α is also involved in the transformation process in ovarian cancer. In murine ovarian surface epithelium (MOSEs), PKC α in cooperation with mutant Ras was able to induce transformation (soft-agar colony formation). Additionally, PKC α RNAi in ovarian cancer cells caused a reduction in anchorage-independent growth (Zhang, 2006). Thus, PKC α cooperates in Ras-induced transformation. Transformation by Ras will be covered in detail in the 'Ras' section below. (3) In NSCLC, PKC α was shown to activate Rac1 through its interaction with PAR-6 and Ect2. Ect2, which is commonly amplified, overexpressed, and correlated with PKC α expression in NSCLC, is a guanine NEF (nucleotide exchange factor) for Rho GTPases. Inhibition of Ect2 by RNAi (similar to PKC α inhibition) resulted in a decrease in transformation and tumorigenesis. PKC α or Ect2 inhibition was reverted upon ectopic expression of Rac1, indicating the role of PKC α in transformation is through the PKC α /PAR-6/Ect2 \rightarrow Rac1 \rightarrow Pak \rightarrow MEK1/2 \rightarrow ERK1/2 pathway in NSCLC (Justilien, 2009). (4) In PDAC cells, PKC α inhibition resulted in decreased transformation. Similarly, in a xenograft mouse model, PKC α inhibition resulted in decreased tumorigenesis. The role of PKC α in pancreatic cancer transformation was determined to be through the Rac-Pak-Mek-Erk pathway, indicating the PKC α -PAR-6

binding is also important in pancreatic cancer transformation (Scotti, 2010). Thus, the role of PKCιota in transformation is through the PAR-6/Rac1-MEK/ERK pathway (Figure 7).

Similarly, the PKCιota-PAR-6-Rac1 complex is also involved with transformation through activation of MMP-10, a matrix metalloproteinase, which breaks down the extracellular matrix (a process necessary for transformation/ cell invasion) (Figure 7). By use of PKCιota RNAi in NSCLC cells, Frederick et al was able to block transformation, as measured by soft-agar colony formation, and downregulate MMP-10 expression. Similar results were observed with RNAi to PAR-6 and Rac1 also resulting in downregulation of MMP-10 expression and decreased transformation. In PAR-6 deficient cells, overexpression of PAR-6, but not mutant PAR-6 that lacks the ability to bind PKCιota, is able to restore MMP-10 expression. Similarly, silencing MMP-10 also blocked transformation in these cells, proving that the decrease in MMP-10 expression due to downregulation of PKCιota, PAR-6, and Rac1 was not merely an artifact but rather the cause of the decrease in transformation. In a reverse experiment, constitutively active MMP-10 restored soft-agar colony formation in PKCιota silenced NSCLC cells. *In vivo*, dominant negative (dn) PKCιota blocked both MMP-10 expression and tumorigenicity. In NSCLC patient samples, PKCιota and MMP-10 expression is correlated, and expression of PKCιota or MMP-10 predicts poor survival. Thus, PKCιota leads to transformation via expression of the matrix metalloproteinase, MMP-10 (Frederick, 2008).

Use of inhibitors of PKCιota PB1:PB1 interactions (aurothiomalate-ATM and aurothioglucose-ATG) block transformation and therefore tumorigenesis. PKCιota binds to PAR-6 via PB1:PB1 interactions (see “PKCιota- Binding Partners: PB1 Domain” section for more details). Use of the PKCιota inhibitor, ATG blocked transformed growth of A549 cells (similar to kd PKCιota); mice treated with ATG exhibited decreased tumor volume (similar to kd PKCιota). In NSCLC cells, ATG was able to disrupt the PKCιota:PAR-6 complex, resulting in decreased active Rac1 (Stallings-Mann, 2006). Also in NSCLC, inhibition of PKCιota by ATM blocked Ras-induced tumorigenesis (Regala, 2009). Thus, PKCιota is involved in transformation, and PKCιota inhibition may decrease this phenotype by blocking the interaction of PKCιota effector molecules.

Figure 7: Mechanism of PKC ϵ -Induced Transformation

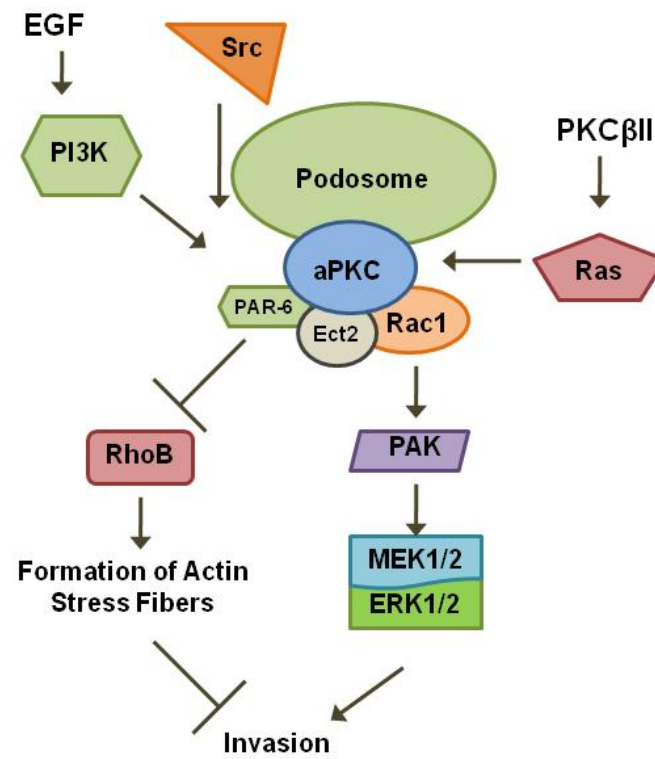


In an alternative model, PKCιota was found to lead to transformation through Src-induced recruitment of PKCιota to the podosome (Figure 7). (Podosome = “invasive adhesions on the ventral surface of the cell that are sites of protease secretion”). In transformed mouse fibroblasts (3T3) (by infection with v-Src), PKCιota was identified as a mediator of Src-induced transformation. PKCιota inhibition (by RNAi and dn) revealed that PKCιota was necessary for Src-induced stress fiber disruption. Specifically, tyrosine phosphorylation of PKCιota by Src was necessary for localization of PKCιota to the podosome and in turn transformation (Rodriguez, 2009). Thus, PKCιota is essential for Src-induced transformation through its localization to the podosomes, leading to degradation of the basement membrane.

Mechanism of PKCιota-Induced Invasion- The mechanism of PKCιota-stimulated invasion seems to occur through the MEK/ERK pathway and involves the usual players: Ras, Src, and PI3K. Another aspect of invasion is the loss of actin stress fibers; this is addressed in detail in the “PKCιota-Functions: Polarity” and “PKCιota- Signaling: Ras” sections (see Figure 8).

As mentioned, PKCιota activates Rac1 through PAR-6 and Ect2 interactions. In NSCLC, like inhibition of PKCιota, inhibition of Ect2 by RNAi resulted in a decrease in transformation, invasion, and tumorigenesis, which was reverted upon exogenous expression of Rac1. In cancer, Ect2 is mislocalized to the cytoplasm (as is the case with PKCιota), where it can then bind to the PKCιota-PAR6 complex. Without mislocalization of PKCιota or PAR-6 to the cytoplasm, Ect2 does not become mislocalized, indicating that the PKCιota-PAR-6 complex recruits Ect2 to the cytoplasm. PKCιota contributes to invasion through the PKCιota/PAR-6/Ect2 → Rac1 → Pak → MEK1/2 → ERK1/2 pathway in NSCLC (Justilien, 2009).

Figure 8: Mechanism of PKC α -Induced Invasion



Another study found that the interaction of PKC δ and Rac1 is important for invasion. Specifically, in RIE (rat intestinal epithelial) cells with stably expressed PKC β II, Zhang et al found that RIE-PKC β II cells exhibit increased invasiveness as compared with RIE parental cells, which was through the Ras-PKC δ /Rac-MEK/ERK pathway. By using specific inhibitors or RNAi (as was the case for PKC δ / lambda), PKC β II-induced invasion, as measured by matrigel invasion chambers, was blocked. Thus, PKC δ / lambda was necessary for PKC β II-induced invasion in the following order PKC β II \rightarrow Ras \rightarrow PKC δ /Rac1 \rightarrow MEK/ERK \rightarrow invasion through matrigel (Zhang, 2004). Additionally, colon tumor formation and progression, seemed to be through cooperation between PKC δ and PKC β II as determined by a double knockout mouse model compared to the single knockouts of either PKC alone (Murray, 2009). Thus, PKC δ and PKC β II both contribute to tumorigenesis and invasion.

In mouse fibroblasts (3T3 cells) that have undergone transformation due to viral infection with v-Src, PKC δ was identified as the driver of Src-mediated transformation and invasion. Using three distinct methods to inhibit PKC δ activity (mPS, RNAi, and dn), Rodriguez et al found that PKC δ was essential for Src-induced transformation, due to the ability of PKC δ to induce stress fiber formation and to polarize at the apical edge. Using a Boyden chamber invasion assay and an *in situ* zymography assay, PKC δ was also found to be essential for Src-induced invasion. Specifically, inhibition of PKC δ tyrosine phosphorylation resulted in podosome defects. In Src-transformed cells, PKC δ localized to the podosomes. Thus, PKC δ is essential for Src-induced transformation and invasion through its localization to the podosomes, leading to degradation of the basement membrane (Rodriguez, 2009).

Induction of invasion upon nicotine exposure seems to be through an alternate mechanism. Nicotine is one of the key carcinogens in cigarette smoke. Using H1299 or H460 human lung cancer cells, Xu et al found that PKC δ was able to phosphorylate both μ - and m-calpain in an *in vitro* assay. Specifically, when PKC δ was activated by nicotine, PKC δ was able to lead to calpain phosphorylation/ activation. Use of PKC δ RNAi was able to block nicotine-induced phosphorylation of the calpains (and thus their activity), migration, and invasion of lung cancer cells. Thus PKC δ is a key effector of nicotine-induced tumorigenesis. One of the potential mechanisms of PKC δ RNAi blocking nicotine-induced invasion could be through downregulation of μ - and m-calpain secretion, which is known to be upregulated upon nicotine exposure in lung cancer cells (Xu, 2006).

Pro-Apoptotic PKC α - Rarely, PKC α has been shown to have pro-apoptotic effects. In both endothelial cells and fibroblasts, PKC α / λ is involved in cPLA2-mediated apoptosis as a result of decreased serum levels. cPLA2-mediated apoptosis is due to a lack of ECM components, such as fibronectin. cPLA2 leads to PKC α / λ activation, which in turn leads to p53-induced apoptosis through a cascade of caspases (SPDC- small prodomain caspases). ECM components are able to inhibit cPLA2 \rightarrow PKC α / λ \rightarrow p53-mediated cell death through expression of FAK (focal adhesion kinase) according to the pathway FAK \dashv cPLA2 \rightarrow PKC α / λ \rightarrow p53-mediated cell death. This function of PKC α / λ is PI3K-independent. This pathway was identified using a DN-FAK (FAT), which, when expressed in the absence of serum, would lead to increased apoptosis. A DN-PKC α was able to revert the phenotype to no increase in apoptosis. Thus, PKC α is involved in the p53-mediated apoptosis that results from a decrease in FAK function (Ilic, 1998).

Another circumstance of pro-apoptotic PKC α involves lipid-induction of PKC α . Upon lipid-induction (addition as fatty acids) in HCAEC (human coronary artery endothelial cells), inhibition of PKC α resulted in a decrease in apoptosis. Similarly, lipid-induction led to activation in PKC α (Staiger, 2009). Thus, PKC α can also be pro-apoptotic in rare occasions.

Anti-Apoptotic PKC α (Survival)- The majority of the studies on PKC α in cell death have been focused around PKC α 's role in survival via NF κ B activation and inhibition of pro-apoptotic Bad Kinase. PKC α is able to phosphorylate IKK β , leading to the phosphorylation/ degradation of I κ B, which then causes release of NF κ B, allowing this transcription factor to translocate to the nucleus where it leads to the expression of anti-apoptotic genes (see "PKC α - Signaling: NF κ B").

PKC α is also able to induce survival through the phosphorylation of Bad Kinase, a pro-apoptotic enzyme. Upon stimulation with NNK (Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), a known potent carcinogen in cigarette smoke, PKC α led to increased survival through phosphorylation of pro-apoptotic Bad, which disrupted Bad/Bcl-XL binding. By use of immunofluorescent microscopy in A549 cells exposed to NNK, Jin et al was able to find that PKC α and Bad co-localize. Also, in both an *in vitro* kinase assay and western blot analysis using phospho-Bad antibodies, PKC α was able to phosphorylate Bad at all three sites, which are characteristic of NNK exposure (Ser-112, Ser-136, and Ser-155). Upon stimulation with NNK in human lung cancer cells, Src becomes activated, which leads to

PKC η activation. Both the Src inhibitor, PP2, and a pan-PKC inhibitor, staurosporine, blocked NNK-induced Bad phosphorylation. More specifically PKC η RNAi was used to block NNK-induced Bad phosphorylation and subsequently block survival. Thus, PKC η activation in lung cancer cells by the carcinogen NNK can lead to Bad phosphorylation and Bad/Bcl-XL disruption, resulting in increased survival and chemoresistance through the pathway: NNK \rightarrow Src \rightarrow PKC η \rightarrow P-Bad \rightarrow survival by Bad not binding Bcl-XL (Jin, 2005).

In prostate cancer cells, silencing PKC η by RNAi resulted in apoptosis. However, induction of apoptosis occurred through two very distinct mechanisms. In RWPE-1 (transformed non-malignant prostate) cells, knockdown of PKC η resulted in apoptosis due to a decrease in phosphoBad (Ser-155, Ser-136) and an increase in Bad/Bcl-xL heterodimerization. No such effect on Bad was observed in DU-145 (androgen-independent malignant prostate) cells. However, RWPE-1 and DU-145 cells both went through apoptosis because of mitochondrial dysfunction/ apoptotic cascades, i.e. cytochrome c release, caspase-7 activation, and PARP cleavage (Win, 2009). Thus, targeting PKC η in cancer treatment is a viable option. Inhibition of PKC η can result in apoptosis in different pathways, potentially due to oncogenic addiction.

In primary cultures of cerebellar granule cells (CGCs), PKC η protein levels decrease with spontaneous cell death implicating a potential role in survival (Lin, 1997). Also, in LNCaP prostate cancer cells, decreased PKC η membrane abundance correlated with increased apoptosis and subsequently antitumor effects (Song, 2004).

Chemoresistance- Survival- Through PKC η , cells have undergone chemoresistance to pro-apoptotic drugs, such as: OA (okadaic acid), taxol (several studies), and cisplatin. This PKC η -mediated chemo-resistance is conferred through NF κ B activation and inhibition of the p38 MAPK pathway.

In human chronic myeloid leukemia cells (K562), PKC η protected these cells from apoptosis induced by OA (okadaic acid) and taxol. Inhibition of PKC η resulted in a re-sensitization of these cells to the apoptotic stimuli; thus indicating PKC η could play a role in survival in leukemia. PKC ζ did not exhibit the same survival characteristics (Murray, 1997).

PKC η is downstream of Bcr-Abl in an NF κ B survival pathway. In K562 leukemia cells (CML), BCR-ABL is involved in chemo-resistance to apoptotic drugs, such as taxol. Upon treatment of K562 cells with taxol, PKC η became activated. Using tyrphostin AG957, which inhibits BCR-ABL, Jamieson et al was able to block taxol-induced PKC η activation and re-sensitize these cells to the apoptotic properties of taxol. Also, constitutively active PKC η

was able to revert the chemo-sensitive phenotype of K562 cells treated with taxol and tyrphostin AG957 back to the chemo-resistant phenotype. Similarly, in a BCR-ABL negative and taxol-sensitive leukemia cell line (HL60), taxol was unable to induce PKC ζ activation, suggesting that PKC ζ would be downstream of BCR-ABL in response to taxol according to the pathways: In K562, CML cells: TAXOL \rightarrow BCR-ABL \rightarrow PKC ζ \rightarrow drug resistance. However, if Bcr-Abl is inhibited (by tyrphostin AG957), the pathway would be as follows: TAXOL \rightarrow BCR-ABL \rightarrow PKC ζ \rightarrow re-sensitization to taxol (Jamieson, 1999). Another study found that PKC ζ was able to stimulate Bcr-Abl-induced chemoresistance through the pathway: Taxol \rightarrow Bcr-Abl \rightarrow PKC ζ \rightarrow NF κ B (due to I κ B degradation and RelA transcriptional up-regulation) \rightarrow Survival (transcriptional activation of anti-apoptotic genes) (Lu, 2001). Using human glioblastoma cells, the expression of PKC ζ was demonstrated in causing partial resistance to the chemotherapeutic agent, cisplatin. PKC ζ RNAi was able to sensitize human glioblastoma cells to the cytotoxic effects of cisplatin. Examining the difference between parental cells and PKC ζ RNAi cells, Baldwin et al found that an enhancer of the p38 MAPK pathway, GMF β exhibited increased expression in PKC ζ silenced cells (Baldwin, 2006). Cisplatin is known to induce cytotoxicity partially through the p38 MAP kinase pathway (Mansouri, 2003). Basically, Baldwin found that chemoresistance to cisplatin was partially through PKC ζ 's downregulation of the p38 MAPK pathway through a decrease in GMF β expression (Baldwin, 2006).

Thus PKC ζ plays an active role in survival and chemoresistance via activation of NF κ B, phosphorylation of Bad Kinase, and inhibition of the p38 MAPK pathway.

Additional Functions of PKC ζ - PKC ζ has also been implicated in other processes such as hypoxia (Gozal, 1998), ischemia (Albert, 1998) and viral propagation. In the parvovirus minute virus of mice (MVM), PKC ζ / lambda phosphorylates the viral protein NS1. Phosphorylation of NS1 at T435 and S473 by PKC ζ / lambda, allows for DNA unwinding (NS1 helicase activity) and subsequently replication (Dettwiler, 1999; Nuesch, 2003). Additionally, HSV-1 (Herpes Simplex Virus-1) infection led to IL-15 upregulation, resulting in increased cell proliferation and prevents apoptosis by upregulation of anti-apoptotic genes. This HSV-1 driven increase in IL-15 expression is dependent on PKC ζ / lambda in monocyte cells (Ahmad, 2007).

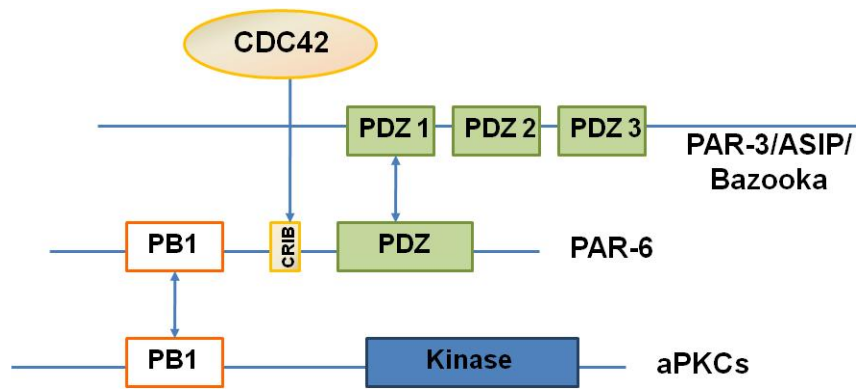
PKC ζ is an important regulator of several essential biological functions, such as: establishment and maintenance of epithelial polarity, microtubule dynamics, membrane trafficking, maintenance of tight junctions, inflammation, proliferation, DNA synthesis, survival

and invasion, to name a few. While PKC ι is able to influence several of these functions, PKC ι is absolutely essential for polarity, as PKC ι is part of a polarity complex being localized to the tight junctions of epithelial cells. In fact, PKC ι /lambda knockout mice are embryonic lethal due to defects in polarity (Bandyopadhyay, 2004), thus indicating the predominant role of PKC ι is in establishment and maintenance of polarity. Similarly, overexpression of PKC ι results in aberrant polarity and increased proliferation, which can lead to transformation (Eder, 2005; Zhang, 2006). Several diseases such as cancer, along with Alzheimer's disease and diabetes, can be linked to aberrant expression or localization of PKC ι , thus indicating the importance of proper regulation of PKC ι . PKC ι is linked to many processes involved in the stages of tumorigenesis, such as: polarity, cytoskeletal reorganization, proliferation, anchorage-independent growth, survival, chemoresistance, invasion, and metastasis. Because PKC ι is involved in so many unique facets of tumorigenesis and tumors are heterogeneous, PKC ι is an ideal potential target for cancer therapy.

PKC IOTA BINDING PARTNERS

The structure of the aPKCs compared to the nPKCs and cPKCs is quite different (see Figure 4). Like the nPKCs and cPKCs, the aPKCs contain a catalytic subunit comprised of the C3 ATP-binding site and the C4 substrate binding site. However, unlike the nPKCs and cPKCs, the aPKCs contain a distinct PB1 domain and a truncated C1 domain (Hirano, 2004). The PB1 domain is distinctly located on the atypical PKCs and allows them to bind effector molecules, such as p62, PAR-6, and MEK5. These effector molecules can then bind other proteins, such as PAR-3 or CDC42, making protein complexes involved in polarity (PKC ι -PAR-6-PAR-3) or invasion (PKC ι -PAR-6-CDC42) (see Figure 9). However, PKC ι is also able to bind proteins through other domains, such as the C1 (zinc-finger) domain or the catalytic (kinase) domain. Table 7 provides a complete list of all the PKC ι binding partners. Knowledge of the PKC ι binding partners is important because the ability of PKC ι to be activated by other proteins via PB1:PB1 interactions is what distinguishes PKC ι (and the other member of the atypical PKCs, PKC ζ) from the nPKCs and cPKCs. While Table 7 provides a comprehensive list of the known functions of PKC ι in binding to its binding partners, Figure OPP provides a schematic of the known roles of PKC ι through its interaction with the proteins listed below. While most PKC ι -binding partners have a defined role, some proteins identified in PKC ι -binding were not further examined for functional relevance (Table 7).

Figure 9: PKCιota Interactions through PAR-6 Linker Protein



PB1 Domain

PKC η interactions with several effector molecules, such as p62/ZIP, PAR-6, and MEK5, are mediated via the PB1 (Phox and Bem 1) domain on the N-terminus of the peptide. The PB1 domain is unique to the atypical PKCs, and allow PKC η and PKC ζ to be activated by protein:protein interactions as opposed to activation by DAG or calcium like the other PKC family members. By use of NMR (nuclear magnetic resonance), Hirano et al was able to determine that the PB1-PB1 interactions that occur between PKC η and its effector molecule require the PKC η PB1 domain to take on a ubiquitin-like fold (Hirano, 2004). Uniquely, PKC η contains both a type I and type II PB1 domain. The type I PB1 domain is acidic, containing both conserved acidic and hydrophobic residues (Nakamura, 1998). This acidic motif in a type I PB1 domain is called a PC motif (from mammalian p40 \textit{phox} and yeast Cdc24p). Other names given to the PKC η PC motif include OPR (octicosapeptide repeat) (Ponting, 1996), AID (atypical PKC interaction domain) (Moscat, 2000), or OPCA (OPR, PC, and AID) motif (Ponting, 2002). The most common name for the PKC η PC motif is the OPCA motif (Ponting, 2002). The type II PB1 domain is basic and contains a conserved lysine residue, which is necessary to bind to the OPCA of other PB1 domain-containing proteins. PKC η contains both the acidic OPCA and the basic lysine residue, indicating that PKC η is able to function as either or a type I or II PB1 domain. For example, in the case of PKC η binding to p62/ZIP, the PKC η OPCA motif binds the lysine residue of p62/ZIP (Wilson, 2003; Lamark, 2003). However, in the case of MEK5, the MEK5 OPCA motif binds the lysine residue on PKC η 's PB1 domain (Noda, 2003). Thus both the type I and type II domains of PKC η are fully functional.

The PB1 domain of PKC η can be targeted therapeutically. Aurothiomalate (ATM) and aurothioglucose (ATG) are two gold compounds that can interrupt the PB1:PB1 interactions of PKC η with their effector proteins but do not disrupt the PB1:PB1 domains of other molecules. This is due to ATM and ATG binding at a conserved cys-residue in the PB1 domain of PKC η (and PKC ζ) that is not present in any other PB1 domain-containing proteins (Stallings-Mann, 2006). See "PKC η -PKC η as a Target for Therapy" section for more details.

Table 7: PKC ζ Binding Partners

PKC ζ Binding Partner	Binds PKC ζ	Region Bound to PKC ζ	Function	Reference
LIP (Lambda Interacting Protein)	No	Zinc-finger Domain	Necessary for PKC ζ -induced NF κ B activation/ Activates PKC ζ	Diaz-Meco, 1996
PAR-4 Partitioning Defective 4	Yes	Zinc-finger Domain	Leads to Growth Inhibition and Cell Death/ Inhibits PKC ζ /zeta Activity	Diaz-Meco, 1996
p62 / ZIP	Yes, but not as well as PKC ζ	PB1 Domain	Involved in Lysosomal-Targeted Endosomes/ Changes PKC ζ Localization	Sanchez, 1998
ASIP/ PAR-3 Atypical PKC isotype- Specific Interacting Protein	Yes	Binds through interaction with PAR-6	PKC ζ /lambda to Localize to the Tight Junctions/ Also involved in PKC ζ Insulin Signaling	Izumi, 1998
p70 S6K p70 S6 Kinase	Yes	The Regulatory and Kinase Domains	Involved in DNA Synthesis	Akimoto, 1998
FRS2 Fibroblast growth factor Receptor Substrate 2	Yes	Catalytic Domain	Upon FGF Stimulation Serves to Localize PKC ζ To the Plasma Membrane	Lim, 1999
CDC42 Cell Division Cycle 42	Yes	Binds through interaction with PAR-6	Necessary for CDC42-induces loss of stress fibers (Required for Ras-Induced Transformation)/ Localizes PKC ζ to the Plasma Membrane	Coghlan, 2000
Adducin	Not Determined	Not Determined	Seem to Localize PKC ζ to the sites of Cell-Cell Contact or Tight Junctions in Adipocytes	Laustsen, 2001
PAR-6 Partitioning Defective 6	Yes	PB1 Domain	Involved in Polarity and Migration/ Links PKC ζ to other Effector Molecules	Noda, 2001
MEK5 MAP Kinase Kinase	Yes	PB1 Domain	Upon EGF Induction binds PKC ζ and Leads To the Transcriptional Activation of Jun	Diaz-Meco, 2001
PSF Polypyrimidine tract-binding protein (PTB)- associated Splicing Factor	Not Determined	Not Determined	Upon IGF-1 Induction Binds Nuclear PKC ζ Transcriptional Activation of Cytochrome P-450(scc)	Urban, 2004
L-selectin A Leukocyte Receptor	Not Determined	Not Determined Most Likely at Catalytic Domain	Phosphorylated on the Cytoplasmic Domain by PKC ζ	Kilian, 2004
Bad Pro-apoptotic when Bound to BCL-XL	Not Determined	Not Determined Most Likely at Catalytic Domain	Interaction with PKC ζ Leads to Increased Survival and Chemoresistance/ Phosphorylated by PKC ζ at Three Sites	Jin, 2005

LIP (Lambda Interacting Protein)

LIP (Lambda-interacting protein) was initially identified as a binding partner to PKC ι /lambda by a yeast two-hybrid screen. LIP, which binds only to PKC ι /lambda constructs that contain the entire regulatory domain or the zinc-finger domain, is specific to PKC ι /lambda and does not bind to PKC zeta. The interaction between LIP and the zinc-finger lipid-binding domain of PKC ι /lambda, which occurs both *in vitro* and *in vivo*, is the first reported protein binding to a lipid-binding region (Diaz-Meco, 1996). LIP has been shown to activate PKC ι .

Activation of PKC ι by LIP leads to activation of NF κ B in a κ B-dependent manner. In both Cos and NIH3T3 cells, PKC ι /lambda exogenous expression led to activation of NF κ B, which was decreased by a dominant negative isoform (pseudosubstrate domain). Exogenous LIP, which binds specifically to PKC ι /lambda, led to κ B-dependent promoter activation similar to that of TNF α . The activation of κ B promoter activity was increased upon co-transfection with the PKC ι /lambda expression construct and decreased upon co-transfection with the dominant negative (kinase dead) PKC ι /lambda expression construct. The decrease in LIP-induced, κ B-dependent promoter activity was not observed upon co-transfection with a dominant negative (kinase dead) form of PKCzeta (Diaz-Meco, 1996). Thus, the LIP-induced transcriptional activation of κ B leading to the activation of NF κ B is PKC ι /lambda specific.

PAR-4

In NIH-3T3 cells, PAR-4, which is induced in apoptotic cells, interacts with both PKC ι and PKCzeta via the zinc-finger motifs of the regulatory domains of these atypical PKCs. Interaction of PKC ι with PAR-4 results in a decrease in its enzymatic activity and thus growth inhibition and cell death. Overexpression of ectopic PKC ι can revert the apoptotic phenotype induced by PAR-4 exogenous expression (Diaz-Meco, 1996).

However, PAR-4 has been deleted in several types of cancers (Cook, 1999). This implies that a method for PKC ι activation in some cancers may be through a deletion of the pro-apoptotic, PKC ι -inhibiting protein, PAR-4.

PKC ι is implicated in the TNF α \rightarrow I κ B \rightarrow Survival (NF κ B) pathway. PAR-4, which can also be activated by TNF α , inhibits the TNF α -PKC ι -I κ B-NF κ B pathway, leading to apoptosis (Diaz-Meco, 1999). Specifically, in NIH-3T3 mouse fibroblasts, upon TNF α induction, the atypical PKCs function in phosphorylation of IKK β (Lallena, 1999), which leads to I κ B phosphorylation and degradation, leading to NF κ B nuclear translocation (survival). The

TNF α -induced activation of NF κ B is in turn inhibited by PAR-4 expression causing onset of apoptosis. Pro-apoptotic PAR-4 expression inhibits translocation of NF κ B and therefore inhibits the transcription of κ B in response to TNF α . PAR-4 is able to inhibit TNF α -induced survival and cause apoptosis by binding PKC ι /lambda and PKC ζ and inactivating their kinase activity. Therefore, addition of constitutively active atypical PKCs revert the effects of exogenous PAR-4 expression, causing complete loss of apoptosis in response to TNF α (Diaz-Meco, 1999).

Similarly, PKC ι has been shown in several models to be necessary for Ras-mediated transformation and tumorigenesis. Ectopic Ras expression, which inhibits endogenous PAR-4 expression (Berra, 1993), leads to survival. Because Ras inhibits expression of PAR-4, addition of exogenous PAR-4 leads to an increase in apoptosis, even in the presence of Ras (Diaz-Meco, 1999).

Thus, PKC ι binding to PAR-4 is important in tumorigenesis. If PKC ι and PAR-4 bind, this causes inhibition of PKC ι (and PKC ζ), resulting in growth inhibition and cell death. However, if PAR-4 is absent or inactive, PKC ι can lead to survival through the TNF α \rightarrow NF κ B pathway and to transformation and tumorigenesis through Ras activation. Thus, the balance between PKC ι and PAR-4 seems to be important for cell fate.

p62 / ZIP

p62 is the human homolog to ZIP- ζ -Interacting Protein (rat). Both p62 and ZIP were identified as aPKC interacting proteins by two separate groups (Sanchez, 1998 and Puls, 1997, respectively). p62 has several functions: (1) at the endosome (a vesicle formed during endocytosis, which is used for trafficking) upon NGF stimulation (2) in a complex with RIP and PKC ι in TNF α -induction of NF κ B and (3) in a complex with TRAF6/PKC ι in IL-6 expression.

Using a yeast two-hybrid system, Sanchez et al was able to identify p62, a phosphotyrosine-independent p56(lck) SH2-interacting protein, as a PKC ι binding partner. p62, which also binds to a lesser extent to PKC ζ , binds to the V1 domain (Sanchez, 1998). The V1 domain of PKC ι is located in the regulatory region of the peptide and is the most variable region of the PKC family members (Nishizuka, 1992). Later, the specific region of the V1 domain was found to be the PB1 domain (Wilson, 2003; Lamark, 2003). PKC ι binding to p62 does not change the activity of PKC ι , indicating that p62 is not a PKC ι substrate. Instead, p62 binding to PKC ι serves as a method for localizing PKC ι to the lysosome-targeted endosomes. p62 leads to EGF endocytic membrane transport; this transport is block

by exogenous expression of a dominant negative form of PKC ϵ (Sanchez, 1998). Therefore, PKC ϵ through binding to p62 likely plays a role in the trafficking of growth factor receptors, such as EGF.

(1) Upon NGF treatment, PKC ϵ / λ localizes with p62 to the endosome (Samuels, 2001). Some of the purposes of the endosome include carrying either proteins or lipids from the plasma membrane to the lysosome, or carrying these molecules to the golgi apparatus for processing. The endosome is also used to secrete proteins/lipids out of the cells. Autophagy uses many of the same regulators as does the process of the endosome to the lysosome (Tooze, 1990). After induction of PC12 cells with NGF, p62 localizes to the endosome, where PKC ϵ / λ is recruited upon tyrosine phosphorylation by Src. PKC ϵ / λ co-localized with p62. This interaction between p62 and PKC ϵ / λ at the endosome had been described previously (Sanchez, 1998). However, Samuels et al identified the mechanism of this interaction in response to NGF induction (Samuels, 2001).

Stimulation of PC12 cells with NGF lead to the binding of PKC ϵ / λ and p62, this was determined by use of immunoprecipitation analysis. While PKC ϵ / λ and p62 expression levels remained constant upon stimulation by NGF, the binding of p62 to PKC ϵ / λ increased 4-times that of non-NGF treated. Kinetics experiments revealed that PKC ϵ / λ peak activation is between 5 to 15 minutes and peak co-localization between PKC ϵ / λ and p62 is between 15 to 30 minutes, indicating that PKC ϵ / λ must first be activated by phosphorylation before it can bind p62.

Using an inhibitor of tyrosine kinase activity (genistein), Samuels et al showed that tyrosine phosphorylation of PKC ϵ / λ was necessary for the interaction between PKC ϵ / λ and p62. However, phosphorylation of PKC ϵ / λ at a tyrosine residue is not necessary for p62 to localize to the endosome. Endosomes were visualized using immunofluorescence with antibodies to Rab7, a known marker of the endosome. Also, fractionation was used to determine the endosomal proteins. Thus, p62 localizes to the endosome, and upon tyrosine phosphorylation of PKC ϵ / λ , PKC ϵ / λ gets recruited shuttle by p62 to the endosome (Samuels, 2001).

To determine the tyrosine kinase responsible for PKC ϵ / λ tyrosine phosphorylation, Samuels et al added increasing amounts of Src and measured the amount of PKC ϵ / λ bound to p62 (Samuels, 2001). Puls et al had already identified a Src phosphorylation site on aPKC at tyrosine 116 (Puls, 1997). Increasing Src expression led to increased PKC ϵ / λ bound to p62, indicating that Src is the tyrosine kinase that activates PKC ϵ / λ , causing PKC ϵ / λ to be recruited to bind p62 and

translocate to the endosome. To determine the specificity of the effect of Src on PKC ι /lambda binding, HEK 293 cells were also given increasing amounts of Src, and as was the case with PC12 cells, increasing Src expression resulted in increased PKC ι /lambda bound to p62 (Samuels, 2001). Because PKC ι had already been established in NGF-induced differentiation (Coleman, 1994), antisense p62 was used to determine the role of p62 in NGF-induced differentiation. They found that antisense p62 blocked NGF-induced neurite outgrowth, indicating PKC ι /lambda binding to p62 is likely necessary for NGF-induced neurite outgrowth (Samuels, 2001). Thus, PKC ι plays an important role in the NGF \rightarrow Src \rightarrow P-PKC \rightarrow recruited to bind p62 at the endosome pathway (Samuels, 2001).

(2) p62 was also determined to be important in NF κ B activation. In the TNF α \rightarrow TNFR1 \rightarrow TRADD \rightarrow RIP/p62/aPKCs (independent of TRAF2) \rightarrow IKK β \rightarrow degradation of I κ B \rightarrow NF κ B (activation/ nuclear translocation) pathway, p62 links PKC ι /lambda and PKC ζ to NF κ B transcriptional activation. Using a dominant negative form of PKC ι /lambda in 293 cells, Sanz et al was able to link both PKC ι /lambda and PKC ζ to NF κ B transcriptional activation through the interaction of PKC ι /lambda or PKC ζ with p62. PKC ι /lambda was not able to bind directly to RIP. Using a series of *in vitro* and *in vivo* binding assays, p62 was shown to bind to RIP, which is a known molecule in the TNF α to NF κ B pathway. The dominant negative PKC ι /lambda construct inhibited RIP- induced NF κ B activation. PKC ι /lambda, p62, and RIP all must be present in a complex in order for PKC ι /lambda to activate IKK β and therefore activate NF κ B. Therefore, while p62 links RIP to PKC ι /lambda, PKC ι /lambda links p62 to IKK β (Sanz, 1999).

(3) PKC ι was found to be in a signalsome with TRAF6, p62, and IKK through a series of immunoprecipitations in response to Thymosin alpha 1, a treatment of cancer in clinical trials. This induction of the signalsome led to IL-6 expression through the Thymosin alpha 1 (Ta1) \rightarrow TRAF6/p62/PKC ι /zeta/ IKK \rightarrow NF κ B \rightarrow IL-6 pathway (Zhang, 2005). The function of p62 in the p62-TRAF6-IKK β -PKC ι complex, which is formed upon stimulation with NGF and leads to NF κ B expression, is to lead to the polyubiquitination of TRAF6 (Wooten, 2005).

PAR-3/ ASIP/ Bazooka

PAR-3, together with PKC ι and PAR-6, makes up the polarity complex that localizes to the tight junctions. See “PKC ι - Functions: Polarity” for a thorough background on polarity and tight junctions. PAR-3 is also known as ASIP (atypical PKC isotype-specific interacting protein) in rats (Izumi, 1998), Bazooka in drosophila (Muller, 1996), XASIP in xenopus

(Nakaya, 2000), and PAR-3 in humans and *c. elegans*. In addition to the role of PKC ι -PAR-3 in polarity, this complex also plays a role in glucose transport.

PAR-3/ ASIP was originally identified as a PKC ι binding protein by Izumi et al. In NIH3T3 cells (mouse fibroblasts), COS (monkey kidney cells), epithelial MDCKII cells (Madin Darby Canine Kidney), and rat intestinal epithelium, both PKC ι /lambda and PKC ζ bind ASIP (atypical PKC isotype-specific interacting protein) (Izumi, 1998). MDCKII cells form a tight cell layer and are used to mimic polarized epithelial cells. ASIP is conserved in *c. elegans* (PAR-3) and *drosophila* (Bazooka), which is where the role of ASIP was found to be in establishment and maintenance of asymmetric cell polarity (Izumi, 1998; Tabuse, 1998; Schober, 1999; Wodarz, 1999). ASIP is commonly referred to as PAR-3 in humans. Both PAR-3 (*C. elegans*) and ASIP (rats) contain PDZ domains, which were originally believed to allow these proteins to bind the atypical PKC members. However, later PAR-6 was determined to be the linker protein that allowed PKC ι and PAR-3 to co-localize (Noda, 2001). When PKC ι / lambda and ASIP are bound in rat intestinal epithelial cells, the complex localizes to the tight junctions, as determined by immunoelectron microscopy (Izumi, 1998). Similarly, exogenous expression of ASIP was able to alter the localization of PKC ι / lambda (Kotani, 2000). Also, in *Xenopus laevis* epithelial cells PKC ι / lambda (XaPKC) and ASIP (XASIP) colocalize at the *Xenopus* equivalent of tight junctions (Nakaya, 2000). Because PKC ι /lambda is localized to the tight junctions, which are found in epithelial cells that have apical-basal polarity, PKC ι /lambda was demonstrated to play a role in asymmetric polarity of epithelial cells.

In 3T3-L1 adipocytes, ASIP interacts with PKC ι / lambda, but not with PKC ϵ or AKT, as determined by a series of *in vitro* immunoprecipitation experiments. While endogenous ASIP could only be found in the LDM fraction, overexpression of ASIP resulted in an increase in the PKC ι / lambda levels in the LDM and PM fractions, but a decrease in the cytosolic fraction. Therefore, ASIP overexpression results in altered localization of PKC ι / lambda in 3T3-L1 adipocytes (Kotani, 2000).

Insulin has been shown to induce glucose uptake and GLUT4 translocation in a PKC ι / lambda dependent manner (see "PKC ι - Signaling: Insulin" for more details). Kotani et al showed that overexpression of the atypical PKC binding protein, ASIP (PAR-3/Bazooka), was able to inhibit both insulin-induced and constitutively active PKC ι / lambda-induced glucose uptake. Specifically, in 3T3-L1 adipocytes, addition of insulin resulted in an increase in glucose uptake as well as an increase in the activity of PKC ι / lambda, as indicated by use of an *in vitro* kinase assay using MBP as the substrate. The effects of insulin-

induction on PKC α / λ activation as well as on GLUT4 translocation and therefore on glucose uptake were reduced upon addition of exogenous ASIP in a dose-dependent manner, indicating that insulin works through PKC α / λ signaling to induce glucose uptake. To test the specificity of insulin-induced glucose uptake, Kotani et al used two other methods of inducing glucose uptake, growth hormone (GH) and hyperosmolarity (sorbitol). However, ASIP did not block GH- or sorbitol- induced glucose uptake, indicating that inhibition of glucose uptake by ASIP is insulin and PKC α / λ - specific. Interestingly, ASIP did not block all insulin-induced signaling. For example, ASIP did not block the translocation of AKT to the PM fraction in response to insulin (Kotani, 2000). Thus, while ASIP blocks PKC α / λ - and insulin-induced translocation of GLUT4 and therefore glucose uptake, ASIP does not block all insulin signaling.

p70 S6 Kinase

p70 S6K, a kinase involved in the PI3K pathway, is involved in cell cycle progress through DNA synthesis (Lane, 1993; Reinhard, 1994; Pullen, 1997; Alessi, 1998). p70 S6K binds to PKC α and PKC ζ via both the regulatory and kinase domains. PKC α is required, but not on its own sufficient, to activate serum-induced DNA synthesis via S6K, which is not a PKC α substrate. PKC α / λ binds to p70 S6K. In both COS and 293 cells, two dominant negative mutants (kinase dead and a truncated regulatory domain) of PKC α / λ were able to suppress the ability of p70 S6K to lead to DNA synthesis in response to serum stimuli, determined by BrdU incorporation. However, a constitutively active form of PKC α was unable to stimulate p70 S6K-induced DNA synthesis, indicating that PKC α is required but not on its own sufficient for the activation of p70 S6K (Akimoto, 1998).

FRS2

In Swiss 3T3 mouse embryonic fibroblasts, upon stimulation with fibroblast growth factor (FGF), FRS2 interacts with both PKC α / λ and PKC ζ . This interaction was identified using a yeast two hybrid assay and validated using co-immunoprecipitation experiments with FGF induction. FRS2 works by binding to FGFR and recruiting proteins to the plasma membrane. While FRS2 and a similar isoform, SNT2, bind to PKC α / λ , these proteins are not a PKC α / λ substrate. However, FGF does increase PKC α / λ kinase activity three fold, using hnRNPA1 as a substrate. Using tagged, truncated forms of FRS2 and PKC α / λ in 293T cells, Lim et al found that the c-terminus of FRS2 binds to the catalytic domain of PKC α / λ . FRS2 binds to PKC α / λ two fold more efficiently

when PKC ι /lambda is in its “open” constitutively active confirmation as compared to the “closed” wild type confirmation. Because PKC ι /lambda does not phosphorylate or activate FRS2, it seems likely that the purpose of FRS2-PKC ι /lambda interaction is in the localization of activated PKC ι /lambda to the plasma membrane (Lim, 1999).

CDC42

Cell Division Cycle 42 (CDC42) is connected to PKC ι in everything from glucose transport, to the cytoskeleton, to transformation and invasion. In rat brain cytosol and NIH 3T3 mouse fibroblasts, PKC ι /lambda and PKC ζ were shown to interact with CDC42 by a series of immunoprecipitation assays. Truncated constructs of these proteins were used to determine the region of interaction between these two proteins. The interaction between PKC ι /lambda (regulatory domain) and CDC42 (V12 region) was shown to be both GTP-dependent (in rat brain cytosol) and indirect, requiring an unknown effector protein (in Cos and 293 cells) (Coghlan, 2000). Later, the unknown protein that links CDC42 to PKC ι /lambda was found to be PAR-6, which also links PKC ι to PAR-3 (Noda, 2001). In a PKC ι /lambda kinase assay, using myelin basic protein as the substrate, results reveal that CDC42 was not able to alter the kinase activity of PKC ι /lambda, thereby implying CDC42s interaction with PKC ι /lambda was to localize PKC ι /lambda to the plasma membrane. In NIH 3T3 cells, PKC ι /lambda translocates from the nucleus to the cytoplasm upon exogenous expression of CDC42. Earlier studies had shown that PKC ι /lambda was translocated from the nucleus to the cytoplasm in response to growth factor stimulation (PDGF and EGF), and this translocation was PI3K- dependent (Akimoto, 1996).

Ras, Rac, CDC42, PKC ι /lambda, and PKC ζ all individually had been implicated in stress fiber loss and actin remodeling (Zhao, 1998; Uberall, 1999). Cells, which have undergone transformation, have alterations in their actin cytoskeleton, one of these alteration is a loss of stress fibers (Mackay, 1998). While Uberall et al linked Ras and PKC ι /lambda and PKC ζ , Coghlan et al linked Ras-CDC42-PKC ι /lambda in stress fiber loss (Ras→ CDC42/PKC ι → loss of stress fibers → transformation) (Coghlan, 2000). Loss of stress fibers is a necessary step in Ras-induced transformation. While Rac expression is able to cause stress fiber loss, this phenotype is through a different pathway than the Ras-CDC42-PKC ι /lambda pathway (Coghlan, 2000) (see “PKC ι -Signaling: Ras” section for details).

In NIH3T3 cells, ectopic expression of constitutively active CDC42 (V12 CDC42) led to a loss in stress fibers (Kozma, 1995). Coghlan et al was able to show that transient exogenous expression of PKC ι /lambda (but not PKC ζ) was able to lead to the same loss of stress

fibers observed in response to CDC42 overexpression. To show that PKC ι / λ was essential for CDC42-induced loss of stress fibers, kinase dead mutants of PKC ι / λ (KD PKC ι / λ) and PKC ζ (KD PKC ζ) were used to block CDC42-induced loss of stress fibers. The KD PKC ι mutant blocked CDC42- induced loss of stress fibers, but had no effect on CDC42- induced membrane ruffling or filopodia formation, indicating that while PKC ι / λ was essential for CDC42-induced loss of stress fibers, CDC42 induced other pathways independently of the PKC ι / λ or PKC ζ pathways. Because Rac-1 had also been implicated in loss of stress fibers, the KD PKC ι mutants were used to block loss of stress fibers in response to Rac. However, the KD PKC ι mutants were unable to block Rac-induced loss of stress fibers (Coghlan, 2000). Uberall et al had published that Ras-induced loss of stress fibers, was not only dependent on PKC ι / λ and PKC ζ , but also dependent on Rac, contrary to the finding of Coghlan et al (Uberall, 1999; Coghlan, 2000). Coghlan et al was also able to link PKC ι / λ and PKC ζ to Ras-induced loss of stress fibers by use of the KD aPKC mutants (Coghlan, 2000). However, contrary to the findings of Uberall, upon induction of NIH 3T3 cells with constitutively active Ras and either dominant negative CDC42 (N17 CDC42) or dominant negative Rac, only N17 CDC42, but not dominant negative Rac, was able to block Ras-induced loss of stress fibers. Thus, according to Coghlan et al at least two pathways exist for loss of stress fibers in transformation, one pathway involving Ras- CDC42- PKC ι / λ and one involving Rac (independently of Ras or the atypical PKCs) (Coghlan, 2000). It is important to note that Coghlan et al did not directly test the KD aPKC mutants on transformation just on indicators of transformation (loss of stress fibers).

CDC42 is also involved in the insulin-PI3K-PKC ι / λ -GLUT-4 transport pathway (Insulin \rightarrow PI3K/CDC42 \rightarrow PKC ι / λ \rightarrow GLUT4 translocation \rightarrow glucose uptake). By use of CDC42 antibody (to block activity) and constitutively active (CA) CDC42 (to activate it) in 3T3-L1 adipocytes, CDC42 was examined in response to insulin induction. CDC42 is involved in the insulin response through direct interaction with PI3K. However, PI3K inhibitors, wortmannin and LY294002, block CA-CDC42 induced GLUT4 translocation to the plasma membrane. Also, inhibition of PKC ι / λ by microinjection of PKC ι / λ antibody and a kinase dead mutant resulted in a block of both insulin and CA-CDC42 induced GLUT4 transport. Thus, insulin \rightarrow PI3K/CDC42 \rightarrow PKC ι / λ are all in the same GLUT4 translocation pathway that leads to glucose uptake (Usui, 2003).

Of course, CDC42 also has PKC ι -independent functions. PKC ι was not involved in other CDC42-induced steps in transformation, such as membrane ruffling and filopodia

formation (Coghlan, 2000). Also, while PKC ι / λ was linked to CDC42-induced loss of stress fibers, PKC ι / λ does not play a role in the previously published CDC42-induced JNK activation or Fos expression (Coso, 1995; Minden, 1995; Chang, 1998; Kaneki, 1999; Coghlan, 2000).

Adducin

In 3T3-L1 adipocytes, adducin was identified as a PKC ι / λ binding protein by use of purified PKC ι / λ antibody in an immunoprecipitation followed by mass spectroscopy to identify the unknown proteins. It is important to note that the antibody used in this immunoprecipitation screen was non-specific among the atypical PKCs and could also bind PKC ζ . However, because PKC ι / λ was the predominant atypical PKC isoform in 3T3-L1 cells, the author assumed binding was due to PKC ι / λ rather than PKC ζ (Laustsen, 2001). Adducin is a cytoskeleton protein involved in the assembly of spectrin-actin complexes (Matsuoka, 2000) and is associated at the site of cell-cell contact in epithelial cells (Kaiser, 1989). PKC ι λ forms a polarity complex at the tight junctions with PAR3 and PAR6 (Izumi, 1998). PKC ι / λ binding to adducin may cause PKC ι to localize to the tight junctions (Abdi, 2008). PKC ι / λ did not seem to phosphorylate adducin in response to insulin stimulation (Laustsen, 2001). However, adducin appears to be constitutively phosphorylated in 3T3-L1 cells (Abdi, 2008).

PAR-6

PAR-6 is the linker protein between the PKC ι and the Rho GTPases (Rac and CDC42) in migration and invasion and between PKC ι and PAR-3 in polarity. *In vitro* and *in vivo*, PAR-6 binds the N-terminal regulatory domain of both PKC ι and PKC ζ . The binding between PKC ι -PAR-6 is through PB1:PB1 protein:protein interactions. In HeLa and COS-7 cells, Noda et al found that PKC ι , PAR-6, and Rac1 co-localize at membrane ruffles, which are found in migrating cells at the leading edge (Noda, 2001). However, a year prior, Coghlan et al found that PKC ι was not involved in other CDC42-induced steps in transformation, such as membrane ruffling and filopodia formation (Coghlan, 2000).

PKC ι and PAR-6 binding requires PAR-3 to bring about polarity in epithelial cells. Thus, PKC ι -PAR-6-PAR-3 forms the polarity complex responsible for the development of the apical membrane in polarized epithelial cells. Using PAR-3 knockdown in MDCK cells, which are polarized and able to form a monolayer in culture, Horikoshi et al found that PKC ι and PAR-6 are unable to localize to the apical domain, a phenotype that was rescued upon re-

addition of PAR-3 (Horikoshi, 2009). However, PKCιota is unable to bind PAR-3 without PAR-6 (Noda, 2001). Thus, PKCιota, PAR-6, and PAR-3 are all required to make the polarity complex.

The PKCιota-PAR-6 interaction can be targeted therapeutically in cancer. ATG (aurothioglucose- a gold compound used in the treatment of rheumatoid arthritis) and ATM (aurothiomalate- a similar gold compound) were identified as compounds which disrupt PKCιota/PAR-6 binding. Use of ATG blocked transformed growth of A549 cells, to the same extent as exogenous overexpression of kinase dead (kd) PKCιota. Also, ATG was able to cause a decrease in tumor volume in mice, much to the same extent as kd PKCιota. Similarly, both ATG and kd PKCιota did not affect the rate of proliferation of adherent cells. This decrease in transformation of NSCLC cells in the presence of ATG was due to a disruption of the PKCιota/PAR-6 complex, resulting in a decrease in the level of active Rac1 (GTP versus GDP-bound) (Stallings-Mann, 2006).

Predictably, PKCιota inhibitors (such as ATG or ATM) do not inhibit soft-agar colony formation in lung cancer cells that do not overexpress PKCιota. More specifically, ATM sensitivity in lung cancer cells is higher in cells overexpressing PKCιota-PAR-6 than in cells with PKCιota-p62. In cells with high levels of PKCιota, the IC50 values of ATM are low (300nM), while in cells with low levels of PKCιota, the IC50 values of ATM are quite high (100μM). Interestingly, ATM sensitivity only correlated with PKCιota levels, it did not correlate with overall sensitivity to chemotherapeutics, such as cisplatin, placitaxel, or gemcitabine. The trend of ATM being most efficacious in PKCιota overexpressing cells corresponds to the trend *in vivo*, with tumors of high PKCιota levels being the most sensitized to ATM. *In vivo*, ATM manifests its effect through inhibition of the PKCιota-PAR6-Rac-PAK-MEK-ERK pathway, as determined by western blot analysis. Thus, ATM treatment both *in vitro* and *in vivo* is most effective in PKCιota overexpressing tumors, indicating the need for a screening technique for lung cancer patients (Regala, 2008). The exact pathway that is targeted by ATM or ATG resulting in decreased tumorigenesis in NSCLC is PKCιota/PAR-6/Ect2 → Rac1 → Pak → MEK1/2 → ERK1/2 (Justilien, 2009). Because the PKCιota-PAR-6 complex recruited Ect2 to the cytoplasm leading to transformation, invasion, and tumorigenesis in a NSCLC model, inhibition of the binding between PKCιota and PAR-6 by ATM treatment caused a decrease in NSCLC incidence through disruption of the PKCιota-PAR-6-Ect2 complex (Justilien, 2009).

Thus, PKCιota binding to PAR-6 is essential in apical-basal epithelial cell polarity in addition to cellular migration and invasion. Unfortunately, the PKCιota-PAR-6 complex can be hijacked, causing cancer cell invasion and eventually metastasis.

MEK5

PKC ι interacts with MEK5 upon stimulation with EGF. PKC ι /MEK5 binding is required for EGF activation of ERK5; yet MEK5 is not phosphorylated by PKC ι . Activation of MEK5 by PKC ι binding leads to downstream transcriptional activation of Jun via the MEF2C element. Through a BLAST search, Diaz-Meco found that MEK5 contains a similar PB1 domain as the PKC ι -interacting protein p62. In 293T cells, through a series of IP-western experiments, MEK5 and PKC ι , as well as PKC zeta, were shown to bind via PB1 domain interactions. By five minutes post EGF treatment, PKC ι (or PKCzeta) and MEK5 interact. In HeLa cells, exogenous expression of both wild-type and kinase dead aPKC lead to increased activation of ERK5 in response to EGF. While ERK5 activation was not dependent on the enzymatic activity of aPKC, ERK5 activation did require MEK5 enzymatic activity. Mutation of the MEK5 kinase domain resulted in a block of EGF induced ERK5 activation. While exogenous expression of PKC ι , both wild type and kinase dead, resulted in an increase in Jun promoter activity, as determined by a Jun-luciferase report gene, dominant negative forms of aPKC and MEK5 inhibited Jun promoter activity (Diaz-Meco, 2001). Thus, EGF stimulation of PKC ι /zeta and MEK5 binding is required for activation of ERK5 and subsequently Jun transcription in the EGF \rightarrow PKC ι \rightarrow MEK5/ERK5 \rightarrow Jun (transcriptional activation through the MEF2C element) pathway (Diaz-Meco, 2001).

PSF (*polypyrimidine tract-binding protein (PTB)-associated splicing factor*)

Interaction of PSF with *nuclear* PKC ι led to transcriptional activation of the cytochrome P-450(scc) (P-450 side-chain cleavage) through the sequestering of PSF binding to the P-450(scc) IGFRE (IGF-1 response element). PSF transcriptionally represses P-450(scc) through its interaction with the IGFRE. In JC-410 (stable porcine granulosa) cells, upon IGF-1 treatment, levels of nuclear PKC ι were increased. Upon IGF-1 treatment or exogenous expression of either constitutively active or kinase dead PKC ι , the levels of IGFRE activity were increased. Thus, the role of PKC ι in activation of the IGFRE is kinase-independent. Examination of nuclear extracts by immunoprecipitation experiments revealed the PKC ι bound PSF, leading to a block of PSF repression of P-450(scc) via the IGFRE. Thus, PKC ι is able to overcome PSF suppression on the cytochrome P-450(scc) promoter IGFRE by interaction with PSF in the nucleus upon IGF-1 treatment (Urban, 2004).

L-selectin

In Jurkat T-cells, PKC ι binds the cytoplasmic portion of L-selectin. L-selectin, a leukocyte adhesion molecule, is involved in the inflammatory response by aiding in the leukocyte extravasation step (Vestweber, 1999). By use of a GST-fusion to the cytoplasmic domain of L-selectin and Jurkat cell lysates, Kilian et al was able to identify PKC ι as an L-selectin binding partner in an immunoprecipitation experiment. A PKC ι pseudosubstrate (dominant negative) mutant blocked L-selectin phosphorylation thus implicating PKC ι as an activator of L-selectin-mediated signal transduction. While PKC ζ was not tested for its ability to phosphorylate L-selectin, PKC θ was found to exhibit the same effects as PKC ι on L-selectin phosphorylation (Kilian, 2004). Thus, PKC ι and PKC θ interact with L-selectin, a leukocyte receptor involved in extravasation, and led to the phosphorylation of its cytoplasmic domain.

Bad

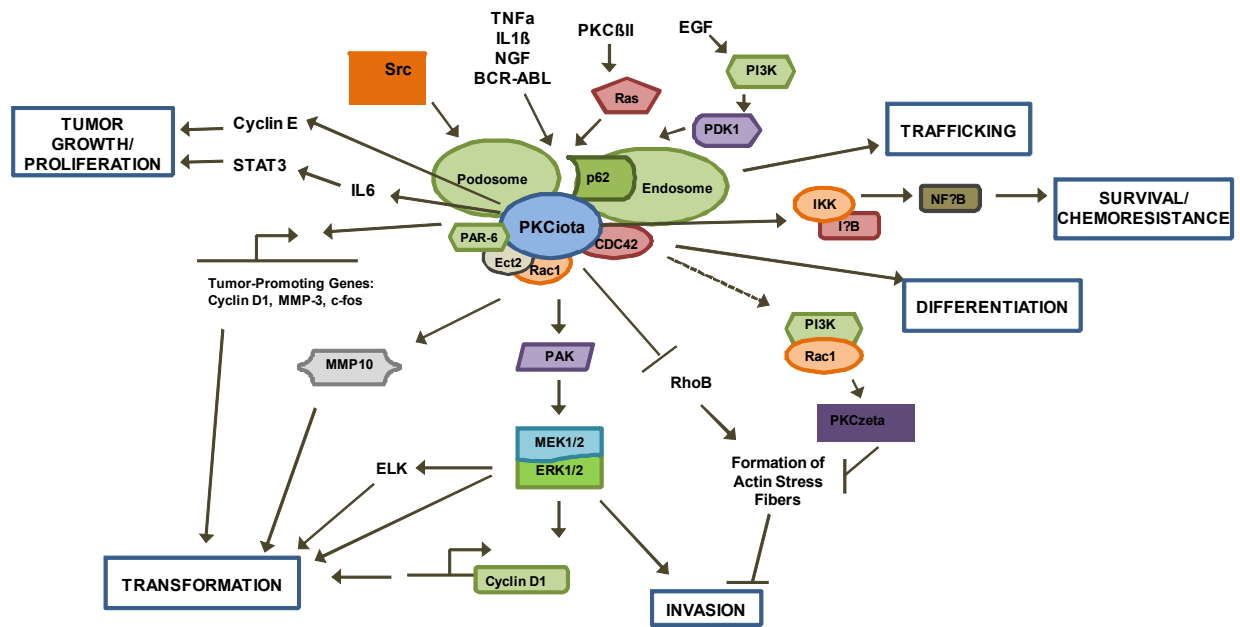
Upon stimulation with NNK (Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), a known potent carcinogen in cigarette smoke, PKC ι led to increased survival through phosphorylation of pro-apoptotic Bad, which disrupted Bad/Bcl-XL binding. PKC ζ was not tested. By use of immunofluorescent microscopy in A549 cells exposed to NNK, Jin et al was able to find that PKC ι and Bad co-localize. Also, in both an *in vitro* kinase assay and western blot analysis using phospho-Bad antibodies, PKC ι was able to phosphorylate Bad at all three sites, which are characteristic of NNK exposure (Ser-112, Ser-136, and Ser-155). Upon stimulation with NNK in human lung cancer cells, Src becomes activated, which leads to PKC ι activation (Jin, 2005). Src had already been shown to bind PKC ζ by affinity chromatography using the regulatory domain of PKC ζ as the bait. This paper also showed that Src was able to tyrosine phosphorylate PKC ζ (Seibenhener, 1999). Both the Src inhibitor, PP2, and a pan-PKC inhibitor, staurosporine, blocked NNK-induced Bad phosphorylation. More specifically PKC ι RNAi was used to block NNK-induced Bad phosphorylation and subsequently block survival. Thus, PKC ι activation in lung cancer cells by the carcinogen NNK can lead to Bad phosphorylation and Bad/Bcl-XL disruption, resulting in increased survival and chemoresistance through the NNK \rightarrow Src \rightarrow PKC ι \rightarrow P-Bad \rightarrow survival by Bad not binding Bcl-XL pathway (Jin, 2005).

In a prostate cancer cell model, silencing PKC ι by RNAi resulted in apoptosis. However, induction of apoptosis occurred through two very distinct mechanisms. In RWPE-1 (transformed non-malignant prostate) cells, knockdown of PKC ι resulted in apoptosis due

to a decrease in phosphoBad (Ser-155, Ser-136) and an increase in Bad/Bcl-xL heterodimerization. No such effect on Bad was observed in DU-145 (androgen-independent malignant prostate) cells. However, RWPE-1 and DU-145 cells both went through apoptosis because of mitochondrial dysfunction/ apoptotic cascades, i.e. cytochrome c release, caspase-7 activation, and PARP cleavage (Win, 2009). Thus, targeting PKC ϵ in cancer treatment is a viable option, and Inhibition of PKC ϵ can result in apoptosis in different pathways, potentially due to oncogenic addiction.

PKC ϵ , unlike the novel and conventional PKC family members, is regulated by protein:protein interactions, many of these interactions are by means of the PB1 domain. This unique ability is what allows PKC ϵ to be involved in so many diverse aspects of the biological functions that occur within a eukaryotic cell, from tumor growth and proliferation (p70 S6K and MEK5) to transformation (CDC42), polarity (ASIP/PAR-3, Adducin, and PAR-6), invasion (PAR-6), differentiation (unknown), survival (LIP and BAD), and membrane trafficking (p62/ ZIP) (see Table 7 above and Figure OPP below). Some PKC ϵ binding partners affect localization (p62/ ZIP, ASIP/PAR-3, FRS2, CDC42, Adducin, PSF, and L-selectin), while others inhibit PKC ϵ activity (PAR-4). By determining which binding partners are overexpressed in tumor cells, this allows for a better understanding of which pathways are predominant in tumorigenesis, in an attempt to discover alternative pathways to be targeted therapeutically in cancer patients. Knowledge of which pathways are active in different cancers allows for specific regimens to be tailor-fitted to each individual patient. For example, the PKC ϵ inhibitor that I will be discussing in detail works by disrupting PKC ϵ PB1:PB1 interactions. However, if the pathway that is active in a particular cancer is not active due to PB1:PB1 interactions, but rather interactions of PKC ϵ at another domain, the PB1-inhibiting drug would be inadequate and ineffective. Thus, a profound knowledge of the pathways involved in tumorigenesis, including protein:protein interactions allows for an improved ability to acquire therapies to target these pathways.

Figure OPP: Overall PKC δ Pathway



SIGNALING: RAS

Ras through PKC α is able to induce transformation through three main pathways: (1) induction of cyclin D1- leading to increased transformed growth, (2) increased transcription of tumor-promoting genes- such as MMP-3 (leading to invasion) and c-fos (a tumor-promoting transcription factor), and (3) re-arrangement of the cytoskeleton- such as stress fiber loss. As always, discrepancies exist in these pathways as indicated by the dashed arrows in the diagram below (see Figure 10). These discrepancies could be present for many reasons including use of different cells types in these studies.

Ras-PKC α in Transformation

Transformation can occur when normal cells assume the ability to survive and grow void of anchorage-dependence. Normal (anchorage-dependent) cells that have detached would undergo anoikis- a specific form of programmed cell death (Grossmann, 2002). Ras is a well known oncogene recognized for inducing transformation and PKC α is necessary for Ras-induced transformation. PKC ζ (the other atypical PKC family member) had been shown to interact with Ras *in vitro* and *in vivo* (Diaz-Meco, 1994).

In NIH 3T3 cells, a dominant negative (kinase-defective) mutant of PKC α / lambda was able to revert the transformed phenotype resultant of either v-Ras or PC-PLC (phosphatidylcholine-hydrolyzing phospholipase C) (Bjorkoy, 1997). Specifically, in v-Ras and PC-PLC transformed NIH 3T3 cells, ERK1/2 (extracellular signal-regulated kinase) are constitutively activated (even upon growth factor deprivation) and localized to the nucleus. Dominant negative PKC α / lambda reverts this phenotype. The reversion of the phenotype along with transient transfection experiments demonstrate that PKC α / lambda is downstream of v-Ras but upstream of ERK1/2 in v-Ras and PC-PLC transformed NIH 3T3 cells (Bjorkoy, 1997). Essentially PKC α was found to be in the Ras/PLC \rightarrow PKC α \rightarrow MAPK/ERK1/2 \rightarrow ELK \rightarrow transformation pathway. This pathway was then disputed by Kampfer et al, stating that the MEK/ERK pathway was involved in oncogenic Ras signaling, but this pathway was PKC α -independent as described below (Kampfer, 2001). Oncogenic Ras has been shown to induce transformation through several mechanisms, two of these mechanisms are (1) through the G1 phase of the cell cycle by myc (Murray, 1983) and cyclin D1 induction (Filmus, 1994; Lovec, 1994) and (2) by phenotypic changes due to alterations in cell-cell adhesion molecules, such as the Rho family: CDC42, Rac1, and RhoA, B, and G (Qiu, 1995; Khosravi-Far, 1995; Prendergast, 1995; Qiu, 1995; Roux, 1997; Lebowitz, 1997) and by

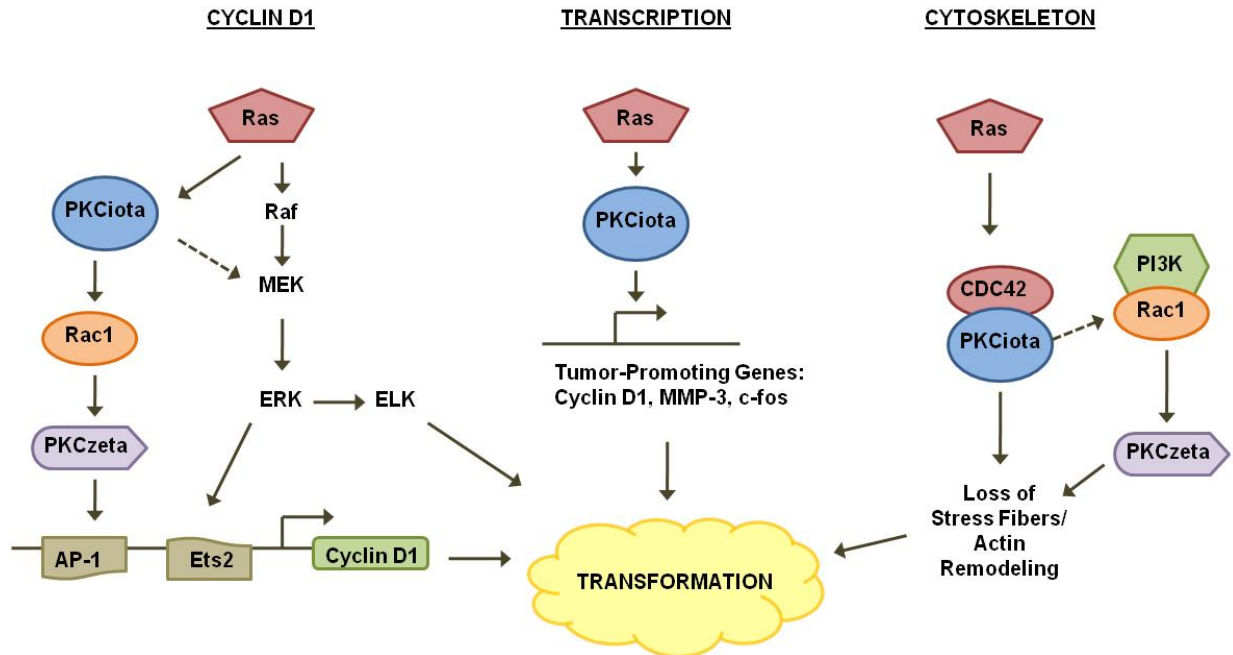
actin stress fiber remodeling (Zohn, 1998; Ridley, 1992; Prendergast, 1993; Dartsch, 1994). PKC δ / λ has been linked to the Rho family members (CDC42 and Rac1), actin filament remodeling, and the induction of cyclin D1 (see below).

Ras-PKC δ in Transformation-Cyclin D1 Induction- In HC11 mouse mammary epithelial cells, dominant negative mutants of PKC δ / λ (or PKC ζ) suppress Ras-induced cyclin D1 expression, indicating that both PKC δ / λ and PKC ζ are essential for cyclin D1 induction by oncogenic Ras. The induction of cyclin D1 was measured using a cyclin D1 luciferase reporter assay. While PKC ζ induction of cyclin D1 is downstream of Raf-1, as in the case of c-fos induction, PKC δ / λ does not seem to be in the Raf-Mek pathway for cyclin D1 induction but rather in a pathway involving Rac1 (Hellbert, 2000). This is not surprising that the two pathways work separately in cyclin D1 induction. The cyclin D1 promoter had already been shown to contain an Ets2 binding site (activated by the Raf/MEK/ERK pathway) in addition to an AP-1 site. PKC δ has been shown to activate AP-1 (see “PKC δ -Signaling: NF κ B” and “PKC δ -Signaling: AP-1” for details).

In a separate study, in HC11 mouse mammary epithelial cells, through the use of a cyclin D1 luciferase reporter gene, Mwanjewe et al was able to show that PLD2 and PKC δ / λ cooperate in the transcriptional activation of cyclin D1 (Mwanjewe, 2001).

The study that disputed the findings of Bjorkoy et al (mentioned above) found that Ras-PKC δ -Rac are in a different pathway than Ras-MEK-ERK. Specifically, transcriptional regulation of cyclin D1 by transforming Ras required two pathways, one PKC δ - dependent and one PKC δ -independent. By using constitutively active Ras, Raf, MEK, and a cyclin D1 reporter gene, in HC11 mouse mammary epithelial cells, Kampfer et al found that PKC δ was not involved in Ras induction of cyclin D1 through the MEK-ERK pathway (however PKC ζ was required). By using constitutively active Ras, Rac, and a cyclin D1 reporter gene, PKC δ was found to be downstream of Ras but upstream of Rac. Interestingly, PKC ζ was found to be involved downstream of Rac (Kampfer, 2001). Thus, Ras transcriptionally activates cyclin D1 in a PKC δ -independent (HA-Ras \rightarrow MEK-1 \rightarrow ERKs \rightarrow cyclin D1 transcription) pathway and a PKC δ -dependent (HA-Ras \rightarrow PKC δ \rightarrow Rac \rightarrow cyclin D1 transcription) pathway.

Figure 10: Ras-PKCiota in Transformation



Ras-PKC α in Transformation- Cytoskeletal Reorganization- Ras, Rac, CDC42, PKC α /lambda, and PKC ζ all individually had been implicated in stress fiber loss and actin remodeling (Zhao, 1998; Uberall, 1999). Cells, which have undergone transformation have alterations, in their actin cytoskeleton, one of these alteration is a loss of stress fibers (Mackay, 1998). While Uberall et al linked Ras with PKC α /lambda and PKC ζ , Coghlan et al linked Ras-CDC42-PKC α /lambda in stress fiber loss. While Rac expression is able to cause stress fiber loss, this phenotype is through a different pathway than Ras-CDC42-PKC α /lambda pathway (Coghlan, 2000), a study that was contradicted by Uberall et al (Uberall, 1999).

In NIH3T3 cells, ectopic expression of constitutively active CDC42 (V12 CDC42) led to a loss in stress fibers (Kozma, 1995). Coghlan et al was able to show that transient exogenous expression of PKC α /lambda (but not PKC ζ) was able to lead to the same loss of stress fibers observed in response to CDC42 overexpression. To show that PKC α /lambda was essential for CDC42-induced loss of stress fibers, kinase dead mutants of PKC α /lambda (KD PKC α /lambda) and PKC ζ (KD PKC ζ) were used to block CDC42-induced loss of stress fibers. The KD PKC α mutant blocked CDC42- induced loss of stress fibers, but had no effect on CDC42- induced membrane ruffling or filopodia formation, indicating that while PKC α /lambda was essential for CDC42-induced loss of stress fibers, CDC42 induced other pathways independently of the PKC α /lambda or PKC ζ pathways. Because Rac-1 had also been implicated in loss of stress fibers, the KD PKC mutants were used to block loss of stress fibers in response to Rac. However, the KD PKC mutants were unable to block Rac-induced loss of stress fibers. Uberall et al had published that Ras-induced loss of stress fibers, was not only dependent on PKC α /lambda and PKC ζ , but also dependent on Rac. In contrast, Coghlan et al reported that, upon induction of NIH 3T3 cells with constitutively active Ras and either dominant negative CDC42 (N17 CDC42) or dominant negative Rac, only N17 CDC42, but not dominant negative Rac, was able to block Ras-induced loss of stress fibers. Thus, according to Coghlan et al at least two pathways exist for loss of stress fibers in transformation, one pathway involving Ras- CDC42- PKC α /lambda and one involving Rac (independently of Ras or the atypical PKCs). It is important to note that Coghlan et al did not directly test the KD PKC mutants on transformation just on indicators of transformation (loss of stress fibers).

CDC42 also exhibits PKC α independent functions. While PKC α /lambda was linked to CDC42-induced loss of stress fibers, PKC α /lambda does not play a role in the

previously published CDC42- induced JNK activation or Fos expression (Coso, 1995; Minden, 1995; Chang, 1998; Kaneki, 1999; Coghlan, 2000).

In NIH3T3 and COS fibroblasts, PKC α / λ is essential for transforming RAS-mediated disassembly of actin fibers. PI3K had already been shown to be necessary for Ras-induced reorganization of the actin cytoskeleton (Rodriguez-Viciano, 1994). Upon addition of transforming Ha-RAS L61, re-organization of the cytoskeleton occurs. RAS activates PKC α / λ , which in turn, activates PI3K/Rac-1, leading to disassembly of actin fibers. This role of PKC α was demonstrated using both dominant negative PKC α / λ (in the presence of transforming Ha-RAS L61) and constitutively active PKC α / λ to mimic the effect of Ha-RAS L61 on cytoskeleton remodeling. Likewise, PKC ζ is involved in RAS-mediated cytoskeleton re-modeling, but unlike PKC α / λ , PKC ζ acts downstream of PI3K/Rac-1 (Uebachs, 1999). Thus, Ras signaling is involved in the remodeling of actin in a PKC α -dependent (Ha-Ras L61 \rightarrow PKC α / λ \rightarrow PI3K/Rac-1 \rightarrow actin remodeling) and PKC α -independent (Ha-Ras L61 \rightarrow PI3K/Rac-1 \rightarrow PKC ζ \rightarrow actin remodeling) manner.

Ras-PKC α in Transformation- Transcriptional Activation- Ras through PKC α is able to increase transcription of several tumor promoting genes, such as MMP-3, cyclin D1, and c-Fos. Transcriptional upregulation of tumor promoting genes, as well as alterations in the cytoskeleton are two of the ways Ras induces transformation through PKC α .

Ras through PKC α transcriptionally activates MMP-3 (matrix metalloproteinase 3), which is involved in breakdown of the extracellular matrix (leading to invasion into surrounding tissue as well as metastasis). In NIH3T3 human fibroblasts, PDGF signaling (as measured by induction of the human stromelysin-1 (MMP-3) gene) was affected by PKC α in a RAF-independent fashion (Kirstein, 1996). Specifically, MMP-3 transcriptional regulation was through two elements on the promoter, a PEA3-AP-1 element and a SPRE-AP-1 element. Raf, in a PKC α -independent manner activated MMP-3 transcription through the PEA3-AP-1 element according to the pathway: PDGF \rightarrow RAS \rightarrow 1) RAF (PEA3-AP-1 Element) \rightarrow MMP-3 transcriptional activation, while PKC α in a Raf-independent manner activated transcription of MMP-3 through the SPRE-AP-1 element according to the pathway: PDGF \rightarrow RAS \rightarrow PKC α (SPRE-AP-1 Element) \rightarrow MMP-3 transcriptional activation (Kirstein, 1996).

Similar to the transcriptional activation of MMP-3, Ras through PKC α is able to transcriptionally up-regulate c-fos, a tumor-promoting transcription factor. Transforming Ras had already been shown to induce c-fos expression (Stacey, 1987). Like MMP-3 transcriptional up-regulation occurs through two pathways, a PKC α -dependent pathway and

a Raf/PKCzeta-dependent pathway. Using a series of PKC ι /lambda antisense, dominant negative, kinase defective, and constitutively active mutants in HC11 mouse mammary epithelial cells, both PKC ι /lambda and PKCzeta were shown to be necessary for transcriptional activation of c-fos by Ha-Ras. The pathway seems to be Ras \rightarrow PKC ι /lambda \rightarrow c-fos transcriptional activation. However, the Raf/PKCzeta pathway appears to be Ras \rightarrow Raf-1 \rightarrow MEK \rightarrow PKCzeta (but not PKC ι /lambda) \rightarrow c-fos transcriptional activation (Kampfer, 1998). This implies that PKC ι /lambda and Raf/PKCzeta are autonomous in their roles in c-fos activation.

Ras leads to co-localization of PKC ι with CDK9/cyclin T1, which is implicated in RNA transcription through phosphorylation of RNA polymerase II. Oncrasin-1 (an inhibitor of mutant Ras (Guo, 2008)) works by inhibition of the phosphorylation of RNA polymerase II and causes PKC ι to coaggregate with splicing factors into what are known as “megaspliceosomes”, which are involved in pre-mRNA processing. Interestingly, PKC ι forms a complex with CDK9/cyclin T1, which is the complex involved in the phosphorylation of RNA polymerase II. The PKC ι /CDK9/cyclin T1 complex is disrupted by oncrasin-1 *in vitro*, thus resulting in a decrease in RNA polymerase II phosphorylation (Guo, 2009). Therefore, PKC ι is likely involved in RNA processing through splicing and transcriptional activation.

The role of PKC ι in transcriptional activation has been addressed in several studies. For example, using meta-analysis of lung adenocarcinomas (LACs), Erdogan et al found that PKC ι expression correlated with COPB2, ELF3, RFC4, and PLS1 expression. By use of RNAi to silence either PKC ι or any of these four other genes, Erdogan found that PKC ι controlled the expression of these four genes (as determined by qRT-PCR), possibly accounting for some of the ability of PKC ι to drive tumorigenesis (Erdogan, 2009). More research needs to be done to determine the exact role of these four genes in PKC ι -driven tumorigenesis.

Ras-PKC ι in Lipid Signaling- PKC ι /lambda is involved in lipid signaling. In HC11 mouse mammary epithelial cells, through the use of the atypical PKC specific inhibitor, Ro-31-8220, PKC ι was identified in Ras-mediated phospholipase D (PLD) signaling. PLD is an important enzyme which is necessary for hydrolysis of membrane phospholipids in the generation of phosphatidic acid (PA), a signaling lipid and precursor for DAG (diacylglycerol), which is involved in the recruitment of cytosolic signaling proteins to the plasma membrane. Through transfection experiments, the order Ras \rightarrow PLD1b \rightarrow PKC ι /lambda \rightarrow PLD2 was established (Mwanjewe, 2001). Thus, PKC ι plays an active role in lipid signaling.

From the literature analyses done here, one can conclude that PKC α is necessary for Ras –induced transformation by induction of cyclin D1 (resulting in an increase in transformed growth), transcriptional activation of genes involved in invasion and tumor-promotion (such as MMP-3 and c-fos), and cytoskeletal changes (such as loss of stress fibers). While these factors taken together could result in transformation, we have found that another protein, cyclin E, appears to be a downstream effector molecule in PKC α -induced transformation. Using a rescue of PKC α knockdown by addition of exogenous cyclin E (EL and LMW-E), we found that cyclin E is necessary for PKC α -induced transformation. The next few questions could be: which pathway is PKC α /cyclin E involved in (expression of cyclin D1, MMP-3, or c-fos) or, is cyclin E affecting cytoskeletal organization? Or rather, is there another pathway that PKC α /cyclin E is in that is resulting in the transformed phenotype? Clinically, the question remains if inhibition of Ras, PKC α , or cyclin E or any combination of these oncogenes result in a synergistic decrease in transformed growth *in vivo*?

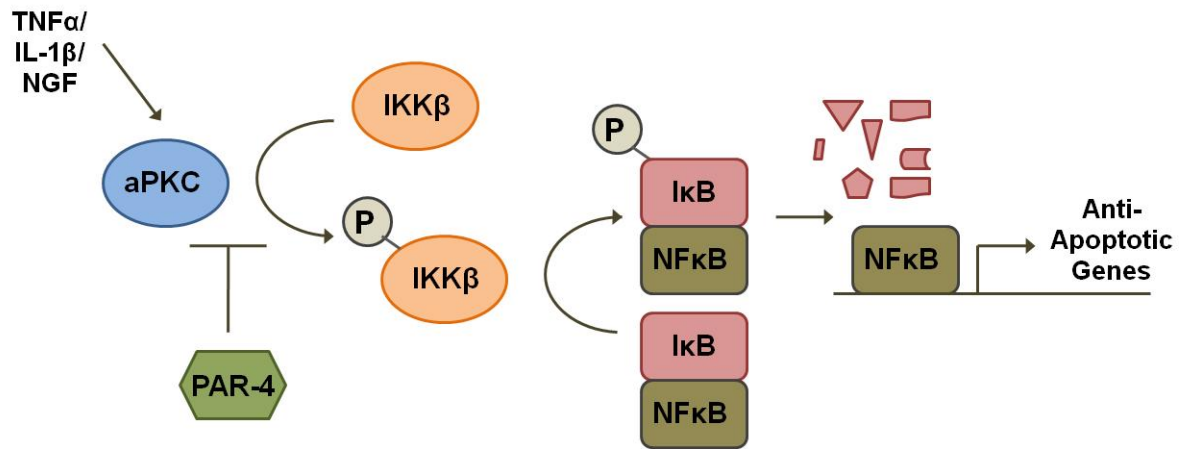
SIGNALING: NF κ B

NF κ B (Nuclear Factor Kappa-light-chain-enhancer of activated B cells) is a transcription factor involved in immune response. However, in cancer cells, NF κ B is linked to survival due to its ability to transcriptionally activate anti-apoptotic genes, thereby suppressing apoptosis. There are of course exceptions. For example, while in epithelial cells NF κ B (and PAR-6) are involved in survival and chemoresistance, in the stromal compartment, the expression of NF κ B (and PAR-6) is correlated with a favorable prognosis (Al-Saad, 2008). However, overall, NF κ B expression leads to survival, an autocrine feedback response, and chemoresistance of tumor cells.

Mechanism of PKC α Activation of NF κ B

Many different laboratories have shown that PKC α leads to activation of NF κ B through the pathway (see Figure 11):

Figure 11: Mechanism of PKC α Activation of NF κ B



Mechanism of PKC ι Activation of NF κ B-TNF α

Diaz-Meco found that PKC ι (but not PKC ζ) led to the activation of NF κ B through its interaction with LIP (Lambda Interacting Protein). In both Cos and NIH3T3 cells, PKC ι /lambda exogenous expression led to activation of NF κ B, which was decreased by a dominant negative isoform (pseudosubstrate domain) of PKC ι /lambda. Exogenous LIP, which binds specifically to PKC ι /lambda, led to κ B- dependent promoter activation similar to that of TNF α . The activation of κ B promoter, which indicated activation of NF κ B, was increased upon co-transfection with the PKC ι / lambda expression construct and decreased upon co-transfection with the kinase dead PKC ι /lambda expression construct. The decrease in LIP-induced, κ B-dependent promoter activity was not observed upon co-transfection with a kinase dead form of PKC ζ (Diaz-Meco, 1996). However, eventually, PKC ζ was shown to phosphorylate and activate IKK β , which in turn phosphorylated I κ B, leading to its degradation, causing a release of cytoplasmic NF κ B, allowing NF κ B to translocate to the nucleus, where it is active (Lallena, 1999). Thus, implying both atypical PKCs possess the ability to activate NF κ B.

In another study, using a dominant negative form of PKC ι /lambda in 293 cells, Sanz et al was able to link both PKC ι /lambda and PKC ζ to NF κ B transcriptional activation through the interaction of PKC ι /lambda or PKC ζ with RIP and p62. PKC ι / lambda was not able to bind directly to RIP. Using a series of *in vitro* and *in vivo* binding assays, p62 was shown to bind to RIP, which is a known molecule in the TNF α to NF κ B pathway. The dominant negative PKC ι /lambda construct inhibited RIP- induced NF κ B activation. PKC ι /lambda, p62, and RIP all must be present in a complex in order for PKC ι /lambda to activate IKK β and therefore activate NF κ B. Therefore, while p62 links RIP to PKC ι / lambda, PKC ι / lambda links p62 to IKK β . Thus, TNF α \rightarrow TNFR1 \rightarrow TRADD \rightarrow RIP/p62/aPKCs (independent of TRAF2) \rightarrow IKK β \rightarrow Degradation of I κ B \rightarrow NF κ B (activation/ nuclear translocation) (Sanz, 1999).

As mentioned, PKC ι is able to lead to NF κ B activation through p62 interaction in response to TNF α . Conversely, the TNF α pathway is also able to activate PAR-4, whose expression inhibits the TNF \rightarrow PKC ι /p62 \rightarrow NF κ B pathway by binding and sequestering PKC ι . Thus, TNF α can induce apoptosis (PAR-4) or survival (NF κ B) (Diaz-Meco, 1999). PAR-4 has been deleted in some cancers (Cook 1999), tipping the balance toward survival. Similarly, Ras has been shown to inhibit PAR-4, also leading toward survival (Berra, 1993). In NIH-3T3 mouse fibroblasts, upon TNF α induction, pro-apoptotic PAR-4 expression inhibits

translocation of NF κ B and therefore inhibits the transcription of κ B in response to TNF α . PAR-4 is able to inhibit TNF α -induced survival and cause apoptosis by binding PKC ι /lambda and PKC ζ and inactivating their kinase activity. Therefore, addition of constitutively active atypical PKCs revert the effects of exogenous PAR-4 expression, causing complete loss of apoptosis in response to TNF α (Diaz-Meco, 1999). Thus, a switch is implicated in NF κ B activation by TNF α between the PKC ι -binding partners, p62 (survival) and PAR-4 (apoptosis).

The TNF α pathway leads to NF κ B activation through the pathway: TNF α \rightarrow TNFR1 \rightarrow TRADD \rightarrow RIP/p62/aPKCs (independent of TRAF2) \rightarrow IKK β \rightarrow Degradation of I κ B \rightarrow NF κ B (activation/ nuclear translocation) (Sanz, 1999). While this pathway is the consensus, several other pathways have been observed in PKC ι activation of NF κ B. Anthonsen et al found that both inflammatory cytokines TNF α and IL-1 β led to NF κ B activation through the TNF α /IL-1 β \rightarrow PI3K \rightarrow PKC ι \rightarrow cPLA2 \rightarrow NF κ B and TNF α /IL-1 β \rightarrow snpPLA2 \rightarrow P-PKC ι \rightarrow P-cPLA2 \rightarrow NF κ B pathways (Anthonsen, 2001). In human keratinocytes, HaCaT cells, Anthonsen et al found that by using a pseudo-substrate inhibitor to the atypical PKCs, TNF α /IL-1 β induced cPLA2 phosphorylation, arachidonic acid release, and NF κ B activation were all decreased. In NIH3T3 cells, the role of PKC ι / lambda in activation of cPLA2 and arachidonic acid release in response to TNF α /IL-1 β was validated by use of a PKC ι / lambda kinase-dead mutant to block response to TNF α /IL-1 β . Interestingly, blocking PI3K activity also blocked PKC ι activation and TNF α /IL-1 β induced NF κ B activation. Thus, PKC ι is involved in TNF α /IL-1 β -induced activation of NF κ B via two unique mechanisms (Anthonsen, 2001).

Mechanism of PKC ι Activation of NF κ B-NGF- Upon stimulation by NGF (nerve growth factor), PKC ι is implicated in several other pathways for NF κ B activation, some leading to survival and some leading to differentiation (Wooten, 2000). NGF activation of NF κ B will be covered in detail below in the section on NGF signaling.

Discrepancies in NF κ B Regulation by PKC ι - While PKC ι activates NF κ B in some cells, PKC ζ activates NF κ B in other cell lines, yet sometimes NF κ B activation is completely independent of the atypical PKCs, even upon addition of the same stimulus (TNF α in these cases).

In NIH3T3 (mouse embryonic fibroblasts) and Jurkat (immortalized T-lymphocytes) but not MCF7 (epithelial breast cancer) cells, PKC ι / lambda is involved in NF κ B transcriptional activation in response to TNF α (in NIH3T3 and Jurkat cells) and IL-1 β (in NIH3T3 cells). In the

TNF α and IL-1 β pathways, PKC ι /lambda is necessary but not sufficient to activate NF κ B. In MCF7 cells, TNF α was able to activate PKC ι / lambda and thus AP-1 but not NF κ B, indicating that the role of PKC ι / lambda in NF κ B activation is cell type specific (Bonizzi, 1999). In these two cell lines, PKC ι is necessary but not sufficient to activate NF κ B (exogenous dominant negative (dn) PKC ι blocked NF κ B activation but wild type (wt) PKC ι did not lead to its activation). Note: The dominant negative used in these experiments might also inhibit PKC ζ .

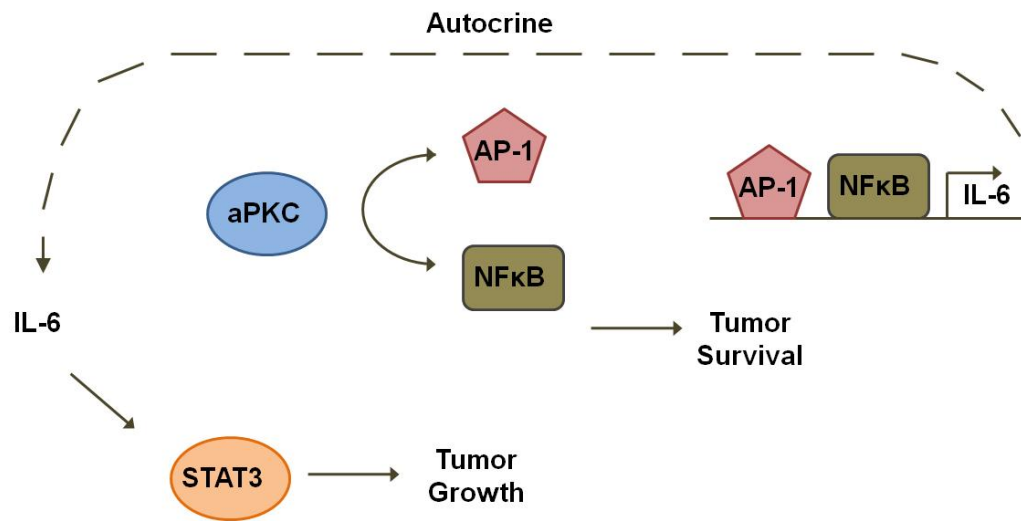
Similarly, in A549 lung cancer cells, exogenous expression of wild type or kinase dead PKC ι did not alter the level of NF κ B expression upon TNF α stimulation. Although, TNF α expression did still cause an increase in the levels of phospho-I κ B α . Thus, in A549 cells, the regulation of NF κ B by TNF α was independent of PKC ι status (Regala, 2005).

In activation of NF κ B in prostate cancer cells by stimulation with TNF α , the role of PKC ι is unclear. In three different prostate cancer cells, three different responses were seen in PKC ι activation. For example, in LNCaP cells, PKC ι did not phosphorylate IKK $\alpha\beta$, a protein that upon phosphorylation gets degraded allowing NF κ B to translocate to the nucleus. However, in DU-145 cells, PKC ι was able to phosphorylate IKK $\alpha\beta$. However, in RWPE-1 cells, the kinase responsible for IKK $\alpha\beta$ phosphorylation is PKC ζ . Thus, the role of PKC ι in the activation of NF κ B in response to TNF α is not well defined in prostate cancer cells (Win, 2008). Because all three of these studies were performed in the same laboratory, it is highly unlikely that lab variation (such as different conditions or different reagents) is the reason for these discrepancies in PKC ι phosphorylation of IKK $\alpha\beta$ in the prostate model. PKC ι and PKC ζ exhibit much functional redundancy, as specified earlier in regard to their overlapping roles and overlapping binding partners. Thus, there is no surprise that PKC ζ is the active kinase in RWPE-1 cells. I speculate that the reason for these discrepancies is more likely due to the differences in the levels of PKC ι and PKC ζ in these prostate cancer cell lines. For example, I would hypothesize that PKC ι levels are high in DU-145 cells, PKC ζ is high in RWPE-1 cells, and another kinase other than atypical PKC is high in the LNCaP cells, thus overriding the ability of PKC ι or PKC ζ to phosphorylate IKK $\alpha\beta$. Another explanation could be that one or all of the kinases that would usually phosphorylate IKK $\alpha\beta$ is low in the DU-145 and RWPE-1 cell lines but not in the LNCaP cell line. I believe that PKC ι and PKC ζ are able to phosphorylate IKK $\alpha\beta$, but only under altered conditions, such as overexpression of PKC ι or PKC ζ .

NFκB and Autocrine Response- PKCιota regulation of NFκB can also stimulate an autocrine response, leading to increased tumor cell proliferation and survival. In an early study, PKCιota was found to be in a signalsome with TRAF6, p62, and IKK through a series of immunoprecipitations in response to Thymosin alpha 1 (Ta1), a treatment of cancer in clinical trials which acts on both T-helper and NK-cells and alters hormone regulation in the hypothalamus. Ta1 was used in this situation to stimulate an immune response. This induction of the signalsome led to IL-6 expression through NFκB according to the pathway: Thymosin alpha 1 (Ta1) → TRAF6/p62/PKCιota/zeta/ IKK → NFκB → IL-6 (Zhang, 2005).

Similarly, in a prostate cancer model, PKCιota expression led to induction of an autocrine response through increased expression of IL-6, resulting in STAT3 phosphorylation/activation and thus proliferation. PKCιota increased the expression of IL-6 through upregulation of the two transcription factors, AP-1 and NFκB (Ishiguro, 2009). PKCιota had previously been shown to activate AP-1 (see below) (Huang, 1996; Huang, 1997; Bonizzi, 1999; Huang, 2000). Thus, not only is NFκB activation PKCιota dependent, but it can stimulate IL-6 production in an autocrine manner (see Figure 12).

Figure 12: PKC α -Induced IL-6 Autocrine Signaling



NFκB and Chemoresistance- Chemoresistance, which is the resistance of a tumor to chemotherapy, to Taxol in Bcr-Abl positive human erythroleukemia K562 cells is mediated via a PKCιota/NFκB-dependent mechanism. In cells that do not contain Bcr-Abl or K562 cells that have been treated with the Bcr-Abl inhibitor AG957, Taxol treatment did not lead to chemoresistance. Also, treatment with Taxol in the presence of antisense or kinase-dead PKCιota did not lead to chemoresistance. Lastly, treatment of Taxol in the presence of NFκB inhibition (by blocking IκB degradation) also did not lead to chemoresistance (Lu, 2001). Thus PKCιota and NFκB are both necessary for Bcr-Abl induced chemoresistance to Taxol.

PKCιota regulation of NFκB via phosphorylation of IKKβ has already been established (Diaz-Meco, 1996; Sanz, 1999; Diaz-Meco, 1999). Lu et al showed that in addition to the regulation of IKKβ by PKCιota, overexpression of a constitutively active PKCιota resulted in an increase in the transcription of RelA, a component of the NFκB complex. Thus, NFκB regulation in response to Bcr-Abl is through the pathway: Taxol → Bcr-Abl → PKCιota → NFκB (due to IκB degradation and RelA transcriptional up-regulation) → Survival (transcriptional activation of anti-apoptotic genes). Similarly, kinase-dead PKCιota partially blocked the transcription of RelA (Lu, 2001). Thus, PKCιota activates NFκB by two mechanisms, (1) phosphorylation of IKKβ and (2) transcriptional activation of RelA. Therefore, Bcr-Abl is capable of mediating survival in response to Taxol in a PKCιota/ NFκB dependent manner. Thus, PKCιota regulation of NFκB due to Bcr-Abl expression induces Taxol-resistance in CML.

The study of PKCιota in NFκB has been very extensive. However, as you read, there are as many studies indicating PKCιota does influence NFκB as there are studies showing PKCιota does *not* influence NFκB. There are many possible explanations and potential reasons for these discrepancies. For example, earlier I mentioned the switch between the two PKCιota binding partners, p62 (which leads to survival through PKCιota activation) and PAR-4 (which leads to cell death through inhibition of PKCιota). Without any fluctuation in PKCιota levels, the outcome could be due to the levels of p62 versus PAR-4. Or, if either p62 or PAR-4 is mutated in that particular cell line, this too could tip the balance of survival versus apoptosis in regard to PKCιota activation. Another possible explanation could be through a threshold effect of PKCιota activity. If a limiting factor other than PKCιota is limiting PKCιota activity, an increase in PKCιota levels would be unable to cause an increase in PKCιota phosphorylation and thus unable to cause an increase in NFκB activation. Because a cell contains so many regulators of different pathways, other regulators of NFκB would also need to be considered

as variables within any given experiment. In closing, I believe that PKC ζ does indeed alter NF κ B activation. However, many other variables need to be considered, including, but not limited to, p62 and PAR-4 status.

SIGNALING: AP-1

Activation of PKC ζ has been shown to lead to AP-1 induction. AP-1 is a heterodimeric transcription factor composed of c-fos, c-jun, or ATF (activating transcription factor) family members. Many studies have been performed using UV (ultraviolet) radiation as a means for induction of AP-1. Thus, this dissertation will examine the role of PKC ζ in UV-induced AP-1 activation.

As mentioned, AP-1 can be composed of c-fos and c-jun family members. Thus, this section will also review the effect of PKC ζ on c-fos and c-jun induction.

PKC ζ Regulates AP-1 Induction

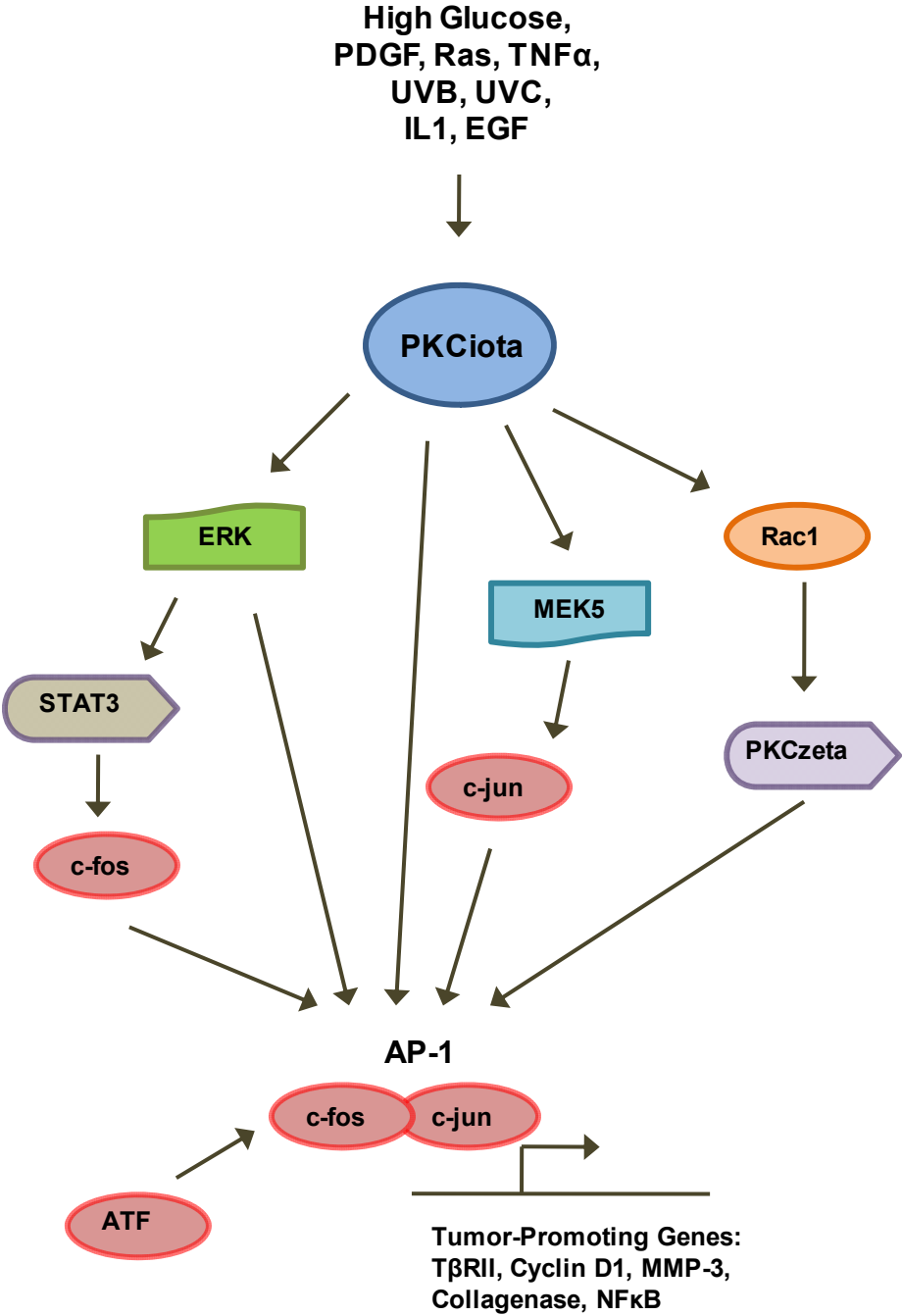
PKC ζ is able to activate AP-1 in various model systems through stimuli such as: high glucose, PDGF, Ras, or TNF α (Kirstein, 1996; Huang, 1996; Huang, 1997; Bonizzi, 1999; Huang, 2000; Hellbert, 2000; Chuang, 2003). Several studies have been performed examining the transcriptional upregulation of various genes through AP-1 activation, including: T β RII (type II transforming-growth-factor-beta receptor), IL-6 (Interleukin-6), cyclin D1, MMP-3 (matrix metalloproteinase 3), and collagenase gene induction (leading to MMP-1 activation- this one will be covered in the c-fos portion of this section) (see Figure 13).

In the presence of high glucose, in MDCK cells, PKC ζ gets translocated into the cytosolic fraction, where it becomes activated. Activated PKC ζ is able to lead to AP-1 activation, which leads to AP-1 transcriptional activation of T β RII (type II transforming-growth-factor-beta receptor) (Chuang, 2003).

PKC ζ increased IL-6 expression through activation of the two transcription factors, AP-1 and NF κ B, which in turn created an autocrine response leading to tumor growth and survival (Ishiguro, 2009).

Oncogenic Ras stimulation led to an increase in cyclin D1 expression through the Ras-PKC ζ -Rac-PKC ζ -AP-1 pathway (Hellbert, 2000). Ras also transcriptionally activates MMP-3 (matrix metalloproteinase 3), through the PDGF \rightarrow RAS \rightarrow PKC ζ (SPRE-AP-1 Element) \rightarrow MMP-3 transcriptional activation pathway (Kirstein, 1996) (see “PKC ζ -Signaling: Ras” section for more details about cyclin D1 and MMP-3 transcriptional upregulation through AP-1 and the implications on transformation).

Figure 13: Mechanism of AP-1 Regulation by PKC ζ



UV-Induced AP-1- AP-1 is necessary for cellular transformation in JB6 cells. Activation of AP-1 by TPA was originally shown to be through EGF (Dong, 1994). In JB6 P⁺ C1 41 mouse epidermal cells, dominant negative expression of PKC ι /lambda resulted in reduced UVB-induced AP-1 activity (Huang, 1996). In the case of UVB-induced activation of AP-1, activation appears to be independent of EGF, but dependent on PKC ι (Huang, 1996). Similarly, in JB6 (mouse epidermal) and B82 (mouse fibroblast) cells, dominant negative expression of PKC ι /lambda resulted in reduced UVC-induced AP-1 activity (Huang, 1997).

Examining the mechanism of UV-induced AP-1 activation through PKC ι revealed that, while AP-1 induction can be through ERK, JNK, or p38 kinase activation, the UV-induced activation of AP-1 by PKC ι /lambda appears to be through ERK in JB6 cells (Huang, 2000).

PKC ι Regulates c-fos Induction- Upon induction with IL-1 (Interleukin-1), PKC ι through the ERK/STAT3 pathway has been demonstrated in c-fos activation. This activation of c-fos, by IL-1 results in an increase in collagenase expression, leading to activation of MMP-1 (matrix metalloproteinase 1) according to the pathway: IL1 \rightarrow PKC ι \rightarrow ERK/STAT3 phosphorylation \rightarrow c-fos expression \rightarrow collagenase gene induction \rightarrow MMP1 (Litherland, 2010) (see “PKC ι - Functions: Inflammation” for details).

Ras through PKC ι is able to transcriptionally up-regulate c-fos, a tumor-promoting transcription factor. Transforming Ras had already been shown to induce c-fos expression (Stacey, 1987). The PKC ι -dependent pathway seems to be Ras \rightarrow PKC ι /lambda \rightarrow c-fos transcriptional activation. However, the PKC ι -independent pathway appears to be Ras \rightarrow Raf-1 \rightarrow MEK \rightarrow PKC ζ (but not PKC ι /lambda) \rightarrow c-fos transcriptional activation (Kampfer, 1998). This implies that PKC ι /lambda and Raf/PKC ζ are autonomous in their roles in c-fos activation.

PKC ι Regulates c-jun Induction

Upon EGF-stimulation, PKC ι through its binding to MEK5 is able to induce c-jun, a component of the AP-1 transcription factor. PKC ι /MEK5 binding is required for EGF activation of ERK5; yet MEK5 is not phosphorylated by PKC ι . While exogenous expression of PKC ι , both wild type and kinase dead, resulted in an increase in Jun promoter activity, as determined by a Jun-luciferase report gene, dominant negative forms of aPKC and MEK5 inhibited Jun promoter activity. Thus, EGF stimulation of PKC ι /zeta and MEK5 binding is required for activation of ERK5 and subsequently Jun transcription in the EGF \rightarrow PKC ι \rightarrow

MEK5/ERK5 → Jun (transcriptional activation through the MEF2C element) pathway (Diaz-Meco, 2001).

PKCιota is able to regulate AP-1 (c-jun and c-fos) in response to several stimuli, including: high glucose, PDGF, Ras, TNFα, UVB and UVC, IL1, and EGF. This regulation of AP-1 by PKCιota results in transcription activation of many genes involved in tumorigenesis: cyclin D1 (transformed growth and proliferation), MMP-3 and collagenase (invasion and metastasis), and NFκB (survival and chemoresistance). Because the ability of PKCιota to transcriptionally activate so many stages of tumorigenesis, from initial transformation of the primary lesion to invasion, metastasis, and survival, this provides sufficient rationale for PKCιota to be targeted therapeutically in cancer independently of the other roles of PKCιota in tumorigenesis. Because tumors are heterogeneous and often self-sufficient in growth signals, blocking PKCιota activation could lead to tumor cell death through sensitization of the tumor cells to chemotherapy by blocking growth (cyclin D1) and survival (NFκB). PKCιota inhibition could also be used as a means of preventing metastasis (MMP-3 and collagenase). Thus, PKCιota, through its activation of AP-1, provides rationale for the use of PKCιota inhibitors in clinical trials.

SIGNALING: INSULIN/ GLUT4 TRANSLOCATION

PKCιota is involved in insulin-induced GLUT4 (a glucose transport molecule) translocation and glucose uptake. This particular role of PKCιota is important for reasons regarding diabetes and insulin resistance. While PKCιota is amplified in cancer, decreased PKCιota can lead to deregulation of the glucose transport pathway that is found in diabetes. For example, a muscle-specific knockout in mice of PKCιota/lambda resulted in reduced glucose transport (through decreased GLUT4 translocation to the plasma membrane), as well as “systemic insulin resistance, impaired glucose tolerance or diabetes, islet beta cell hyperplasia, abdominal adiposity, hepatosteatosis, elevated serum triglycerides, FFAs (free fatty acids), and LDL-cholesterol, and diminished HDL-cholesterol” (Farese, 2007). Similarly, a decrease in PKCιota in muscle resulted in insulin-resistance in humans (Farese, 2007; Beguinot, 2008). Also, insulin-resistant type 2 diabetic patients exhibited reduced PI3K and PKCιota activation in response to insulin (Bandyopadhyay, 2005). Thus, PKCιota plays a vital role in insulin-induced glucose transport (see Figure 14).

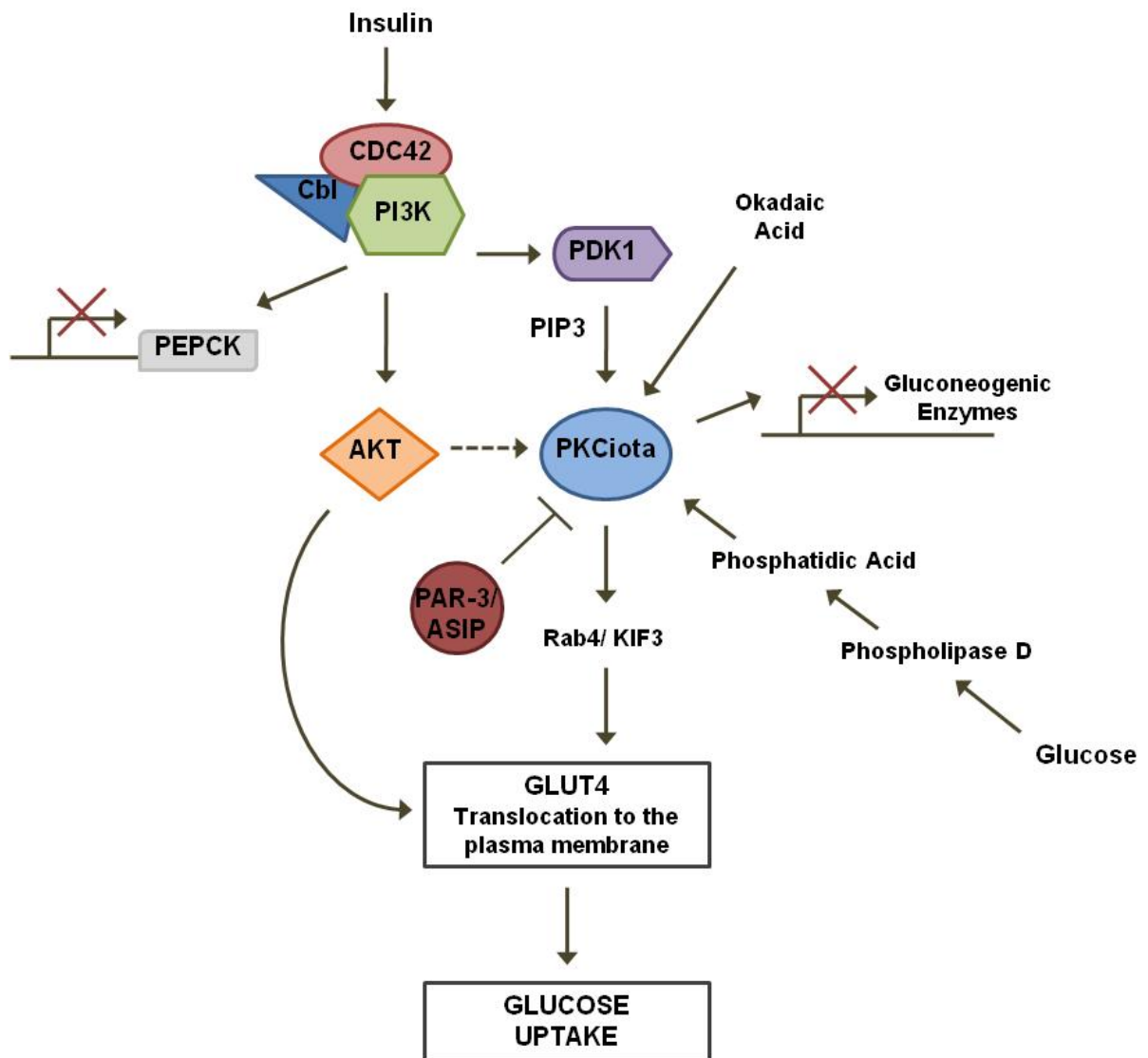
Reducing the levels of PKCιota expression, which would ideally occur upon treatment of cancer patients with PKCιota inhibitors, could then results in an onset of diabetic concerns: such as insulin resistance, glucose tolerance, increases in triglycerides, FFAs, and LDL

cholesterol, diabetes, or similar issues. Therefore, complications of PKC α -targeted treatment of cancer arise. Thus, treatment of cancer with any type of PKC α inhibitor should be short-term, in order to sensitize the cancer cells to other chemotherapeutics, rather than long-term treatment which could result in diabetes-related issues.

Insulin-Stimulated Glucose Transport- Mechanism

Several laboratories using different model systems have identified PKC α -dependent and PKC α -independent mechanisms for insulin-induced GLUT4 (a glucose-transported protein) translocation and glucose uptake. GLUT4 translocation refers to the movement of GLUT4 from the perinuclear compartment to the plasma membrane (cell surface). Overall, a majority of the studies indicate PKC α is necessary for insulin-induced GLUT4 translocation, but several laboratories have found that AKT is the molecule responsible for this translocation. Thus, the PKC α - dependent mechanism and the discrepancies in this mechanism will be covered in the *Discrepancies in PKC α 's Role in Insulin-Stimulated Glucose Transport* section below. For the most part, two pathways have been identified for insulin-stimulated glucose transport, which may or may not overlap (one PKC α -dependent, one AKT-dependent; but both PI3K-dependent):

Figure 14: Mechanism of Insulin-PKCiota Stimulated Glucose Transport



Two distinct pathways:

Insulin-stimulated GLUT4 uptake occurs via two pathways, PI3K→PKC ι and PI3K→AKT. PKC ι as a downstream effector in the PI3K-PDK1 pathway had already been studied quite meticulously (Chou, 1998; Akimoto, 1998; Standaert, 1999; Kanoh, 2003). Using 3T3-L1 adipocytes, insulin was used to stimulate the PI3K pathway. With both exogenous dominant negative and constitutively active forms of PKC ι /lambda, PKC ι /lambda was demonstrated to be involved in insulin-stimulated glucose uptake as well as the translocation of GLUT4 to the plasma membrane. While PI3K also stimulates AKT upon insulin stimulation in adipocytes, these two pathways (PI3K → AKT and PI3K → PKC ι / lambda) appear to be distinct non-overlapping pathways in 3T3-L1 adipocytes (Kotani, 1998). However, contrary to Kotani et al, in L6 myotubes, Bandyopadhyay et al showed that PKC ι was necessary for AKT-dependent, insulin-induced glucose uptake (Bandyopadhyay, 2000). Thus, PKC ι and AKT may or may not be in the same pathway in insulin-stimulated GLUT4 translocation.

Cbl, which is phosphorylated by the insulin receptor in response to insulin stimulation, activates PI3K and subsequently glucose transport. Mutation of Cbl at the pYXXM motif prevents glucose transport. Specifically, mutation of Cbl at the pYXXM motif inhibited 3T3-L1 adipocyte response to insulin by blocking binding of Cbl to PI3K, activation of PI3K, activation/translocation of PKC ι / lambda to the plasma membrane, GLUT4 translocation to the plasma membrane, and thus glucose transport. Thus, Cbl binding to PI3K is necessary for insulin-induced glucose transport through the PKC ι / lambda/GLUT4 pathway (Standaert, 2004).

The activation of PKC ι / lambda and PKC ζ in response to insulin requires PIP3 (phosphatidylinositol-3,4,5-(PO₄)(3)) and PDK-1 phosphorylation at the activation loop sites. The phosphorylation of PKC ι / lambda by PDK-1 leads to PKC ι / lambda activation and subsequently autophosphorylation. Activated PKC ι / lambda in response to insulin can then lead to the translocation of GLUT4 to the plasma membrane (Standaert, 1999). Kanoh et al also found that PKC ι activation by insulin-stimulated PI3K requires PIP3 in an *in vivo* rat model (Kanoh, 2003). Thus, PDK1 and PIP3 are likely required in the insulin-PI3K-PKC ι -GLUT4 pathway.

CDC42, one of the PKC- ι many binding partners, is also involved in the insulin-PI3K-PKC ι / lambda-GLUT-4 transport pathway (Insulin → PI3K/CDC42 → PKC ι / lambda → GLUT4 translocation → glucose uptake). By use of CDC42 antibody (to block activity) and constitutively active (CA) CDC42 (to activate it) in 3T3-L1 adipocytes, CDC42 was

examined in response to insulin induction. CDC42 is involved in the insulin response through direct interaction with PI3K. However, PI3K inhibitors, wortmannin and LY294002, block CA-CDC42 induced GLUT4 translocation to the plasma membrane. Also, inhibition of PKC α / λ by microinjection of PKC α / λ antibody and a kinase dead mutant resulted in a block of both insulin and CA-CDC42 induced GLUT4 transport. Thus, insulin \rightarrow PI3K/CDC42 \rightarrow PKC α / λ are all in the same GLUT4 translocation pathway that leads to glucose uptake (Usui, 2003).

Downstream from PKC α in the insulin-PI3K-PKC α -GLUT4 pathway, Rab4, a small GTPase-binding protein, and KIF3, the motor protein kinesin, are involved in GLUT4 translocation. Upon insulin stimulation of 3T3-L1 adipocytes, as visualized by photoaffinity labeling, Rab4 becomes activated, and this activated form of Rab4 binds KIF3. Upon binding to KIF3, the complex associates with microtubules, as determined by a microtubule capture assay. The interaction of Rab4 with KIF3 is necessary for insulin-stimulated GLUT4 translocation via microtubule reorganization. The role of Rab4/KIF3 is dependent on PI3K/ PKC α / λ activation by insulin. Rab4/KIF3 localization to the microtubules and subsequently GLUT4 translocation and exocytosis upon insulin stimulation was blocked by both a PI3K inhibitor, LY294002, and by ectopic expression of a dominant negative mutant PKC α / λ (Imamura, 2003). Thus, Rab4 and KIF3 appear to have a role downstream of PI3K and PKC α / λ in GLUT4 translocation and exocytosis.

Okadaic acid (which inhibits protein phosphatase 1 and 2A) causes insulin-like effects, such as glucose transport and GLUT4 translocation. Much like insulin, in both 3T3/L1 and rat adipocytes, okadaic acid was used to stimulate the PI3K pathway. With ectopically expressed dominant negative PKC α / λ , PKC α / λ was demonstrated to be involved in okadaic acid-stimulated glucose uptake as well as the translocation of GLUT4 (Standaert, 1999).

GLUT1 translocation has also been examined in response to insulin. Bosch et al found that glucose uptake involved two pathways. While PKC α / λ was required for GLUT1 translocation to the plasma membrane, the MAPK pathway was required for GLUT1 expression in 3T3-L1 adipocytes upon PMA treatment. Bosch found that PKC α / λ co-immunoprecipitated with PKC β II. However, PKC α / λ needed to be released from PKC β II before it could lead to GLUT1 translocation and glucose uptake (Bosch, 2004).

Inhibition of PKC α Inhibits GLUT4 Translocation

Insulin has been shown to induce glucose uptake and GLUT4 translocation in a PKC α / λ dependent manner. Kotani et al showed that overexpression of the atypical PKC binding protein, ASIP (PAR-3/Bazooka), was able to inhibit both insulin-induced and constitutively active PKC α / λ -induced glucose uptake. Specifically, in 3T3-L1 adipocytes, addition of insulin resulted in an increase in glucose uptake as well as an increase in the activity of PKC α / λ , as indicated by use of an *in vitro* kinase assay using MBP as the substrate. The effects of insulin-induction on PKC α / λ activation as well as on GLUT4 translocation and therefore on glucose uptake were reduced upon addition of exogenous PAR-3/ASIP in a dose-dependent manner, indicating that insulin works through PKC α / λ signaling to induce glucose uptake. To test the specificity of insulin-induced glucose uptake, Kotani et al used two other methods of inducing glucose uptake, growth hormone (GH) and hyperosmolarity (sorbitol). However, PAR-3/ASIP did not block GH- or sorbitol- induced glucose uptake, indicating that inhibition of glucose uptake by PAR-3/ASIP is insulin and PKC α / λ - specific. Interestingly, PAR-3/ASIP did not block all insulin-induced signaling. For example, PAR-3/ASIP did not block the translocation of AKT to the PM fraction in response to insulin (Kotani, 2000). Thus, while PAR-3/ASIP blocks PKC α / λ - and insulin-induced translocation of GLUT4 and therefore glucose uptake, PAR-3/ASIP does not block all insulin signaling.

Glucose Induction of GLUT4 Translocation

In rat adipocytes and skeletal muscle, PKC α / λ was activated in response to glucose induction. The activation of PKC α / λ by glucose was PI3K independent. Upon glucose stimulation, the ERK pathway was activated along with phospholipase D, which lead to an increase in phosphatidic acid, and thus activation of PKC α / λ (Bandyopadhyay, 2001). This glucose/phospholipase D/ phosphatidic acid pathway resulted in the translocation of GLUT4 to the plasma membrane, similar to the PI3K-dependent insulin-induction of GLUT4 translocation. Both pathways Insulin/PI3K and glucose/phospholipase D/ phosphatidic acid require PKC α / λ for GLUT4 translocation.

Transcriptional Repression

Insulin stimulation has also been shown to lead to PKC α -dependent and PKC α -independent transcriptional repression. Knockout of PKC α / λ in beta pancreas cells in mice reveal that PKC α / λ is important in glucose-stimulated insulin secretion.

However, this role of PKC ζ seems to be through lack of gene expression of known insulin secretion genes. Thus, PKC ζ / λ is involved in gene repression in response to insulin stimulation in pancreatic beta cells (Hashimoto, 2005; Yang, 2005).

Addition of either metformin, an anti-diabetic drug, or insulin can lead to CREB binding protein (CBP) phosphorylation at Ser436 by PKC ζ . This phosphorylation of CBP causes dissociation of the CREB-CBP-TORC2 complex, which is involved in the transcriptional activation of gluconeogenic enzymes. Thus, PKC ζ is also able to cause a decrease in transcription of several gluconeogenic genes (He, 2009).

While PKC ζ is involved in insulin and PI3K signaling, another molecule besides PKC ζ is involved in the transcriptional inhibition of PEPCCK (phosphoenolpyruvate carboxykinase), an enzyme involved in gluconeogenesis, by insulin and PI3K. In HL1C (rat hepatoma) and 293 cells, PKC ζ / λ dominant negative mutants were unable to revert the inhibitory effect of insulin on transcription of PEPCCK (Kotani, 1999).

Insulin Stimulated Glucose Transport Varies by Cell Type

It seems that insulin-stimulated GLUT4 translocation can vary from cell type to cell type. In accordance with previous findings, PKC ζ / λ ^{-/-} embryonic stem cells (ES) exhibit impaired glucose transport. PKC ζ / λ ^{-/-} ES cells, which were made by recombinant DNA techniques, do not exhibit glucose transport in response to insulin unlike their PKC ζ / λ ^{+/+} counterparts. Also, ectopic expression of wild type PKC ζ / λ in PKC ζ / λ ^{-/-} ES cells reverted the insulin resistant phenotype. However, the effect of insulin on PKC ζ / λ dependent glucose transport was not through PI3K, but rather dependent on TYK2 (tyrosine protein kinase 2), PLD (phospholipase D), and the ERK pathway, as determined by use of the PI3K inhibitor, wortmannin, the MEK1/ERK inhibitor, PD98059, and the PLD inhibitor, 1-butanol, on insulin stimulated glucose uptake and PKC ζ / λ activation (Bandyopadhyay, 2004). Thus, PKC ζ / λ was essential for insulin-stimulated glucose transport in embryonic stem cells.

Surprisingly, differentiation of the ES PKC ζ / λ ^{-/-} and PKC ζ / λ ^{+/+} cells into adipocytes revealed that PKC ζ / λ activation in response to insulin was once again PI3K-dependent and independent of TYK2, PLD, and ERK. Similar to the PKC ζ / λ ^{-/-} ES cells, PKC ζ / λ ^{-/-} adipocytes also exhibited impaired glucose uptake upon insulin stimulation when compared to PKC ζ / λ ^{+/+} adipocytes, which was reverted upon ectopic expression of PKC ζ / λ (Bandyopadhyay, 2004). Thus, PKC ζ / λ is

required for insulin-induced glucose transport whether PI3K signaling is involved or not, as ES cells did not but adipocytes did require PI3K.

As mentioned earlier in this section, in 3T3-L1 adipocytes, two distinct mechanisms (PI3K → AKT and PI3K → PKCιota/ λ) appear to be non-overlapping pathways in insulin-stimulated glucose transport (Kotani, 1998). However, in L6 myotubes, Bandyopadhyay et al showed that PKCιota was necessary for AKT-dependent, insulin-induced glucose uptake (Bandyopadhyay, 2000). Thus, PKCιota and AKT are likely both involved in insulin-stimulated GLUT4 translocation but their roles are cell type specific.

In a rat model system, Campbell et al found that the liver and muscle tissues exhibited different pathways upon IRS- phosphorylation by stimulation with DHEA (5-Dehydroepiandrosterone), a naturally occurring steroid hormone. In the rat muscle, IRS-1 was tyrosine phosphorylated and the PI3K/PKCιota pathway was activated. However, in the rat liver, IRS-2 was tyrosyl phosphorylated and the PI3K/AKT pathway was activated. Activation of the AKT and PKCιota pathways were determined by western blot analysis with antibodies to the phospho (active) forms of these proteins (Campbell, 2004).

Similarly, Miura et al found that both IRS1 and IRS2 were necessary for Cbl-PI3K binding and activation of the PKCιota/ λ/ GLUT4 pathway. Miura's study was performed in immortalized brown adipocytes (Miura, 2004).

In both normal and high-fat fed rats, PKCιota/λ, PKCζeta, and AKT become activated in response to insulin stimulation. However, only PKCιota/ λ and PKCζeta get recruited to the plasma membrane upon insulin-induction in rodent skeletal muscle. However, this activation of PKCιota/ λ, PKCζeta, and GLUT4 were all reduced in high fat fed rats as compared to the rats on a normal diet (Herr, 2005). Thus, many factors can affect insulin-stimulated glucose transport.

Discrepancies in PKCιota's Role in Insulin-Stimulated Glucose Transport

Besides the discrepancies mentioned in the "PKCιota- Signaling: Insulin/GLUT4- Insulin-Stimulated Glucose Transport Varies by Cell Type" section, others have shown that PKCιota is dispensable for glucose uptake. AKT was identified as the insulin-stimulated regulator of glucose uptake. While PKCιota and AKT are both activated by insulin, they appear to be in distinct pathways in 3T3-L1 adipocytes (Kotani, 1998). Also in 3T3-L1 adipocytes, PP2A was identified as being an inhibitor of insulin-induced glucose transport. While small t antigen, which induces glucose transport by inhibiting PP2A, was not able to activate glucose transport in the presence of dominant negative or AKT RNAi, dominant negative and PKCιota

RNAi had no effect on glucose transport. Thus, PP2A blocks glucose transport through its inhibition of the AKT pathway rather than through PKC α inhibition (Ugi, 2004).

Also, in fully differentiated 3T3-L1 adipocytes, using electroporation methods to transfect with various siRNAs, Zhou et al found that PKC α was dispensable in insulin-induced GLUT4 transport. However, AKT2 (and to a lesser extent AKT1) were necessary for insulin-stimulated GLUT4 transport (Zhou, 2004). Thus, PKC α in these two particular studies was found to be dispensable for insulin-induced glucose transport.

PKC α in Glucose Transport and Uptake-Summary: Tumors exhibit upregulated metabolic activity compared to adjacent normal tissue. This principle is the basis of PET (Positron Emission Tomography) imaging, which measures glucose metabolism (and uptake), which is detectably higher in tumors. As mentioned, PKC α plays an essential role in glucose metabolism, where in response to either insulin or glucose, PKC α leads to GLUT4 translocation to the plasma membrane, resulting in glucose uptake, which is necessary to generate energy (in the form of ATP- adenosine triphosphate) for cellular function. Because PKC α is vital in this metabolic function and the metabolism is upregulated in tumors, inhibition of PKC α should result in a decrease in tumor cell metabolism and growth, through inhibition of tumor cell glucose uptake. This would indicate PKC α inhibition in tumors should result in a decrease in glucose uptake, resulting in the inability of tumor cells to generate ATP for cellular functions. In a way, PKC α inhibition would result in starvation of tumor cells, which need high levels of glucose for survival. The role of PKC α in metabolism is another testimony to the statement that PKC α inhibitors should be considered for clinical trials for the treatment of cancer.

SIGNALING: NGF (Nerve Growth Factor)

PKC α is involved in NGF signaling, particularly in NGF-induced differentiation, survival, and trafficking. In rat pheochromocytoma cells (PC12), which differentiate in response to NGF (nerve growth factor), PKC α / λ is involved in both differentiation and survival via NF κ B activation. More specifically, overexpression of PKC α / λ resulted in increased differentiation, neurite outgrowth, JNK activation, NF κ B activation, and subsequent c-Jun phosphorylation in response to NGF stimulation, while antisense PKC α / λ blocked NGF-induced NF κ B activation (Wooten, 1999). As mentioned, upon NGF-stimulation, PKC α through its binding to p62 is involved in (1) NGF-induced trafficking through its localization to the endosome, a vesicle formed for trafficking during endocytosis (Samuels,

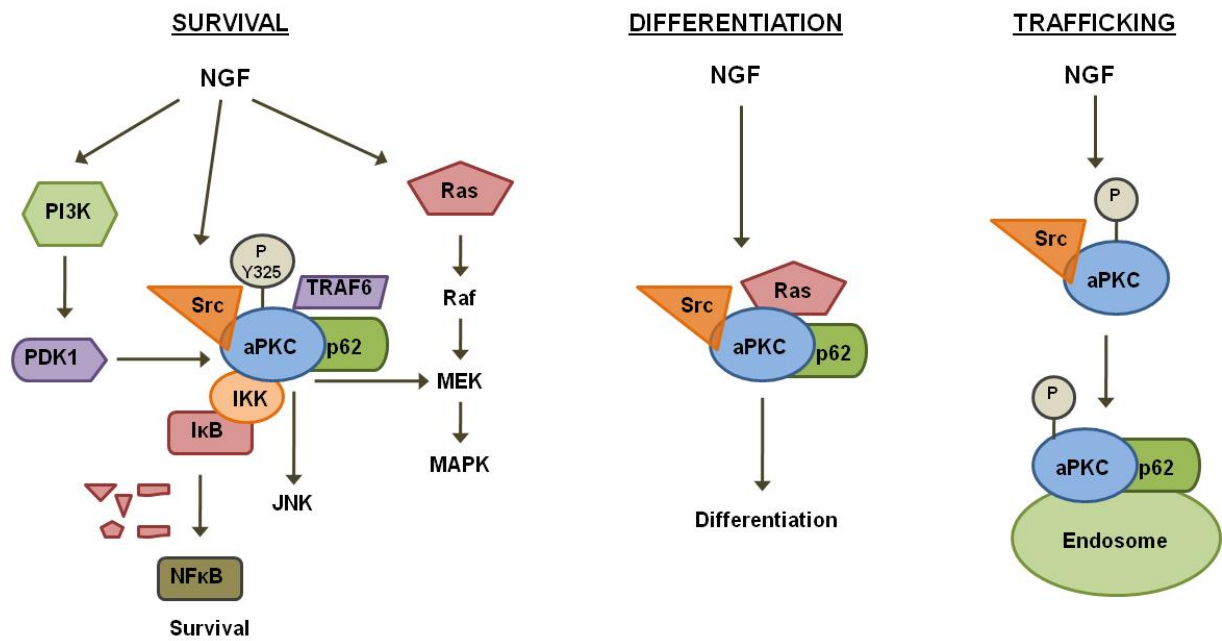
2001) and (2) leads to survival via NF κ B (Samuels, 2001; Wooten, 2005). Thus, redundancy in effector molecules occurs in NGF-stimulated PKC ζ activation, leading to survival, differentiation, and trafficking (see Figure 15).

NGF-PKC ζ in Survival

PKC ζ plays a vital role in NGF-induced survival via activation of NF κ B. PI3K was also required for NGF-induced survival (Yao, 1995). Particularly, induction of survival in PC12 differentiated cells in response to NGF had been shown to be mediated by the atypical PKCs through NF κ B (Wooten, 1999). PKC ζ / λ had already been shown to be downstream of PI3K (Akimoto, 1996). Also, Wooten et al showed that while Ras was required for activation of Raf1 in response to NGF, both PI3K and PKC ζ inhibitors had no effect on the activation of Raf1. Using an *in vitro* kinase assay, Wooten et al was able link PI3K and PKC ζ to the Ras-Raf-MEK-MAPK cascade by showing that aPKC could directly phosphorylate MEK, and this phosphorylation required Src, PI3K, and aPKC. Ras and Src were tested using knockout PC12 cells, while PI3K and PKC ζ were tested using the specific inhibitors PI3K inhibitors (wortmannin and LY294002) and PKC ζ inhibitor (chelerythrine chloride). Using these same methods, they found that activation of JNK in response to NGF required Ras, Src, PI3K, and aPKC. Similarly, activation of NF κ B (and therefore survival) required Src, PI3K, PKC ζ , and JNK, but did not require Ras (Wooten, 2000). Thus the role of Ras in response to NGF is predominantly in differentiation, while Src, PI3K and aPKC are required for both NGF-induced differentiation and survival.

While Src is able to lead to NGF-induced activation of PKC ζ /NF κ B, this function of Src is PI3K-independent. The PI3K and PKC ζ inhibitors mentioned above had no effect on Src activity, indicating that Src regulation of aPKC is independent of PI3K. Using Src knockout PC12 cells, Wooten et al was able to find that PKC ζ could only activate NF κ B if Src was found in complex with PKC ζ (Wooten, 2000). Similarly, in PC12 cells, PKC ζ / λ was shown to interact with Src, leading to PKC ζ / λ mediated activation of NF κ B and consequentially survival. IKK and I κ B were also in the Src- PKC ζ / λ complex. Atypical PKC had been shown to associate with IKK and I κ B to induce NF κ B activation and nuclear translocation, and Src had been found to be necessary to induce PKC ζ activation of NF κ B (Wooten, 2000). PKC ζ had also been shown to be phosphorylated by Src. Specifically, in the PC12 cell line, upon NGF-treatment, PKC ζ was phosphorylated by Src at three sites (Tyrosines 256, 271, 325-only 325 was important for the activation of NF κ B) (Wooten, 2001).

Figure 15: Mechanism of NGF-PKCiota Signaling



PKC δ is found to be in a signalsome in response to NGF. Through IP studies, PKC δ , along with p62, TRAF6, and IKK, was found to be in a signalsome, which led to IL-6 expression via NF κ B activation (Zhang, 2005). Similarly, NGF-stimulation results in assembly of the p62-TRAF6-IKK β -PKC δ complex leading to NF κ B expression. The role of p62 in the signalsome is to cause the polyubiquitination of TRAF6 (Wooten, 2005). Thus, PKC δ , through a series of interactions, leads to NGF-induced survival through NF κ B activation.

NGF-PKC δ in Differentiation

PKC δ is also involved in NGF-induced differentiation. In PC12 rat pheochromocytoma cells, which are a well characterized model system for studying differentiation, induction with nerve growth factor (NGF) causes both differentiation and survival via Src and Ras (Kremer, 1991). NGF activation leads to the induction of the MAPK cascade (Kremer, 1991), p38 (Morooka, 1998), c-Jun N-terminal kinase (JNK) (Heasley, 1996; Minden, 1994), PI3K (Kimura, 1994), and the atypical PKCs (Coleman, 1994; Wooten, 1994). PKC δ activation in response to NGF had been measured using a kinase assay using MBP as the substrate. PKC δ activity due to addition of NGF was blocked using a series of PI3K inhibitors (wortmannin and LY294002) and PKC δ inhibitor (chelerythrine chloride) (Wooten, 2000). Thus PKC δ is downstream of PI3K in NGF signaling. In PC12 cells, differentiation due to NGF had been shown to be mediated through Raf-1 (Wood, 1993), the MEK-MAPK cascade (Pang, 1995), PI3K signaling (Jackson, 1996; Kimura, 1994; Kobayashi, 1997), and through the atypical PKCs (Coleman, 1994). Using co-IP experiments, Wooten et al found that both Ras and Src associate with PKC δ . Thus, Ras, Src, and aPKC are required for NGF-induced differentiation (Wooten, 2000).

Because PKC δ had already been established in NGF-induced differentiation (Coleman, 1994), antisense p62 was used to determine the role of p62 in NGF-induced differentiation. Antisense p62 blocked NGF-induced neurite outgrowth, indicating PKC δ /lambda binding to p62 is likely necessary for NGF-induced neurite outgrowth (Samuels, 2001). Thus, Ras, Src, p62, and aPKC are required for differentiation in response to NGF, and only Src, PI3K, p62, and aPKC are required for survival (via NF κ B).

NGF-PKC δ in Trafficking

As summarized previously, upon NGF treatment, PKC δ /lambda localizes with p62 to the endosome, a vesicle formed during endocytosis, which is used for trafficking (Samuels,

2001). The endosome functions in (1) carrying either lipids or proteins or to the lysosome (from the plasma membrane), (2) carrying lipids/proteins to the golgi apparatus for decomposition, and (3) secreting lipids/proteins out of the cells.

As demonstrated by IP assays in response to NGF in PC12 cells, PKC α / λ bound p62 4-times higher than non-NGF stimulated cells, in spite of the fact that PKC α / λ and p62 levels remained unchanged. Through kinetics experiments, PKC α / λ was determined to first be tyrosine phosphorylated/ activated prior to p62 binding. However, while tyrosine phosphorylation of PKC α / λ was necessary for PKC α / λ -p62 binding, this phosphorylation was not necessary for the localization of p62 (alone) to the endosome, as determined by IF with antibodies against Rab7 (an endosomal marker) and validated by fractionation analysis. Therefore, upon NGF-stimulation, p62 localizes at the endosome, followed by tyrosine phosphorylation of PKC α / λ , and lastly followed by the recruitment of tyrosine-phosphorylated PKC α / λ by p62 to the endosome (Samuels, 2001). Src was identified as the kinase responsible for the tyrosine phosphorylation of PKC α / λ that resulted in its binding to p62 and subsequently translocation to the endosome. This was determined by addition of exogenous Src leading to the binding of PKC α / λ -p62 in a dose-dependent manner in both PC12 and HEK 293 cells. Thus, Src activation of PKC α in response to NGF results in recruitment of PKC α to the endosome by p62.

SIGNALING: EGF

EGF, PDGF, and Insulin have all been shown to activate PKC α . The growth factors, EGF and PDGF, activate PKC α / λ through PI3K. Upon stimulation of rat 3Y1 fibroblasts cells with EGF and PDGF, there was an increase in PKC α / λ *in vivo* phosphorylation. Also, upon addition of EGF and PDGF, PKC α localization changed from nuclear to cytosolic. The activation of PKC α / λ by PDGF was shown to be dependent on PI3K in this rat fibroblast model system according to the pathway: PDGF \rightarrow PI3K \rightarrow PKC α / λ \rightarrow TRE (TPA Response Element) (Akimoto, 1996). Also, EGF is able to induce PKC α according to the pathway: EGF \rightarrow PKC α \rightarrow MEK5/ERK5 \rightarrow Jun (transcriptional activation through the MEF2C element) (Diaz-Meco, 2001) (see “PKC α -Binding Partners: MEK5” for details). Thus, EGF, PDGF, and insulin can all individually activate PKC α .

PKC IOTA AS A TARGET FOR THERAPY

As described in detail in this chapter, PKC ι is involved in nearly every stage of tumorigenesis, from the initial transformation of normal tissue to a malignant state, to migration, invasion, and in turn metastasis. PKC ι is involved in several facets of cellular functions including cell growth (and tumor growth), proliferation, transformation and anchorage-independent growth, invasion, migration, protein/lipid trafficking, survival, chemoresistance, and glucose metabolism. This diversity in the functions of PKC ι is due to an assortment of PKC ι activators, binding partners, and downstream effector molecules. Several of the activators of PKC ι have already been linked to tumorigenesis and quite a number of these are considered oncogenes. Activators of PKC ι include: Ras, PI3K, PDK1, growth factors (NGF, PDGF, EGF), TNF α , Src, IL1, Bcr-Abl, glucose, and insulin to name a few. With this range of oncogenic upstream effector molecules of PKC ι , PKC ι is an excellent potential therapeutic target. Due to the participation of PKC ι in so many diverse pathways, PKC ι -inhibition provides a means of targeting several cancer pathways simultaneously. As you will read, several PKC ι inhibitors are available, but the need for more specific inhibitors exists.

Inhibition of PKC ι has been demonstrated through several mechanisms, including specific inhibitors (see below), kinase dead and dominant negative PKC ι constructs, or RNAi. For example, because the pro-apoptotic effect of PAR-4 is through the sequestration of oncogenic PKC ι /lambda, PKC ι /lambda could be a possible therapeutic target, sensitizing cancer cells to apoptosis (Diaz-Meco, 1999). Also, inhibition of PKC ι by use of a kinase dead construct in A549 lung cancer cells decreased soft agar colony formation and decreased tumor volume *in vivo* by four-fold, thus implicating PKC ι as a potential target in cancer therapeutics (Regala, 2005). See Table 8 for a complete list of small molecule PKC ι inhibitors used.

To date, the most specific inhibitors of PKC ι work through inhibition of PB1:PB1 interactions between PKC ι and other PB1-domain containing molecules such as p62/ZIP, PAR-6, and MEK5. However, the disruption of the PKC ι :PAR-6 complex seems to be the most anti-tumorigenic. Using a FRET-based assay (Fluorescence Resonance Energy Transfer), Stallings-Mann et al screened 960 compounds from the GenePlus chemical compound library to identify a compound, which disrupted PKC ι /PAR-6 binding. Using this screening technique, two gold compounds were identified as disrupting the PKC ι /PAR-6 complex. ATG (aurothioglucose- a gold compound used in the treatment of rheumatoid arthritis) and ATM (aurothiomalate- a similar gold compound) were identified as compounds

that disrupt PKC α /PAR-6 binding. To test the relevance of these two gold compounds, the effects of ATM and ATG were examined on adherent cells growth, transformation (soft agar colony formation), and tumor growth (volume) (Stallings-Mann, 2006), similar to a previous study using (kinase dead PKC α in non-small cell lung cancer) (Regala, 2005; Regala, 2005). These experiments revealed that the PKC α inhibitors, ATM and ATG, were indeed able to cause a decrease in transformation and tumor growth while having no effect on adherent growth. These results using ATM and ATG were the same as the previous study using kd PKC α (see below for the details of these studies). Thus inhibition of PKC α therapeutically seems like a plausible mechanism for the treatment of NSCLC (Stallings-Mann, 2006).

Figure 16: Structures of PKC α Inhibitors: ATM and ATG
Adapted from (Stallings-Mann, 2006)

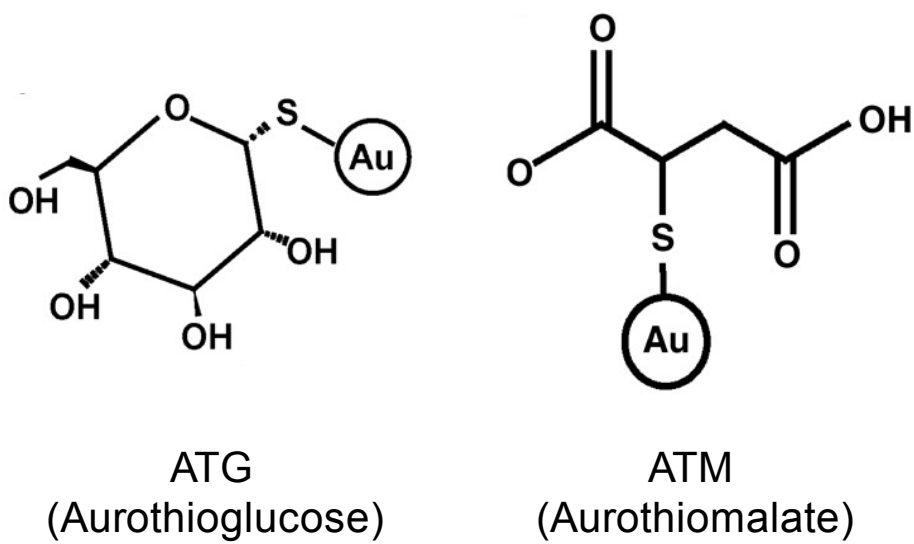


Table 8: PKC α Small Molecule Inhibitors

PKC α Inhibitor	Type of Cancer	Model System	Result	Effector Molecules	Reference
ATG-Aurothioglucose	NSCLC	A549 Cells	No Change in Proliferation; 4-Fold Reduction in Transformed Growth	Rac1	Stallings-Mann, 2006
		Xenograft A549 Cells	4-Fold Reduction in Tumor Volume		
ATM-Aurothiomalate	NSCLC	H1703 Cells	Dose-dependent Decrease in Transformed Growth	PKC α via Cys69	Erdogan, 2006
ATM	NSCLC	A427 (ATM-Sensitive) and H460 (ATM-Insensitive)	Inhibition of Anchorage-Independent Growth in A427 but not H460 Cells	ERK	Regala, 2008
		Xenograft A427 and H460 Cells	Decrease in Proliferation in both Cell Lines; No Change in Apoptosis or Angiogenesis in either Cell Line 4-Fold Reduction in Tumor Size in A427 Cells Only		
ATM	Prostate Cancer	PC3U (Prostate Cancer) Cells & PrEC (Non-Transformed Prostate Epithelium)	3-Fold Increase in Apoptosis (only in tumor cells)	Caspase3 and ERK	Trani, 2009

ATM is particularly specific to PKC α complexes by inhibiting PKC α -PAR-6 or PKC α -p62 complex formation. While ATM was able to disrupt the PB1:PB1 complexes PKC α -PAR-6 and PKC α -p62, ATM failed to disrupt other PB1:PB1 complexes, such as p62-p62, p62-NBR1, and MEKK3-MEK5. This specificity to PKC α PB1:PB1 complexes was due to the presence of a cysteine residue (cys-69) in the PB1 domain of PKC α that formed a thio-gold adduct when bound to ATM. Disruption of PKC α -PAR-6 binding by ATM is diminished in a PKC α cys-69 mutant. Thus, ATM is a specific inhibitor of PKC α complex formation via the PKC α PB1 domain cys-69 residue (Erdogan, 2006).

Unfortunately, these two particular compounds caused a vast number of side effects in the treatment of rheumatoid arthritis and many people withdrew from the study (van Roon, 2005). Specifically, in a Dutch clinical trial, ATG, which had to be replaced with ATM, was withdrawn from the trial due to quality control issues. After the switch to ATM, nearly 25% of the patients withdrew from the study due to inefficiency of the drug or side effects (ADR- adverse drug reactions), such as pruritus (an itching sensation), dermatitis (skin inflammation/rash), stomatitis (skin inflammation/rash in the mouth), and chrysiasis (a change in skin pigmentation due to the exposure of gold salts), and hyperpigmentation (darkening of skin/nails due to increased melanin). While these patients experienced inefficiency or ADR with ATM, many of these patients experienced better results with ATG prior to ATM treatment (van Roon, 2005). The van Roon study is the only published study to date looking at ATM and ATG in humans. However, phase I clinical trials have begun for the treatment of lung cancer with ATM at Mayo Clinic's Arizona and Minnesota facilities. While the details and results of these clinical trials are not yet published, phase II clinical trials are in preparation. Similarly, at the Mayo Clinic in Florida, phase I clinical trials are about to begin using ATM in the treatment of pancreatic cancer (Mayo_Clinic, 2010). Therefore, while PKC α would make an excellent target for the treatment of some classes of cancer patients, according to the van Roon study, less toxic compounds need to be generated. However, the clinical trial reports of the Mayo Clinic studies mentioned above when published may indicate otherwise. Generation of more specific compound is possible. The structures of the catalytic (crystal structure) and PB1 (NMR) domains have been determined, making the synthesis of PKC α -targeted compounds a possibility in the near future (Messerschmidt, 2005, and Hirano, 2004, respectively).

However, use of a myristoylated pseudosubstrate (mPS), a synthetic myristoylated peptide inhibitor (such as mPS-PKC ζ), which is cell-permeable, should not be used to inhibit PKC α . In the case of mPS-PKC ζ , addition of mPS-PKC ζ led to eNOS

(endothelial nitric oxide synthase) activation in cultured pulmonary artery endothelial cells (PAEC). This increased activation of eNOS in response to PKC ζ inhibition was also coupled with increased AKT, ERK1/2, and p38 MAPK (Krotova, 2006). This coincides with the paper by Baldwin showing that silencing PKC ι by RNAi led to an increase in p38 (Baldwin, 2006). However, this result opposes the findings of Ramzy et al that PKC ι expression could lead to increased nitric oxide (NO) synthesis (Ramzy, 2006). Thus, the effect of mPS on the production of eNOS could be non-specific rather than through the downregulation of atypical PKC. Therefore, inhibition of PKC ι activity or binding is likely a better method of inhibition than decreasing PKC ι expression levels, which seems to result in increases in other tumorigenic pathways.

Predictably, PKC ι inhibitors (such as ATM) do not inhibit soft-agar colony formation in lung cancer cells that do not overexpress PKC ι . More specifically, ATM sensitivity in lung cancer cells is higher in cells overexpressing PKC ι -PAR-6 than in cells with PKC ι -p62. In cells with high levels of PKC ι , the IC₅₀ values of ATM are low (300nM), while in cells with low levels of PKC ι , the IC₅₀ values of ATM are quite high (100 μ M). Interestingly, ATM sensitivity only correlated with PKC ι levels, it did not correlate with overall sensitivity to chemotherapeutics, such as cisplatin, paclitaxel, or gemcitabine. The sensitivity to ATM and not other compounds implies that PKC ι does not predict response to chemotherapeutics, in spite of other studies that suggest PKC ι is linked to chemoresistance (as mentioned above). The trend of ATM being most efficacious in PKC ι overexpressing cells corresponds to the trend *in vivo*, with tumors of high PKC ι levels being the most sensitized to ATM. *In vivo*, in a mouse xenograft model, two NSCLC cell lines, A427 and H460 were injected subcutaneously into nude mice. These two particular cell lines were chosen, as A427 cells were very sensitive to ATM treatment *in vitro* (IC₅₀ = ~0.5 μ M), as measured by soft agar colony formation, compared with H460 cells which were insensitive (IC₅₀ = ~70 μ M). In the A427 xenografts, even by 40 days, the average tumor volume of ATM treated mice (ATM was as low as 2 mg/kg) were 500 mm³ as compared to the vehicle treated mice, which were averaged at 2000 mm³. However, ATM insensitive cells, H460, in the xenograft model were not as sensitive to ATM treatment *in vivo* as the A427 cells, with the average tumor volume of ATM-treated mice being 1000 mm³ at 16 days of treatment and 60 mg/kg (and nearly 1500 mm³ with 20 mg/kg). Thus, response to ATM *in vitro* predicts response to ATM *in vivo*. ATM manifests its effect through inhibition of the PKC ι -PAR6-Rac-PAK-MEK-ERK pathway, as determined by western blot analysis using A427 (ATM sensitive) lung cancer cells compared to H460 (ATM insensitive) lung cancer cells (Regala, 2008). Thus, ATM treatment both *in vitro*

and *in vivo* is most effective in PKC α overexpressing tumors, indicating the need for a screening technique for lung cancer patients.

PKC α inhibitors have been studied more extensively in mouse models. Use of the PKC α inhibitor, ATG, blocked transformed growth of A549 cells, to the same extent as exogenous overexpression of kinase dead (kd) PKC α . Also, ATG was able to cause a decrease in tumor volume in mice, much to the same extent as kd PKC α (Stallings-Mann, 2006). Similarly, both ATG and kd PKC α did not affect the rate of proliferation of adherent cells. This decrease in transformation of NSCLC cells in the presence of ATG was due to a disruption of the PKC α /PAR-6 complex, resulting in a decrease in the level of active Rac1 (GTP versus GDP-bound) (Stallings-Mann, 2006). Similarly, PKC α is essential for Ras-induced tumorigenesis *in vivo*. Using a transgenic mouse model overexpressing oncogenic Ras in the lungs with or without PKC α expression, Regala et al found that inhibition of PKC α decreased the Ras-induced hyperplasia and tumor formation. Importantly, inhibition of PKC α by the small molecule inhibitor, ATM, blocked Ras-induced tumorigenesis (Regala, 2009). Thus, PKC α is essential for tumor initiation in a lung cancer model, and PKC α inhibition is a feasible method of treatment in Ras-induced tumorigenesis.

Because the PKC α -PAR-6 complex recruited Ect2 to the cytoplasm leading to transformation, invasion, and tumorigenesis in a NSCLC model, inhibition of the binding between PKC α and PAR-6 by ATM treatment would cause a decrease in NSCLC incidence through disruption of the PKC α -PAR-6-Ect2 complex. This pathway is as follows: PKC α /PAR-6/Ect2 \rightarrow Rac1 \rightarrow Pak \rightarrow MEK1/2 \rightarrow ERK1/2 (Justilien, 2009). Thus, PKC α :PAR-6 binding appears to be a practicable target in the treatment of PKC α -overexpressing cancers.

PKC α inhibitors were able to sensitize cancer cells to pro-apoptotic drugs, such as taxol, cisplatin, and okadaic acid. However, PKC α inhibitors alone were ineffective. For example, in a mouse model, Regala et al found that ATM only influences the proliferation index (BrdU positive cells) of the tumor without an effect on apoptosis or vascularization (Regala, 2008). Thus, PKC α inhibition is alone insufficient as a single agent, but needs to be used to sensitize tumors to chemotherapeutics. Essentially, the role of PKC α in chemoresistance in tumors in response to apoptotic stimuli is likely through PKC α 's role in survival, through phosphorylation/ inhibition of pro-apoptotic Bad Kinase, activation of anti-apoptotic NF κ B, and inhibition of the pro-apoptotic p38 MAPK pathway.

In summary, PKC α provides a novel therapeutic target for the treatment of cancer due to the convergence of so many cancer pathways at PKC α . These cancer pathways

cover a spectrum of stages in tumorigenesis, from transformation to metastasis, and involve a plethora of cellular functions, including polarity, cytoskeletal organization, proliferation, cellular growth, migration, invasion, induction of anchorage-independent growth, trafficking, survival, and metabolism. However, as with most targeted therapies, the need for screening tools for cancers that would express those target proteins exists. Luckily, PKC α overexpression or mislocalization, which is detectable by simple IHC, is sufficient to identify subgroups that would likely respond to PKC α inhibition. This principle has been validated in a lung cancer model, where PKC α levels statistically correlated with the sensitivity of these tumor cell lines to a PKC α inhibitor, aurothiomalate, as determined by transformation assays (Regala, 2008). While PKC α provides a potential therapeutic target, the need for more specific, less toxic inhibitors exists. For the time being, a gold compound, aurothiomalate (ATM) is in phase I clinical trials at the Mayo Clinic for the treatment of both NSCLC and pancreatic cancer. However, PKC α was identified as a *bona fide* oncogene in several other cancer types, including ovarian cancer, which is the focus of this dissertation, and thus the possibility of these clinical trials being extended to other cancer types of epithelial origin is highly probable.

CYCLIN E AND PKCIOTA IN OVARIAN CANCER

Cyclin E in Ovarian Cancer

In 1996, in a screen of ovarian cancer patient samples, Courjal et al identified cyclin E as being genetically amplified and overexpressed in many ovarian cancer patients (Courjal, 1996). Similarly, in 1998, Marone et al showed that cyclin E was amplified in 21% of ovarian cancer patients tested, and CDK2 was amplified in 6.4%. Testing the relevance of this study on protein expression, Marone et al found this amplification translated to the higher expression of cyclin E levels with 29.5% of ovarian cancer samples examined had overexpressed cyclin E protein and 6.5% had increased CDK2 protein expression (Marone, 1998). In 2003, Farley et al found that cyclin E overexpression correlated with poor survival and that this overexpression was due to gene amplification (Farley, 2003). While Courjal found that there was no correlation between cyclin E gene amplification and tumor type, stage, or grade, Bedrosian et al found there was a correlation between expression of low molecular weight forms of cyclin E (LMW-E) and a more advanced stage and grade of ovarian cancer (Courjal, 1996 and Bedrosian, 2004, respectively). LMW-E is described in more detail below. Bedrosian et al also found that ovarian tumors that overexpress LMW-E demonstrated higher kinase activity, which translated to a decrease in doubling time, an increase in S phase fraction, an increase in colony formation, and a better response to platinum-based chemotherapies (Bedrosian, 2004; Bedrosian, 2007). The increase in cyclin E-associated kinase activity due to the presence of LMW-E could also potentially lead to an increase in transformation and metastasis (Fang, 1996; Kang, 1996; Akli, 2007) (see the “Cyclin E- Cyclin E and Transformation” section). Specifically examining serous epithelial ovarian cancer, which is the most lethal and common form of ovarian cancer, Nakayama et al found amplification of the cyclin E gene in high grade serous epithelial ovarian tumors (Nakayama, 2007). Targeting cyclin E (and LMW-E) in the ovarian cancer cell line, OVCAR-3, Todd et al showed that knockdown of cyclin E by RNAi resulted in a restored G1-S checkpoint (Todd, 2009). These summarized results insinuate that cyclin E could be a potential therapeutic target in the treatment of ovarian cancer. Because cyclin E is regulated predominately through the overexpression of the protein product, a potential therapy could be to decrease cyclin E protein levels or inhibit its function by inhibiting cyclin E-associated kinase activity.

PKCιota and Ovarian Cancer:

Several studies have identified the role of PKCιota in ovarian cancer. The first study identified PKCιota as being differentially expressed between ovarian cancer and normal epithelial ovarian tissue (Weichert, 2003). The second study found that PKCιota was amplified in ovarian cancer, which correlated with elevated PKCιota expression and a poor prognosis (Eder, 2005). The third study designated PKCιota as being a *bona fide* oncogene in ovarian cancer (Zhang, 2006). PKCιota has since been labeled an oncogene in several types of cancer, see “PKCιota- Functions: Disease- Cancer” for a detailed overview.

Weichert et al, by use of immuno-histochemistry (IHC) in tissue samples of ovarian cystadenomas, borderline ovarian tumors, primary tumors, and recurrent invasive ovarian cancer that had been formalin-fixed and paraffin-embedded, found that PKCιota was expressed in half of the primary and recurrent malignant ovarian tumors, but not in the normal ovarian epithelial tissue, benign cystadenomas, or borderline tumors, thus implicating the role of PKCιota in the transformation stage of tumorigenesis. Similarly, PKCιota was not expressed in the normal cell line HOSE (human ovarian surface epithelial cells), but PKCιota was overexpressed in the tumor cell lines, OVCAR-3, SKOV-3, and OAW-42. Clinically, PKCιota showed a positive correlation with histopathological grading, stage, and the proliferation index. Specifically, in primary ovarian tumors, the expression of PKCιota exhibited a significant correlation with a reduced median survival time (Weichert, 2003).

In the second study, PKCιota was found to be genomically amplified leading to its over expression in ovarian cancer. In serous ovarian carcinomas, an increase in PKCιota DNA copy number correlated with a decreased progression free survival. Because the drosophila eye is polarized in an apical-basal orientation much like the epithelial tissue that makes up the human ovaries, the drosophila eye provides a useful model system for the manipulation of proteins involved in asymmetrical cell polarity. Thus, in a drosophila eye, which was used as a model of epithelial cell apical-basal polarity and normal PKCιota localization, overexpression of two different constitutively active PKCιota mutants (DaPKM-which lacks the PAR-6 binding site and the pseudosubstrate domain and rPKCζ- which has a deletion in the pseudosubstrate domain) resulted in a disruption in polarity (as determined by mislocalization of the tight junction marker, Patj), an increase in proliferation (as determined by BrdU incorporation), and an increase in cyclin E levels (as determined by immunofluorescence) (Eder, 2005).

Eder et al also determined the role of PKCιota localization in ovarian cancer. Using IHC, both normal ovarian surface epithelial cells and benign serous or mucinous cysts, PKCιota was localized to the apical membrane. However, all serous ovarian patient samples

that were evaluated by IHC (322 total) exhibited a loss of PKC α localization at the apical membrane (Eder, 2005).

In the Zhang study, PKC α was identified as a *bona fide* oncogene in ovarian cancer (Zhang, 2006). By examining 89 ovarian cancer patient samples, Zhang et al found that PKC α had an increased copy number in 43.9% of the patient samples (Zhang, 2006). Also, by examining normal ovarian tissues versus ovarian cancer tissue, Zhang found that PKC α mRNA expression (as determined by a microarray and validated by qRT-PCR) was also increased in the tumor samples. The results found in tumor samples were then validated in 18 ovarian cancer cell lines, versus 6 HOSEs (Human Ovarian Surface Epithelium- normal cells). Similarly, protein levels were also elevated in normal versus tumor tissue samples. Protein levels of PKC α were examined using a tissue array and validated with IHC in normal ovarian epithelium versus ovarian tumor tissue. Thus, these studies have all identified PKC α to be specifically relevant in ovarian cancer.

Additionally, these studies also show that overexpression of PKC α correlated with the ovarian tumor stage and grade. Because PKC α has established roles in proliferation, apoptosis, and transformation, Zhang et al examined each of these characteristics in ovarian tissues as well. While PKC α did not play a role in ovarian cancer proliferation or apoptosis, PKC α was involved in transformation (Zhang, 2006). However, in the study by Eder et al, the role of PKC α in ovarian cancer was partly through an increase in proliferation, as determined by BrdU incorporation (Eder, 2005). Specifically, in Zhang's study, the use of siRNA to silence PKC α in ovarian cancer cells exhibited a reduction in anchorage-independent growth. Additionally, in murine ovarian surface epithelium, PKC α in cooperation with mutant Ras was able to induce transformation. The increase in PKC α mRNA expression that was increased in ovarian cancer patient samples in Zhang's study was not increased in the other types of cancer examined (Zhang, 2006). Eder et al also found PKC α to be amplified at a higher level in ovarian cancer (specifically serous epithelial ovarian cancer) than in other epithelial cancers, such as prostate, lung, and colon. However, since these two studies, PKC α has been demonstrated to be amplified in several types and subsets of cancer.

Link Between PKC α and Cyclin E in Ovarian Cancer

The link between PKC α and cyclin E in ovarian cancer has also been established. Using drosophila epithelial tissue as a models system, Eder et al found that expression of persistently active PKC α (DaPKM and rPKC ζ) resulted in increases in cyclin E levels, increased proliferation, and a disruption of apical-basal polarity. By examining ovarian cancer patient tissues, Eder et al also found a correlation between PKC α protein levels, cyclin E levels, and proliferation (Eder, 2005). Because overexpression of the cyclin E/CDK2 inhibitor, p21/p27 (Drosophila ortholog is Dacapo) reverted the PKC α phenotype, cyclin E is implicated as the effector molecule responsible for the PKC α overexpression phenotype. As mentioned, this correlation between increased PKC α and cyclin E expression also occurred in ovarian cancer patient samples. Specifically, overexpression of PKC α in serous epithelial tumors correlated with the expression of the tumor-specific isoforms of cyclin E (LMW-E) that have been implicated in transformation, genomic instability and metastases (Eder, 2005). Similar to findings with cyclin E, increased PKC α expression correlated with poor survival (Farley, 2003; Eder, 2005).

Rationale:

As you have read, PKC α is involved in a large number of the steps involved in tumorigenesis. The overexpression or mislocalization of PKC α , observed in various types of cancer, results in aberrant epithelial cell polarity, migration/invasion, proliferation, protein/lipid trafficking, cellular metabolism, anchorage-independent growth, angiogenesis, survival, and metastasis, making PKC α a prime target for inhibition in epithelial cancers. PKC α can be mislocalized to the cytoplasm, amplified at the genomic level (which correlates with poor survival), or PKC α can be activated by gain of function mutations in several oncogenic pathways, such as Ras, PI3K, or Src. Rationally, in numerous model systems, PKC α inhibition via various methods resulted in a reversion of the transformed phenotype, both *in vitro* and *in vivo*. Because defining cancer pathways is essential for understanding cancer, the mechanism of PKC α -induced oncogenesis, while well published, still needs to be absolved. PKC α regulates several other oncogenic proteins, including ERK, MEK, Rac1, NF κ B, ELK, IL-6, MMP-3, MMP-10, STAT3, c-fos, cyclin D1, and cyclin E. Because cyclin E is also oncogenic, leading to proliferation, transformation and anchorage-independent growth, and these two proteins have been correlated in ovarian cancer and hepatocellular carcinoma (HCC), we hypothesized that the role of PKC α in transformation is through cyclin E. In Chapter 2 of this dissertation, we examine the regulation of cyclin E by PKC α and *vice*

versa, and we identified the means of this regulation (transcription versus protein stability). In Chapter 3, we examine the functional implications of the regulation of cyclin E by PKC ϵ in terms of proliferation, transformation, and migration.

In an ovarian cancer model system, we identified the regulation of cyclin E by PKC ϵ . In IGROV and 293T cells, knockdown of PKC ϵ leads to decreased protein stability of cyclin E and LMW-E, while not affecting cyclin E transcription. Conversely, in these two cell lines, overexpression of PKC ϵ results in an increase in full-length cyclin E and LMW-E. However, in four ovarian cancer cell lines under both transient and stable ectopic cyclin E expression, PKC ϵ protein levels remain stable, indicating the regulation of cyclin E by PKC ϵ is unidirectional. By examination of the protein expression pattern of PKC ϵ -overexpression (Wt-wildtype, CA-constitutively active, and DN-dominant negative) in 293T cells, we found that several effector molecules in the PI3K pathway are affected by PKC ϵ expression, implying that the regulation of cyclin E by PKC ϵ is via the PI3K pathway. In fact, inhibition of PI3K by PI-103 and GDC-0941 resulted in decreased phospho-PKC ϵ (autophosphorylation) and decreased cyclin E protein levels without a change in PKC ϵ expression. Thus, the regulation of cyclin E by PKC ϵ is at the level of protein stability and occurs through the PI3K signaling cascade.

PKC ϵ and cyclin E are both individually implicated in proliferation, transformation, and anchorage-independent growth. These two oncogenic proteins are often overexpressed in ovarian cancer, and their expression correlates with poor survival. Using IGROV serous ovarian cancer cells, we have found that inhibition of PKC ϵ by RNAi or small molecule inhibitors (aurothiomalate-ATM and aurothioglucose-ATG) results in a decrease in proliferation as indicated by a change in growth curves, and that this decrease in proliferation is via down-regulation of CDK2, CDK4, cyclin D1, and cyclin E and an increase in p27. In IGROV cells, PKC ϵ RNAi resulted in a decrease in transformed cells, a phenotype that was reverted upon infection with Ad-cyclin E or Ad-LMW-E. Similarly, inhibition of cyclin E-CDK2 activity (with Roscovitine) resulted in a decrease in the number of transformed colonies. However, PKC ϵ knockdown resulting in a decrease in cell motility was not reverted by exogenous cyclin E. Thus, PKC ϵ -induced motility is cyclin E-independent, and cyclin E is implicated as the downstream effector molecule responsible for PKC ϵ -mediated transformation.

CHAPTER 2:

Regulation of Cyclin E Stability by PI3K/ PKC α

CHAPTER 2: INTRODUCTION

The leading cause of death among the gynecologic malignancies is ovarian cancer, which due to lack of both obvious symptoms or a screening test for early detection, results in a poor prognosis (CDC, 2010; CF&F, 2010). An effective target in ovarian cancer is yet to be identified. In this chapter, we show that several oncogenes that are overexpressed in ovarian cancer, PI3K, PKC α and cyclin E, all of which have been shown to lead to a poor prognosis (Courjal, 1996; Marone, 1998; Farley, 2003; Weichert, 2003; Eder, 2005) converge into a single pathway that could potentially be targeted therapeutically.

PKC α , an atypical member of the PKC family of serine/ threonine kinases, is an oncogene in ovarian cancer (Zhang, 2006). In normal ovarian epithelial cells, as well as in benign serous or mucinous cysts, PKC α is localized to the apical membrane, however, in all serous epithelial ovarian cancer patient samples examined (n = 322), PKC α was either mislocalized or overexpressed (Eder, 2005). PKC α is involved in actin remodeling, apical-basal polarity, endosomal trafficking, glucose uptake, proliferation, transformation, migration, invasion, differentiation, and survival (see Chapter 1). PKC α has major potential as a therapeutic target due to its oncogenic role in ovarian cancer and high potential of being overexpressed. PKC α is the second most overexpressed oncogene in ovarian cancer, by being overexpressed in 78% of all epithelial ovarian cancers (Bast, 2009). PKC α is activated by PDK1 downstream of PI3K (Chou, 1998; Akimoto, 1998; Standaert, 1999; Kanoh, 2003). The PI3K pathway, either through activation of PI3K, AKT, or inhibition of PTEN, is activated in approximately 70% of ovarian cancers (Bast, 2009). Thus, the PI3K/ PDK1/ PKC α pathway is an excellent example of an ovarian cancer pathway to be targeted therapeutically. In this chapter, we demonstrate that the PI3K/ PKC α pathway converges with the cyclin E-CDK2 pathway in serous ovarian cancer.

Cyclin E, the regulatory subunit in the cyclin E/CDK2 complex, is also an often overexpressed oncogene in ovarian cancer (Bast, 2009). Cyclin E through the activation of CDK2 leads to several aspects of tumorigenesis including increased proliferation, anchorage-independent growth, centrosome amplification, genomic instability, and metastasis (Chapter 1). In the most lethal and common form of ovarian cancer, serous epithelial ovarian cancer, cyclin E is overexpressed in high grade serous epithelial ovarian tumors (Nakayama, 2007). In several cancer types, including ovarian cancer, cyclin E is cleaved post-translationally into oncogenic, tumor-specific low molecular weight isoforms, termed LMW-E (Keyomarsi, 1993; Porter, 2001; Bedrosian, 2004; Davidson, 2007). These LMW-E forms of cyclin E are tumor-

specific and are characterized by the ability to hyperactivate CDK2, even in the presence of CKIs, such as p21 or p27 (Akli, 2004; Wingate, 2005). The overexpression of PKC α in serous epithelial tumors correlates with the presence of LMW-E, which as mentioned in Chapter 1 is likely involved in transformation, genomic instability and metastases (Eder, 2005). Thus, PKC α is a feasible target for therapy in serous epithelial ovarian cancer, potentially through downregulation of other oncogenes, such as cyclin E.

In this chapter, we demonstrate that cyclin E is downstream of PKC α and not *vice versa* in serous ovarian cancer cells. We also show that the effect of PKC α on cyclin E expression is at the level of protein stability. Conversely, altered cyclin E expression does not alter PKC α activity or levels. Using an RPPA assay to identify possible downstream effector molecules of PKC α overexpression, we determined that the regulation of cyclin E by PKC α is via the PI3K pathway. The PI3K pathway is activated in approximately 70% of epithelial ovarian cancers. Thus, because PKC α is mislocalized in serous epithelial ovarian cancer leading to an increase in cyclin E, inhibition of either PI3K or PKC α could be a possible approach used in the treatment of serous epithelial ovarian cancers.

CHAPTER 2: METHODS

Methods are in the order of appearance in this chapter.

Cell lines and other reagents. A complete list of cell lines used in this dissertation along with their source, histotype, and media used is shown in Table 1.

Table 1: Cell Lines Used in Chapter 2

Cell Lines	Availability	Organ	Source	Histotype	Media Used
59M	ECACC	Ovary	Ascites	Endometriod Carcinoma with Clear Cell Areas	Alpha Complete Media
293T/17	ATCC	Kidney	n/a	Adherent	Alpha Complete Media
A2780CP	ECACC	Ovary	Tumor Tissue	Ovarian Carcinoma	Alpha Complete Media
DOV13	Rauh-Adelmann, 2000	Ovary	Ascites	Epithelial Ovarian Cancer	Alpha Complete Media
EF0-27	DSMZ	Ovary	Solid Omental Metastasis	Mucinous Papillary Adenocarcinoma	Alpha Complete Media
FU-OV-1	DSMZ	Ovary	Post-Hysterectomy Tumor Tissue	Serous Papillary Adenocarcinoma	Alpha Complete Media
HeyA8	Moore, 1997	Ovary	Not Specified	Serous Papillary Adenocarcinoma	Alpha Complete Media
HOC1	Buick, 1985	Ovary	Not Specified	Serous Adenocarcinoma, Well Differentiated	Alpha Complete Media
HOC7	Buick, 1985	Ovary	Not Specified	Serous Adenocarcinoma, Well Differentiated	Alpha Complete Media
IGROV1	MIISB	Ovary	Primary Tumor	Serous, Endometriod, and clear cell features	Alpha Complete Media
IOSE29	Maines-Bandiera, 1992	Ovary	Not Specified	Immortalized Ovarian Epithelium	MCDB105:M199
MDAH2774	ATCC	Ovary	Not Specified	Adenocarcinoma	Alpha Complete Media
OAW42	ECACC	Ovary	Ascites	Cystadenocarcinoma	Alpha Complete Media
OC316	ICLC	Ovary	Ascites	Adenocarcinoma	Alpha Complete Media
OVCA 420	Karlan, 1988	Ovary	Ascites	Serous Carcinoma	Alpha Complete Media
OVCA 429	Karlan, 1988	Ovary	Ascites	Serous Carcinoma	Alpha Complete Media
OV-CA 432	MIISB	Ovary	Not Specified	Serous Carcinoma	Alpha Complete Media
OVCA 433	Karlan, 1988	Ovary	Ascites	Serous Carcinoma	Alpha Complete Media
OVCAR-3	ATCC	Ovary	Ascites	Epithelial Adenocarcinoma	Alpha Complete Media
SFHUC	Bedrosian, 2004	Ovary	Not Specified	Not Specified	Alpha Complete Media
SKOV-3	ATCC	Ovary	Ascites	Adenocarcinoma	Alpha Complete Media
SW-626	ATCC	Ovary	Tumor Tissue	Ovarian Carcinoma	Alpha Complete Media
UPN251	Selvakumaran, 2001	Ovary	Not Specified	Serous Carcinoma	Alpha Complete Media

Tissue Culture.

All cell lines (listed in Table 1) were cultured and treated at 37°C in a humidified incubator containing 6.5% CO₂–93.5% air.

Alpha Complete Media: HyQ MEM alpha-modification cell culture medium (HyClone) was supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 10 mM HEPES, non-essential amino acids, 2 mM L-glutamine, sodium pyruvate, hydrocortisone, and 10 µg/mL Ciprofloxacin.

DMEM Complete Media: DMEM (Dulbecco's Modification of Eagle's Medium) (Cellgro) was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10µg/mL Ciprofloxacin.

MCDB105:M199 Media: 1:1 ratio of MCDB 105 (Sigma) and Medium 199 (Sigma) was supplemented with 10% FBS, 2mM L-glutamine, and 12.5 µg/mL EGF.

Western Blot Analysis.

Total cell lysates were prepared/ analyzed using western blot analysis as previously described (Keyomarsi, 1995). For each sample 35µg of protein was subjected SDS-PAGE. The protein were then transferred at either overnight at 35mV or for 2 hours at 85mV at 4°C to Immobilon P (Millipore) membranes. The blots were then blocked for 1 hour at room temperature in blocking buffer (5% nonfat dried milk in TBST- 20 mM Tris, 137 mM NaCl, 0.05% Tween, pH 7.6). After being washed in TBST, the blots were incubated in primary antibodies (see below) for 1-2 hours, depending on the antibody. For the mouse primary antibodies, a secondary antibody from eBioscience (called Mouse TrueBlot) was used. For the rabbit primary antibodies, a secondary antibody from Thermo-Scientific (Goat anti-rabbit HRP) was used. Blots were incubated with either Mouse IgG TrueBlot or anti-Rabbit at a 1:1750 dilution in blocking buffer for 45 minutes to 1 hour at room temperature, then washed 6 times (ten minutes each wash), and developed using the Renaissance chemiluminescence system according to the manufacturer (Perkin-Elmer Life Sciences, Inc.).

Primary Antibodies Used: Primary antibodies used were PKC α (BD Transduction Laboratories- 610176- 1:250 in Blotto), cyclin E (HE-12, Santa Cruz Biotechnology 1:1250 in Blotto), p53 (Ab-6, Calbiochem 1:100 in Blotto), Elafin/SKALP (HyCult Biotechnology 1:2000 in Blotto), Phospho-PKC α (Abcam: Phospho T555 + T563 1:1000 in Blotto), p21 (Ab-1, Calbiochem 1:200 in Blotto), Cdk2 (Santa Cruz Biotechnology 1:200 in Blotto), actin (C4, Chemicon International 1:1000 in Blotto), CDK4 (Santa Cruz Biotechnology 1:200 in Blotto), cyclin D1 (Santa Cruz Biotechnology 1:100 in Blotto), p27 (K25020, BD Biosciences-

Transduction Laboratories 1:250 in Blotto), Phospho-AKT (Ser 473 Cell Signaling 1:750 in 5% BSA), AKT (Cell Signaling 1:1000 in Blotto)

Densitometry.

Densitometric analysis was performed using ImageQuant 5.2 software with freshly scanned TIF images of the films obtained from western blot analysis. All quantitative numbers (with background subtracted out) were normalized to the control of that particular experiment for each antibody. After normalization, each number was divided by the normalized actin numbers to control for differences in loading. These values were then plotted using Microsoft Excel in bar-graph form for visualization purposes with the y-axis being the normalized, controlled for loading values (protein to actin ratio) for each lane.

Statistical Consideration.

Linear regression curves were plotted on Microsoft Excel using the x-y scatter graph function with the y-axis (usually cyclin EL, LMW-E, or total LMW-E) being a function of the x-axis (usually PKC α or phospho-PKC α). A linear trendline was established for each, and the correlation coefficient was determined by Pearson test for the correlation coefficient (r). The p-value was determined using a significance calculator examining the r-value and the number of trials. *The student t-test (two-tailed, equal variance)* was employed to derive the p-value of the experiments with a normal distribution, i.e. all experiments in Chapters 2 and 3 except the linear regression.

Stable Clone Selection.

Stable Clone Selection: MDAH2774 and SFHUC Clones. MDAH2774 and SFHUC, ovarian cancer cell lines (see Table 1), were transfected with pcDNA 3.1 plasmids (Invitrogen) with cyclin EL or cyclin E constructs Truncation 1 (T1) or Truncation 2 (T2) described elsewhere (Porter, 2001) using FuGene Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Stable clones were selected for using G418 (Gibco) 48 hours post transfection. Positive clones were determined by western blot analysis using the cyclin E (HE-12; Santa Cruz Biotechnology) antibody (see western blot analysis).

Stable Clone Selection: IGROV and OVCAR3 Clones. IGROV and OVCAR3, ovarian cancer cell lines, were transfected with pRS-shPKC α (Origene #TR320472) using GeneJuice Transfection Reagent (Novagen) according to the manufacturer's protocol. PKC α shRNA consisted of 4 individual constructs. However, upon BLAST analysis, only constructs

shPKC ι 7 and shPKC ι 8 were determined target only a single ssequence. shControl (shRNA 3) contained shGFP. However, these cells did not express GFP, so this made an excellent control. Stable clones were selected for using Puromycin (IGROV = 1 μ g/mL; OVCAR = 0.1 μ g/mL) 48 hours post transfection. Positive clones were then determined by Western blot analysis using the anti-PKC ι antibody (BD Transduction Laboratories).

Infection with Ad-Cyclin E/ Ad-E2F1.

Cyclin E (EL, LMW-E-T1, and LMW-E-T2), E2F1, and LacZ adenoviruses were constructed using the AdEasy XL adenoviral vector system kit (Stratagene). FLAG-tagged cyclin E was subcloned into the multiple cloning site (MCS) of the Ad-easy shuttle vector, followed by linearization with PmeI. BJ5183-AD-1 bacteria were then transformed with the Ad-Shuttle-Cyclin E constructs and selected for with kanamycin. *In vivo* homologous recombination into the pAdEasy-1 vector occurred in these cells. The recombinant DNA was then digested with PacI. The pAdEasy-1-cyclin E constructs were then transfected into Ad-293 cells, which provide the packaging machinery for mature adenovirus. Supernatant was then harvested for mature Ad-cyclin E adenovirus. IGROV cells were plated at 1×10^6 cells / 10 cm plate overnight before being infected with 1000 MOI of either Ad-Cyclin EL, Ad-LMW-E-T1, or Ad-LMW-E-T2. Ad-Cyclin E was kindly provided by Said Akli, as published in (Bagheri-Yarmand, 2010; Bagheri-Yarmand, 2010). Cells were then harvested at time points indicated for each experiment for examined by Western blot analysis with indicated antibodies.

siRNA Transfection.

siRNA to PKC ι in OVCAR3, IGROV, A2780CP, and SKOV3 was performed using siPORT amine reagent (Ambion) using the reverse transfection protocol according to the manufacturer's instructions. 2.3×10^5 cells were used per well of 6 well plates. 2.5 μ L of 20 μ M (20nM total) siRNA was used per well. PKC ι siRNA was purchased from Ambion. Control siRNA was Silencer Negative Control Number 1 (cat# 4611) and PKC ι siRNA was Silencer Validated PKC ι siRNA (gene code 309 and 311). If only one siRNA was used in this experiment, the 309 siPKC ι oligo was used. 311 was used to confirm the results of 309 and rule out the possibility of non-specific effects. Sequences would *not* be released by Ambion upon inquiry, but are guaranteed to be unique and specific sequences.

siRNA to PKC ι in 293T cells was performed using X-tremeGENE siRNA transfection Reagent (Roche) according to the manufacturer's protocol. Cells were plated at 5×10^5 cells

per well in 6-well plates. Twenty-four hours later, cells were transfected with the Ambion siRNA oligos mentioned above and harvested at the time indicated on each experiment.

Generation of PKC α Constitutively Active (CA) and Dominant Negative (DN)

Constructs.

pCMV6-XL5 PKC α wildtype (Wt) cDNA was purchased from Origene (SC118455) and mutated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The CA mutation (A129E) and the DN mutation (K122STOP) were performed using primers:

CA Sense: 5'-CCG TAG AGG TGA ACG CCG CTG GAG AAA GC-3'

CA Antisense: 5'-GCT TTC TCC AGC GGC GTT CAC CTC TAC GG-3'

DN Sense: 5'-GCC TTG TCC AGG AGA AGA TTA ATC CAT CTA CCG-3'

DN Antisense: 5'-CGG TAG ATG GAT TAA TCT TCT CCT GGA CAA GGC-3'

These mutants have been validated in the literature: (Jamieson, 1999 and Regala, 2005, respectively). Confirmation of the targeted mutation was through sequencing using the T7 primer provided by the DNA core sequencing facility followed by transient transfection into 293T cells.

Subcloning of pCMV6-XL5-PKC α Wt, CA, and DN into the pcDNA3.1+ Vector.

pCMV6-XL5-PKC α and pcDNA3.1 constructs were digested with NotI. The vector (pcDNA3.1+) and inserts (PKC α Wt, CA, or DN) were gel purified using the QIAEX II Gel Extraction Kit (Qiagen). A 1:3 ratio of vector to insert was ligated (using T4 DNA ligase-Roche), and the clones that transduced were subject to sequencing using the T7 primer (as above) to determine orientation of insert as well as confirm the mutations within PKC α .

Cell Sorting using GFP.

Transient expression of PKC α . IGROV cells were plated at 1×10^6 cells into 10 cm plates. 24 hours post-plating, these cells were cotransfected with CMV-GFP and the pcDNA3.1-PKC α Wt or CA constructs (or control = pcDNA3.1 alone) using LipoD 293 Transfection reagent (Signagen) according to the manufacturer's protocol. At 24 hours post-transfect, cells were trypsinized, washed with PBS, and sorted based on GFP expression. Cell sorting was performed using the BD FACSAria. Approximately 1×10^6 cells per condition were added with sheath fluid to the nozzle. As individual cells pass through the nozzle, a fluorescent laser is passed across the cells and detected by a photomultiplier attached to a readout device thus

allowing the GFP positive cells to be deflected into a unique collection tube. The GFP cutoff value was determined for each experiment based on transfection with GFP alone. GFP-negative cells were discarded and GFP-positive cells were examined by Western blot analysis. Less than 5% of the original 1×10^6 cells were GFP positive (Vector = 298,000 GFP positive cells; Wt = 146,660; and CA = 128,000 for the experiment displayed, which also was an indication of other experiments).

Generation of stable PKC α expressing IGROV cells. IGROV cells were transfected and sorted as above. Following cell sorting, cells were re-plated in alpha complete media containing the mammalian selection marker G418. At one week post-selection, single clones remained on the tissue culture plate. These clones were picked, grown up, and analyzed by Western blot. Of the clones picked, approximately a dozen were designated as 'potential positives' and were re-examined by Western blot analysis.

Luciferase Assay on Cyclin E-Luciferase.

The luciferase gene under the control of either the cyclin E promoter (bases -207 to +79 ; termed pE) or a mutated cyclin E promoter at two E2F sites (pME) were transfected into IGROV shControl or shPKC α cells using LipoD 293 Transfection Reagent (Signagen) according to the manufacturer's instruction. These two cyclin E reporter constructs, which were originally generated as CAT (chloramphenicol acetyltransferase)- reporters (Ohtani, 1995), were subcloned into luciferase reporter constructs (see Figure M1) (Bresnahan, 1998).

Lysates were harvested 48 hours post-transfection, and a luciferase assay was performed using the “Luciferase Assay System” luciferase kit (Promega). According to the protocol, each lysate was resuspended in 100 μ L of Luciferase Assay Lysis Buffer. Cells were lysed for 15 minutes, followed by addition of 20 μ L of the cell lysates to a siliconized polypropylene tube. 1 μ L Renilla Luciferase Assay Substrate was added per 100 μ L of Luciferase Assay Buffer to a separate tube. Immediately before reading, 100 μ L of the substrate/ assay buffer was added to the cell lysates and read in a luminometer (Monolight 3010- Pharmingen). The controls consisted of no cells, no substrate, or mock transfection. Similarly, because pE should be activated by E2F1 but pME should be unresponsive, Ad-E2F1 (provided by Dr. Kelly Hunt) at 1000 MOI was used to validate the findings of the luciferase assay.

qRT-PCR, RNA Isolation, cDNA Synthesis.

RNA was isolated as previously describe (Chirgwin, 1979). cDNA was synthesized from purified RNA using the Transcription First Strand cDNA Synthesis Kit (Roche). This cDNA synthesis kit allows for mRNA (with a polyA tail) to be reverse-transcribed with a polyT primer into a cDNA transcript. In this kit, 1 μ g of mRNA was combined with dT-oligo primer (50 pmol/ μ L), Transcriptor Reverse Transcriptase Reaction Buffer (5X), Protector RNase Inhibitor (40 U/ μ L), deoxynucleotide mix (10mM), Transcriptor Reverse Transcriptase (20 U/ μ L), and water. These samples were then run for 60 minutes at 50°C in a thermal cyclers (PTC-200, Peltier Thermal Cycler- MJ Research) to convert the mRNA to cDNA. Then, the cDNA samples were diluted at a 1:50 ratio with autoclaved water and used as a template for qRT-RPR analysis. Template, SYBR Green, and forward/ reverse primers were added to 96 tube plates and subjected to qRT-PCR analysis. Forward/ Reverse primers are as follows:

Cyclin E Primer 1 (Lin, 2006): 5'- GTC CTG GCT GAA TGT ATA CAT GC -3'

Cyclin E Primer 2: 5'- CCC TAT TTT GTT CAG ACA ACA TGG C -3'

PKC α 1 (Self-designed using Pubmed Primer Design Tool): 5'-CGG CAT GTG TAA GGA AGG AT-3'

PKC α 2: 5'-CAT CTG GAG TGA GCT GGA CA-3'

FBW7 Total 1 (Ekholm-Reed, 2004): 5'-ATGGGCCCTGCTCTTCACTTCATGTCC-3'

FBW7 Total 2: 5'-CACTGTGCGTTGTATGCATC-3'

FBW7 Isoform Primers were from (Sangfelt, 2008)

FBW α Primer 1: 5'-GAG CAC ACT GCA AGG AAT GGT GAA GTT-3'

FBW β Primer 1: 5'-GTT GCC GGT TCT GCT CCC TAA TCT-3'

FBW γ Primer 1: 5'-CCA TGG CTT GGT TCC TGT TGA TCT T-3'

FBW alpha, beta, gamma Primer 2: 5'-CCT GTA GGT GGC TGG ACA GAT GT -3'
p53 Primer 1 (Pattyn, 2003): 5'-TCA ACA AGA TGT TTT GCC AAC TG-3'
p53 Primer 2: 5'-ATG TGC TGT GAC TGC TTG TAG ATG-3'

MG132 Treatment.

IGROV shPKCiota clones were cultured for 24 hours at which point they were treated with 5 μ M MG132 (Sigma) for 24 hours. 293T cells were plated at low confluency 24 hours prior to siRNA transfect (see above). Twenty-four hours post transfection, cells were treated with 5 μ M MG132. Cells were harvested as above and subjected to western blot analysis with indicated antibodies.

RPPA Analysis.

RPPA (Reverse Phase Protein Array), a high-throughput technique of quantitated proteomic analysis, was performed as previously published (Tibes, 2006). The RPPA assay measures the change in protein levels (and phospho-protein levels) of hundred of proteins in a high-throughput manner with little lysate needed. 293T cells were plated at 0.5×10^6 cells per 10 cm plate. 24 hours post plating, 293T cells were transfected with either vector alone, PKCiota Wt, CA, or DN, and harvested 48 hours later as for a Western blot as above. IGROV lysates (7 shControl and 7 shPKCiota) were prepared as for Western blot analysis (see above). Lysates were printed onto nitrocellulose-coated glass slides using an automated robotic arrayer (GeneTac). Probing these slides is similar to immunoblotting, where slides are subjected to a blocking step (for endogenous peroxidase and biotin protein activity). Primary antibody treatment was followed by washes and a secondary antibody incubation (biotinylated secondary antibody, either anti-mouse or anti-rabbit). The secondary antibody was followed by a biotiny-tyramide deposition (causes amplification of the signal). The amplified signal was detected by a readout device (ImageQuant- Molecular Dynamics) and quantitated using an automated RPPA module (MicroVigene- VigeneTech). Analysis of the quantitated RPPA data was performed using Microsoft Excel.

MEK/ PI3K Inhibition.

MEK Inhibition. IGROV cells were plated at 0.5×10^6 cells per 10cm plate, and 24 hours later were serum starved for 18 hours (using alpha media plus 0.5 % FBS). Following serum starvation, the MEK inhibitor, U0126 (10 μ M in DMSO) (Promega) was used in the presence of alpha complete media alone or supplemented with EGF (100 ng/mL) for 1, 12, and 24 hours.

Cells were then harvested and processed for Western blot analysis using antibodies against PKC α , phospho-PKC α , and cyclin E. Results were quantitated by densitometric analysis (as above).

PI3K Inhibition. 293T cells were plated at 0.5×10^6 cells per 10 cm plate, and were treated with PI3K inhibitors GDC-0941 (Selleck Chemicals) (0, 0.5, 1, 5, 10, and 20 μ M) and PI-103 (Selleck Chemicals) (0, 0.1, 0.5, 1, 1.5, 2.5, 5, and 10 μ M) 24 hours post plating. Following 24 hours of treatment with each PI3K inhibitor, cells were harvested as above and subjected to Western blot analysis using antibodies against phospho-PKC α , PKC α , and cyclin E.

CHAPTER 2: RESULTS

Cyclin E and PKC α Exhibit an *in vivo* and *in vitro* Correlation

PKC α and cyclin E overexpression correlate in serous ovarian cancer patient samples, and this correlation results in decreased survival and a poor prognosis (Eder, 2005). In order to study the effect of PKC α on cyclin E or *vice versa*, the correlation between these two oncogenic proteins *in vitro* needed to be established. To this end, a panel of nineteen ovarian cancer cell lines (see Table 1) were examined by Western blot analysis to PKC α and cyclin E. The PKC α levels were dichotomized into high and low phospho-PKC α (the active form of PKC α) groups. In these two groups, cyclin E levels were quantitated by densitometric analysis and plotted as a function of PKC α to examine the correlation of these two proteins *in vitro*. As a control for these analyses, we also examined the expression of cyclin E and PKC α in nine randomly chosen tumor tissue samples (obtained from Dr. Isabelle Bedrosian) by Western blot analysis (Figure 1a). PKC α and cyclin E (both full-length and LMW-E) levels exhibited similar overexpression patterns (high PKC α / cyclin E in lanes 1,2, and 5; low in lanes 3, 6-9) consistent with similar findings in high grade serous epithelial ovarian cancer patient samples reported by Eder et al (Eder, 2005). p53 levels also appeared to be elevated in the high PKC α / high cyclin E samples, such as lanes 1, 2, 5, and 6. However, elafin, the protein responsible for inhibition of the elastase-mediated generation of LMW-E, exhibited no correlative pattern, being only expressed in lane 5. We included the expression patterns of both p53 and elafin in this study to rule in/out the involvement of these proteins in the pathway between cyclin E and PKC α . The results of the western blot analysis using the 19 different ovarian cancer cell lines described in Table 2 revealed that similar to the *in vivo* data, PKC α levels had a similar expression pattern to that of cyclin E. However, unlike the *in vivo* data, only the phospho-PKC α rather than total PKC α was correlated to cyclin E. Total levels of PKC α remained unchanged amongst cell lines examined, which could be due to the sensitivity of the PKC α antibody. The following cell lines, FUOV, OVCAR3, IGROV, and OAW42 exhibited the highest levels of phospho-PKC α and total cyclin E (full-length, LMW-E-Truncation T1, and LMW-E-Truncation T2), while 59M and OVCA420 had the reverse pattern, with low expression of both PKC α and cyclin E. The level of expression of p53, p21, and CDK2 did not correlate to those of PKC α and cyclin E in any of the cell lines. While protein levels of any given protein vary between cell lines, this lack of expression patterns could also be due to mutations within these genes. For example,

mutations in p53 could lead to p53 accumulation without subsequent activation of p21. We next separated cells into two groups of high and low phospho-PKCiota levels, with the cutoff value being the average densitometric value of all cell lines. When the levels of high/low phospho-PKCiota were compared to cyclin E, a significant correlation between these two proteins was noted (Figure 1c). Panel 1 of figure 1c displays the names of the cell lines in these two groups, while panel 2 exhibits the averages of the densitometry values of both phospho-PKCiota and cyclin E. In the high phospho-PKCiota group, full-length cyclin E (cyclin EL) levels are high, while in the low phospho-PKCiota group, cyclin EL levels are low. We limited our analysis between phospho-PKCiota and cyclin E to the EL and not the LMW-E forms of cyclin E. We believe that because LMW-E is derived from full length cyclin EL, and elafin was unaffected, we will likely notice the most significant change in cyclin EL that would correspond with further changes in the low forms of cyclin E. Cyclin EL levels as a function of phospho-PKCiota were plotted in a linear regression plot, and the correlation co-efficient was determined (by the Pearson method) (Figure 1d). A positive and significant correlation ($p = 2.5 \times 10^{-5}$; $r = 0.793$) was established for the relationship between PKCiota and cyclin E. Therefore, while PKCiota and cyclin E levels correlate *in vivo*, phospho-PKCiota and cyclin EL protein levels correlate *in vivo*. These initial results raise the following questions: are these two important oncogenic proteins directly related in the same pathway, or is there something upstream of both of these factors driving the expression of cyclin E and the activity of PKCiota?

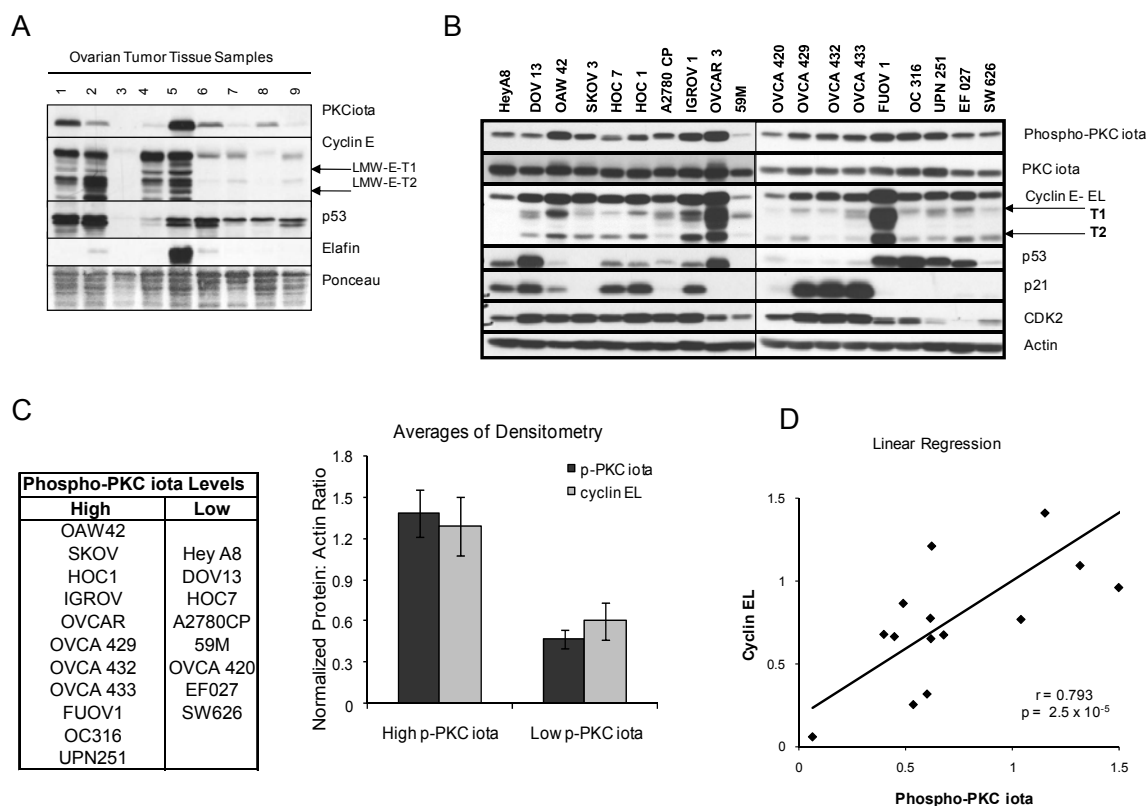


Figure 1: Correlation between phospho-PKCiota and Cyclin E *in vitro*

(A) Nine ovarian cancer patient samples were analyzed by Western blot analysis. PKC*iota*, cyclin E (HE-12, which detects full-length (EL), LMW-E-T1 and LMW-E-T2), p53, and elafin antibodies were examined. Ponceau stain was used as a loading control. (B) Nineteen ovarian cancer cell lines of differing histological backgrounds were examined by Western blot analysis. Phospho-PKC*iota*, PKC*iota*, Cyclin E (HE-12), p53, p21, CDK2, and actin antibodies were examined. Actin was used as a loading control. (C) The protein expression levels of phospho-PKC*iota* and cyclin EL were analyzed by densitometry of the Western blot in (B). The cell lines were then dichotomized into a low phospho-PKC*iota* and a high phospho-PKC*iota* group, and plotted accordingly. The averages of the phospho-PKC*iota* and cyclin EL levels were plotted and revealed a potential correlation. (D) Cyclin EL levels as a function of phospho-PKC*iota* revealed a significant positive correlation ($r = 0.79$; $p = 2.5 \times 10^{-5}$).

PKC α Expression/Activity is Unaffected by Changes in Cyclin E Expression

PKC α overexpression correlates with LMW-E expression in serous ovarian cancer patient samples leading to a poor prognosis (Eder, 2005). Similarly, phospho-PKC α levels correlate with total cyclin E expression levels *in vitro* (Figure 1). While a correlation was established between PKC α and cyclin E, the connection between these oncogenic factors was not explored in a cause and effect relationship. To establish the effect of cyclin E (full-length and LMW-E) on PKC α levels and activity, stable cyclin E overexpressing clones were generated in SFHUC and MDA-2774 ovarian cancer cell lines. These two particular ovarian cancer cell lines were chosen due to their use in the characterization of LMW-E in ovarian cancer as previously published (Bedrosian, 2004; Bedrosian, 2007). However, these cell lines were not examined in the initial panel of 19 cell lines (Figure 1) because they were not as well characterized for protein expression as the Mills panel of ovarian cancer cell lines. So, in addition to the SFHUCs and the MDA-2774 cell lines, we also examined the effect of cyclin E on PKC α in a transient model using IGROV cells. IGROV cells, which have been characterized both by our laboratory as well as that of the Mills' lab, were chosen due to their subtype. IGROV cells are serous epithelial ovarian cancer cells, which is the subtype that exhibited the initial correlation between PKC α expression and LMW-E expression (Eder, 2005). IGROV cells then were infected with Ad-cyclin EL and Ad-LMW-E followed by analysis of PKC α /phospho-PKC α by Western blot analysis.

SFHUC and MDA-2774 cells were chosen due to their inability to process cyclin E into the LMW-E isoforms (Bedrosian, 2004; Bedrosian, 2007). Upon transfection with pcDNA3.1 vector alone, or cyclin EL, LMW-E-T1, or LMW-E-T2 clones were isolated by selection with G418. To generate the SFHUC cyclin E clones, multiple clones were picked and analyzed by Western blot analysis using a cyclin E antibody that detects all forms of cyclin E (Figure 2a,b). Clones 3.1-3 and 3.1-5, transfected with empty vectors, were chosen as the control clones. EL-10 and EL-11 were chosen for their high full length cyclin E expression. LMW-E T1-4, T1-22, T2-4, and T2-10 were chosen as high LMW-E overexpressing clones. When the stably transfected clones from each cell line were subjected to western blot analysis with cyclin E and PKC α the results revealed that PKC α levels did not change upon stable cyclin E overexpression (Figure 3a). MDA-2774 stable cyclin E overexpressing cells exhibited similar pattern of expression to those of the SFHUC cyclin E clones. Both PKC α and phospho-PKC α levels were unaltered by stable overexpression of either cyclin EL or LMW-E. OVCAR-3, which expresses both cyclin E and LMW-E was used to compare the level of overexpression to endogenous levels of cyclin EL and LMW-E.

Since cyclin E levels are subject to cell cycle regulation and its levels can be altered if the cells are too confluent, MDA-2774 stable cyclin E overexpressing cells were also analyzed by Western blot at varying confluencies (Figure 3b). The results showed that while cyclin E, even exogenous cyclin E, was higher in the cells at 70% confluency compared to the cells harvested at 40% confluency, this increase in expression did not translate to any change in PKC α or phospho-PKC α levels. Thus, unlike cyclin E, PKC α levels are not likely to be altered by changes in confluency.

Since stable clones can have secondary oncogenic and/or signaling hits that could mask a potential direct and causal relationship between the transgene and endogenous protein being examined, it was important to also examine the relationship between cyclin E and PKC α in a transient system. For these studies, cyclin E overexpression was examined transiently in the serous ovarian cancer cell line, IGROV and OVCA420 ovarian cancer cells (which have low endogenous levels of PKC α and cyclin E). Corresponding to the finding in stable SFHUC and MDA-2774 that cyclin E overexpression does not affect PKC α levels or activity, IGROV cells infected transiently with cyclin EL or LMW-E did not exhibit changes in PKC α levels/activity even at 48, 72, and 144 hours post infection (Figure 3c). Similarly, OVCA420 cells either transiently infected or transfected with cyclin E did not exhibit changes to the level or activity of endogenous PKC α (Figure 3d). Collectively our results show that in four ovarian cancer cell lines under both transient and stable expression of cyclin E, no change to PKC α protein levels or activity was observed. Thus, we conclude that cyclin E is likely not a regulator of PKC α , either directly or in a feedback loop. Although cyclin E overexpression can not change the expression of PKC α , a correlation still exists between these two oncogenic proteins. Thus, the question that still subsists, is PKC α a regulator of cyclin E or are these proteins both regulated by an upstream effector molecule that controls the up-regulation of both?

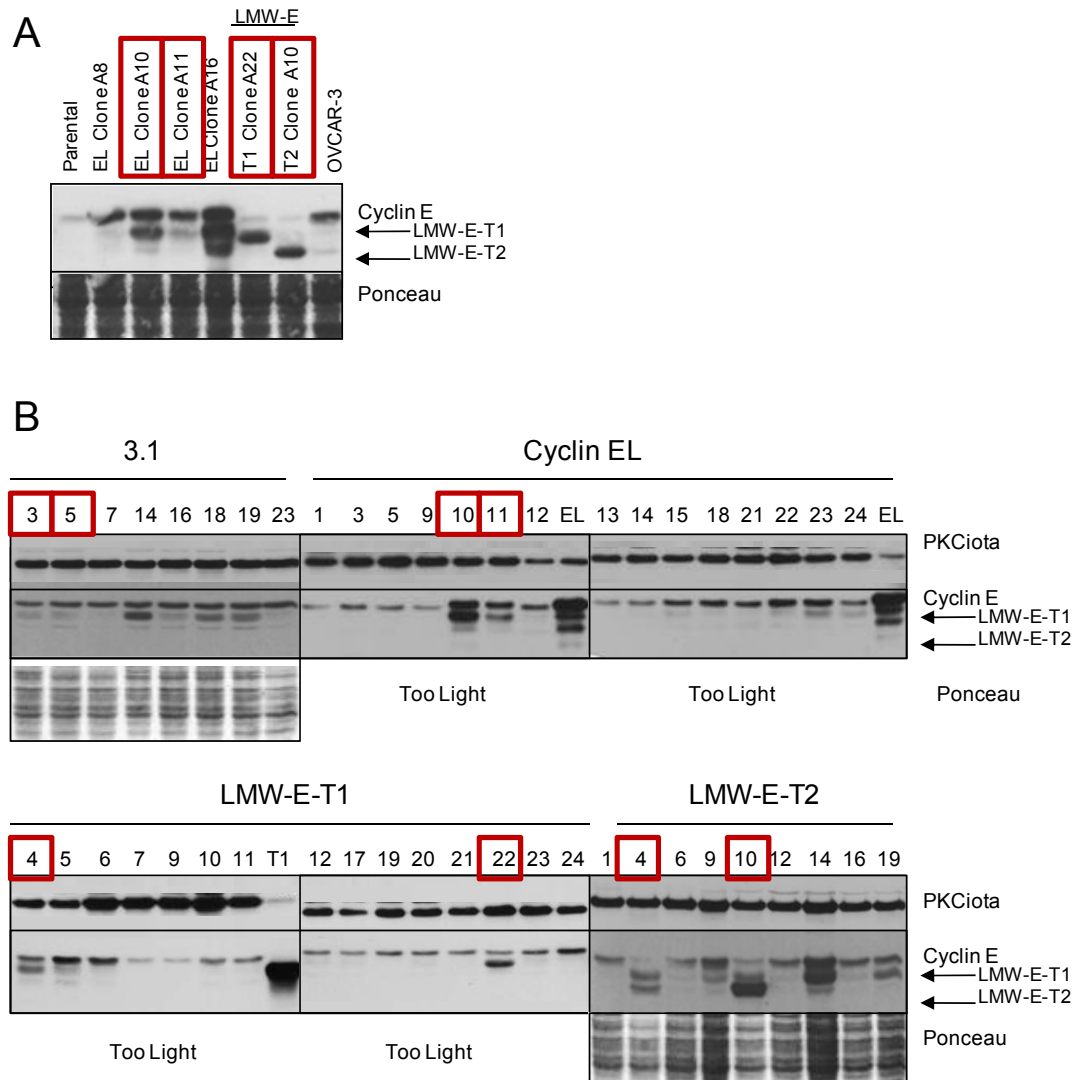


Figure 2: Screening the Ovarian Cancer Cell Line, SFHUC, for Stable Cyclin E Overexpression

(A) Western blot analysis of SFHUC clones generated by Dr. Isabelle Bedrosian. (B) SFHUC cells were transfected with vector alone (3.1), full-length cyclin E (EL) or LMW-E (LMW-E-T1 and LMW-E-T2). Selection of clones by G418 was performed, and results were analyzed by Western blot analysis using cyclin E (HE-12) antibody. PKCιota antibody was also examined but revealed no change in levels upon exogenous cyclin E overexpression. Ponceau stain was used as a loading control. Clones highlighted with red boxes were considered potential positive clones and were further analyzed for expression.

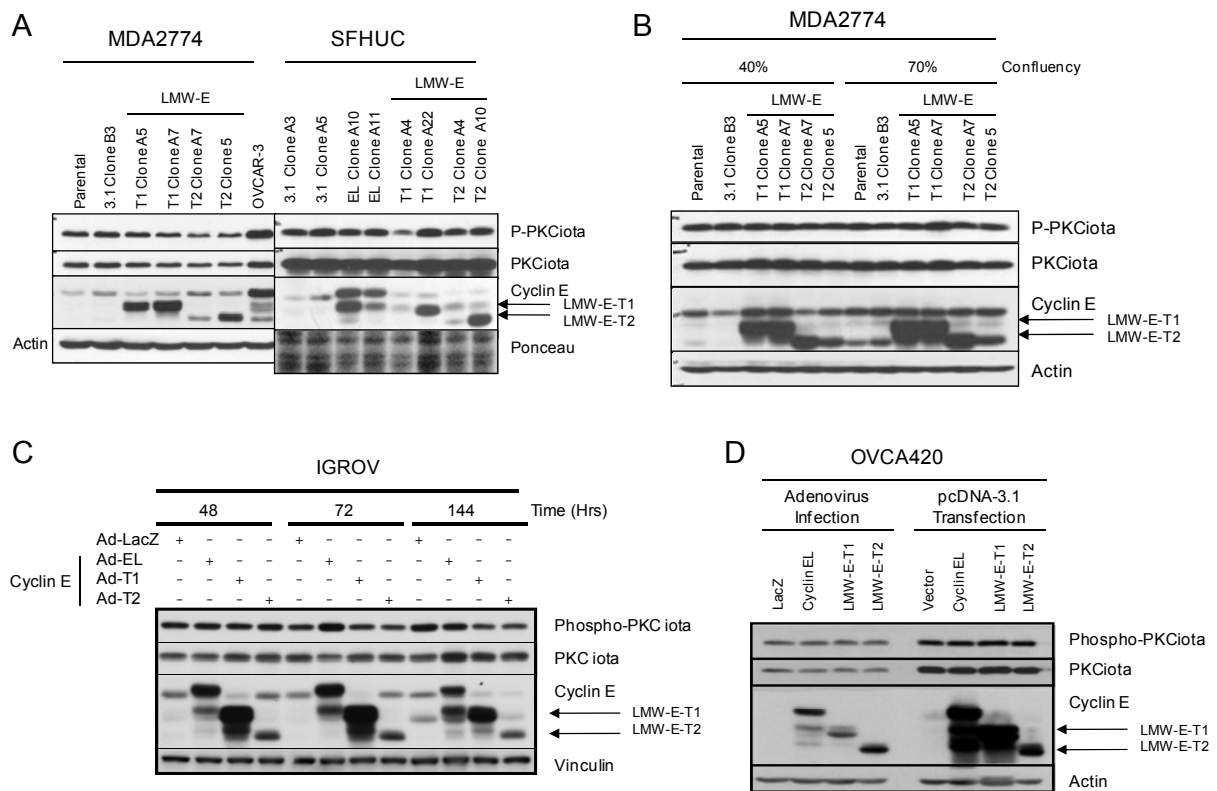


Figure 3: Exogenous Expression of Cyclin E does not Alter PKCiota Levels/ Activity
 (A) MDA2774 and SFHUC cells were transfected with vector alone (3.1), full-length cyclin E (EL) or LMW-E (LMW-E-T1 or LMW-E-T2). Stable clones were selected by G418 resistance and were subject to Western blot analysis using phospho-PKCiota, PKCiota, cyclin E (HE-12), and actin. Actin and Ponceau stain were used as loading controls. Analysis revealed no change in PKCiota levels or activity (as determined using a phospho-PKCiota antibody) upon ectopic cyclin E overexpression. (B) MDA2774 stable cyclin E clones were examined by Western blot analysis under the same conditions of part (A) but at different confluencies. Confluency did not change the lack of effect of cyclin E overexpression of PKCiota. (C) IGROV cells were infected with Adenovirus expressing either LacZ (control), EL, LMW-E-T1, or LMW-E-T2. Transient expression of cyclin E (at 48, 72, and 144 hours post-infection) was examined by Western blot analysis as above. Transient ectopic expression of cyclin E did not alter PKCiota protein levels or activity. Vinculin was used as a loading control. (D) OVCA420 cells were infected with Adeno-virus expressing either LacZ (control), EL, LMW-E-T1, or LMW-E-T2 or were transfected with pcDNA3.1 vector, EL, LMW-E-T1, or LMW-E-T2. Transient expression of cyclin E (at 72 hours post-infection/transfection) was examined by Western blot analysis as above. Again, transient ectopic expression of cyclin E did not alter PKCiota protein levels or activity. Actin was used as a loading control.

Cyclin E is Down-regulated by PKCιota Knockdown

While PKCιota and cyclin E overexpression correlate both *in vitro* and *in vivo*, cyclin E overexpression fails to influence the levels of PKCιota (Figure 1-3). Thus, if these two proteins are in the same pathway, the next question is, does PKCιota expression modulate cyclin E's expression? More specifically, if PKCιota is down-regulated, does this translate to a decrease in cyclin E expression? To test the effect of PKCιota down-regulation on cyclin E levels, an ovarian cancer cell line was chosen as a model system based on high expression of both phospho-PKCιota and cyclin E. To choose a cell line with this criteria, we referred to our panel of 19 ovarian cancer cell lines presented in Table 1 and figure 1 and chose IGROV and OVCAR3 ovarian cancer cells based on their high levels of both phospho-PKCιota and cyclin E. In these two ovarian cancer cell lines, PKCιota was down-regulated by both stable shRNA and transient siRNA and the effect on cyclin E was examined by Western blot analysis to cyclin E.

Once the appropriate cell lines were chosen for high expression of phospho-PKCιota and cyclin E, PKCιota was down-regulated by two mechanisms: stable silencing with shRNA to PKCιota (shPKCιota) and transient silencing with siRNA (siPKCιota). Stable silencing of PKCιota was achieved by transfection with 4 shRNA constructs to PKCιota (shPKCιota 4-8), each coding for a unique sequence within PKCιota. Upon examination of the 4 sequences with a Pubmed BLAST search, shPKCιota constructs 5 and 6 were determined to have greater than one hit and were thus not used in the selection of stable shPKCιota clones in IGROV and OVCAR3 cells (See Table 2). shPKCιota construct 7 (against exon 14) and construct 8 (against exon 13) were used to generate stably-silenced shPKCιota clones. shControl is directed again GFP.

Table 2: shRNA Sequences and Information

shRNA Construct	Against	Sequence	Exons	Blast Hits
3	GFP (-control)	Not Determined	-	
5	PKCιota	CGGCATTCTTTGCCACAGGAACCAAGTGAT	6,7	4
6	PKCιota	CAACAGGCGTGCTCACTGTGCCATCTGCA	5,6	4
7	PKCιota	TGACCAGAACACAGAGGATTATCTCTTCC	14	1
8	PKCιota	CAGGAGATACAACCAGCACTTTCTGTGGT	13	1

Before stable shRNA clones were selected for in IGROV and OVCAR3 cell lines, the transient down-regulation of PKCιota by shRNA was examined by Western blot analysis with phospho-PKCιota and PKCιota (Figure 4). No significant change in PKCιota levels were

detected in IGROV, OVCAR3, or FUOV cell lysates 24, 48, or 72 hours post transfection. Selection for stable shPKCiota clones using Puromycin was still performed as the efficiency of transfection in these cells is very low, requiring the generation of pure clones. While shPKCiota constructs 4 and 5 were examined transiently, only shControl and shPKCiota constructs 7 and 8 were selected for generation of stable clones in IGROV and OVCAR3 cell lines.

To generate the IGROV shPKCiota stable clones, the concentration of Puromycin to use in the selection of clones needed to be established in order to pick the most stringent conditions, to allow for best chance of selecting the most downregulated PKCiota clones. IGROV cells transfect with shControl, shPKCiota constructs 7, 8, or 7 and 8 combined (7/8), were exposed to 0.25, 0.5, or 1 $\mu\text{g/mL}$ Puromycin (Figure S1a). 1 $\mu\text{g/mL}$ Puromycin was determined to be the best concentration based on the limited number of clones that remained on the plate in comparison to the 0.25 and 0.5 $\mu\text{g/mL}$ Puromycin plates, which still had a relatively high number of cells left on the plate one week post-selection. Single colonies were picked and cultured in the presence of Puromycin until enough cells were present to make lysates. 24 IGROV shControl clones were examined by Western blot analysis with antibodies directed against PKCiota and cyclin E (Figure S1b). While the PKCiota levels exhibited very little variability, the cyclin E levels varied considerably. This variability in the cyclin E levels was likely due to harvesting clones at different confluencies, which would be controlled for in future experiments with these cells. 96 shPKCiota construct 7 (24 per plate, plates A, B, C, and D) clones were examined (Figure S1c), as well as 24 shPKCiota construct 8 clones (Figure S1d), and 72 shPKCiota constructs 7/8 clones (24 per plate, plates A, B, and C) (Figure S1e). Of the almost 200 shPKCiota clones examined, over 50 clones displayed knockdown of PKCiota to nearly undetectable levels.

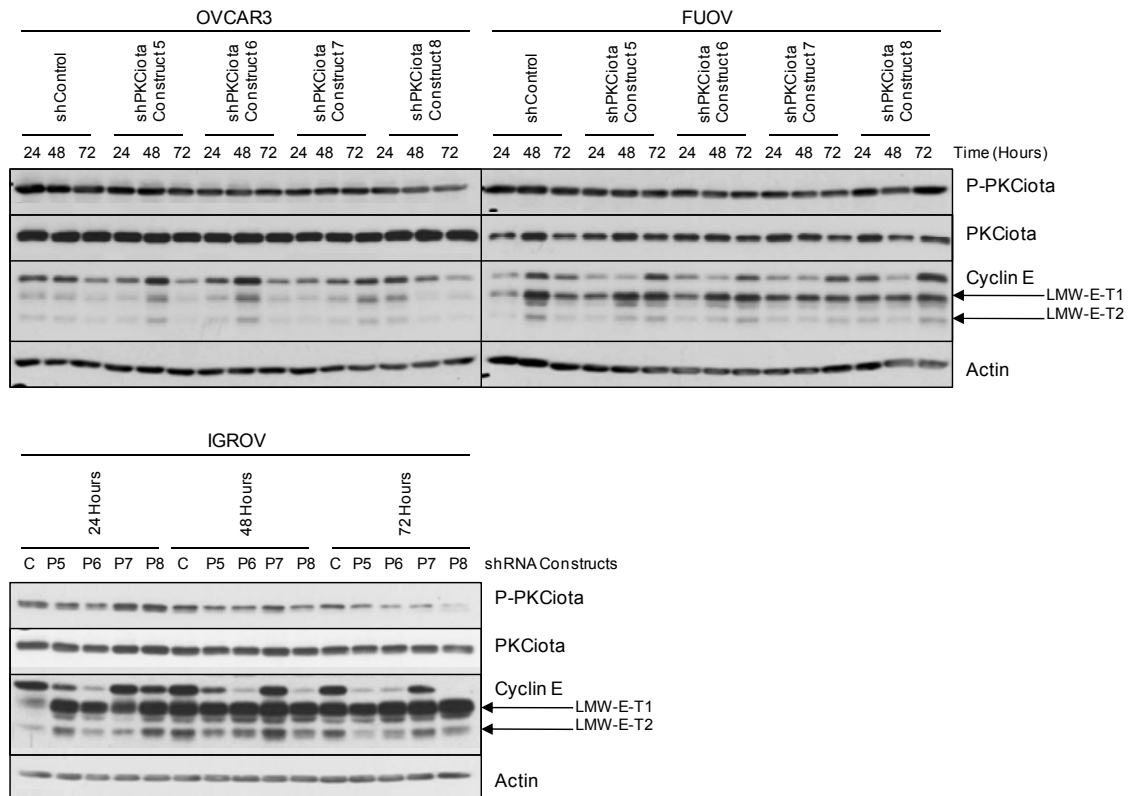


Figure 4: Transient Overexpression of shPKCiota

Four shRNA constructs to PKCiota (P5, P6, P7, and P8) and shControl (C) were transfected into OVCAR3, FUOV, and IGROV ovarian cancer cell lines. Transient knock-down was examined over time (24, 48, and 72 hours) by Western blot analysis with antibodies against phospho-PKCiota, PKCiota, cyclin E, and actin (which was the loading control). No significant changes in PKCiota levels were detected.

Over 50 IGROV shPKCiota clones were determined to be 'potential positives.' However, these clones had been cultured under slightly uncontrolled conditions, such as freshly made media made versus 2 weeks old media or harvested at 90% versus 40% confluency. Thus, in order to rule out the variability of the environmental conditions on cyclin E expression, potential positive clones were plated at the same density and harvest 48 hours post plating. Seven shControl clones, three clones for shPKCiota construct 7 (the most silenced clone per plate), one for shPKCiota construct 8, and three for shPKCiota constructs 7/8 were chosen for analysis. These lysates were then analyzed by Western blot analysis using antibodies to PKCiota, phospho-PKCiota and cyclin E (Figure 5a). When PKCiota was stably silenced by shRNA, phospho-PKCiota, cyclin E, CDK4, and cyclin D1 levels decreased considerably. Cyclin D1 had already been shown to be regulated transcriptionally by PKCiota (Hellbert, 2000; Kampfer, 2001), thus cyclin D1 was used as a control for PKCiota knockdown. To quantitatively measure the effect of PKCiota knockdown on cyclin E expression (cyclin EL, LMW-E, and total cyclin E), densitometric analysis was performed on all lanes of Figure 5a (Figure 5b). The levels of cyclin E varied from clone to clone in the shControl as well as the shPKCiota lanes. However, overall, the average cyclin E level in the shPKCiota clones was lower than the average cyclin E level in the shControl clones, and this difference was statistically significant ($p = 0.0021$) (Figure 5e). Upon silencing PKCiota, full length (cyclin EL) as well as LMW-E decreased (Figure 5c). This led me to believe that cyclin E is a downstream mediator of PKCiota. If LMW-E levels varied without much effect on cyclin EL, one could conclude that the cyclin E cleavage machinery (either elastase, which cleaves cyclin E, or elafin, which inhibits elastase) was being targeted. Similarly, cyclin EL as a function of PKCiota expression exhibited a significant positive correlation ($r = 0.803$; $p = 0.0005$) (Figure 5d). Therefore, because cyclin EL seemed to be down-regulated at a level comparable to LMW-E (Figure 5a,b,c) and the levels of cyclin EL correlated with PKCiota expression (Figure 5d), we conclude that knockdown of PKCiota, in IGROV serous ovarian cancer cells, is able to induce a decrease in cyclin EL and LMW-E expression levels.

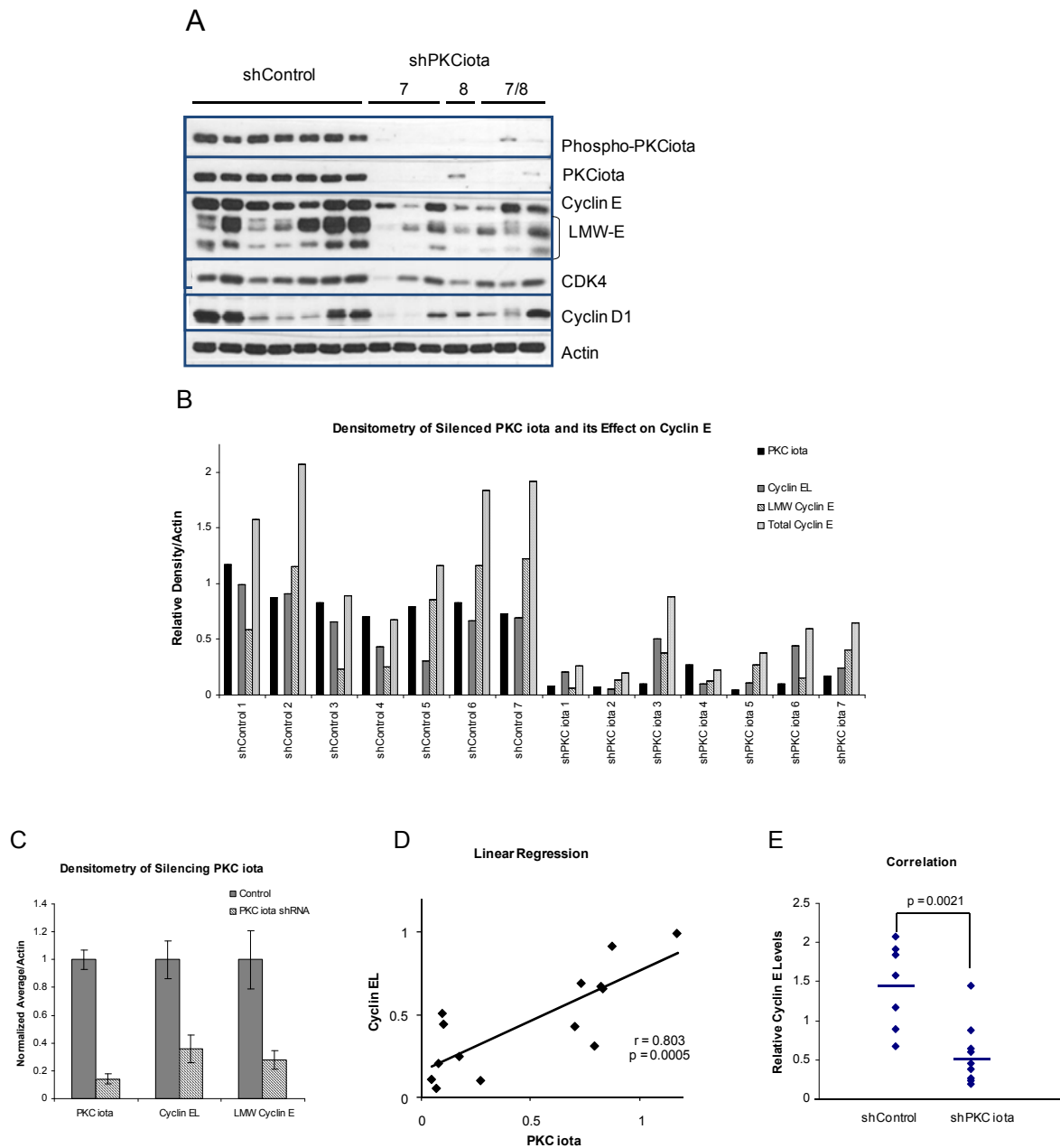


Figure 5: Analysis of Stable IGROV shPKCiota Clones Indicates Correlation between PKCiota and Cyclin E

(A) IGROV stably silenced shControl and shPKCiota clones (constructs 7, 8, and 7/8) were subjected to Western blot analysis using phospho-PKCiota, PKCiota, cyclin E, CDK4, cyclin D1, and actin (as loading control). Cyclin E, CDK4, and cyclin D1 seem to decrease in shPKCiota clones as compared to shControl clones. (B) Densitometry of the PKCiota, cyclin EL (full-length), total cyclin E, or LMW-E to actin ratios were plotted for each individual clone. Averages of all shControl and shPKCiota densitometry data was plotted in (C). As PKCiota decreased, so did both cyclin EL and LMW-E. (D) Cyclin EL levels were plotted as a function of PKCiota. The result of the linear regression plot indicates a significant positive correlation ($r = 0.803$; $p = 0.0005$) between PKCiota and cyclin E. The dot-plot in (E) demonstrates that although there is a significant correlation, some overlap occurs between cyclin E levels in the shControl versus the shPKCiota clones.

To confirm the ability of PKC α knockdown to down-regulate cyclin E, a shPKC α reverted clone (shPKC α clone 7B20) was utilized to examine the resulting change on cyclin E upon PKC α re-expression. After only one passage (passage 2), the expression of PKC α (and phospho-PKC α) returned in IGROV clone 7B20 (Figure 6). In this reverted clone, cyclin E, CDK2, and CDK4 returned. However, no change in p27 or cyclin D1 was observed in the reverted clone. Clone shPKC α 7/8A5 passage 2, which did not revert, was used as a control. Therefore we can conclude that cyclin E, CDK2, and CDK4 are likely regulated by PKC α expression/activity in IGROV cells. The ability of PKC α inhibition to cause a decrease in these vital drivers of the cell cycle could potentially be important when utilizing PKC α inhibitors as targeted agents in ovarian cancer.

The effect of PKC α knockdown on cyclin E expression needed to be validated in another cell line. Initially we used different ovarian cancer cell lines for these experiments. OVCAR3, A2780CP, and SKOV3 cells, all of which have relatively high levels of PKC α and cyclin E, were transfected with siRNA to PKC α (Figure 7a- also see supplemental figure S2, S3). However, despite several attempts (see supplemental figures S2, S3) not one of the three cell lines exhibited consistent results. Where PKC α was silenced by siRNA, cyclin E was not consistently downregulated, possibly due to a combination of low transfection efficiency coupled with the instability of siRNA oligos. We next examined transient PKC α knockdown in 293T cells, which were chosen for siRNA analysis due to their extremely high transfection efficiencies. siRNA to PKC α was transfected into 293T cells, in the presence or absence of EGF and insulin (two known regulators of PKC α) (Figure 7b). When PKC α was transiently knocked down in 293T cells, cyclin E levels corresponded to the decrease in PKC α levels. The results in the 293T cells, reflected the results observed in the IGROV shPKC α clones, that PKC α knockdown led to a decrease in cyclin E levels (both full length and LMW-E). To further validate this result in 293T cells, a second siPKC α oligo was obtained from Ambion (Ambion siPKC α #311), the company from which the first siPKC α oligo was obtained (Ambion siPKC α #309), and transfected into these cells (Figure 7c). At 72 hours post-transfection, both siPKC α oligos resulted in PKC α knockdown and subsequent cyclin E down-regulation compared to the siControl (Ambion-Scramble). Thus, 293T cells validated our results from IGROV cells showing that silencing of PKC α corresponds with a decrease in cyclin EL and LMW-E levels.

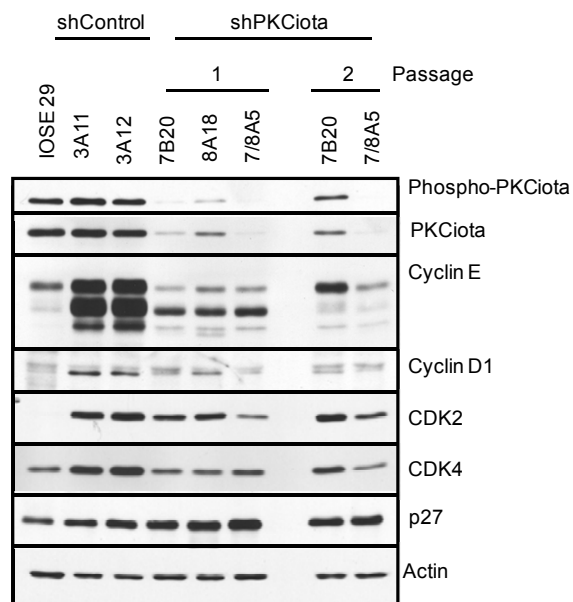


Figure 6: Reversion of shPKCiota in IGROV Clone 7B20

Western blot analysis was performed over several passages of clones using phospho-PKCiota, PKCiota, cyclin E/D1, CDK2/4, p27, and actin (loading control). Immortalized ovarian surface epithelium (IOSE29) were used as a control cell line. As shPKCiota clone 7B20 was passaged, PKCiota (and phospho-PKCiota) returned causing re-expression of cyclin E, CDK2, and CDK4. No reversion of cyclin D1 or p27 occurred.

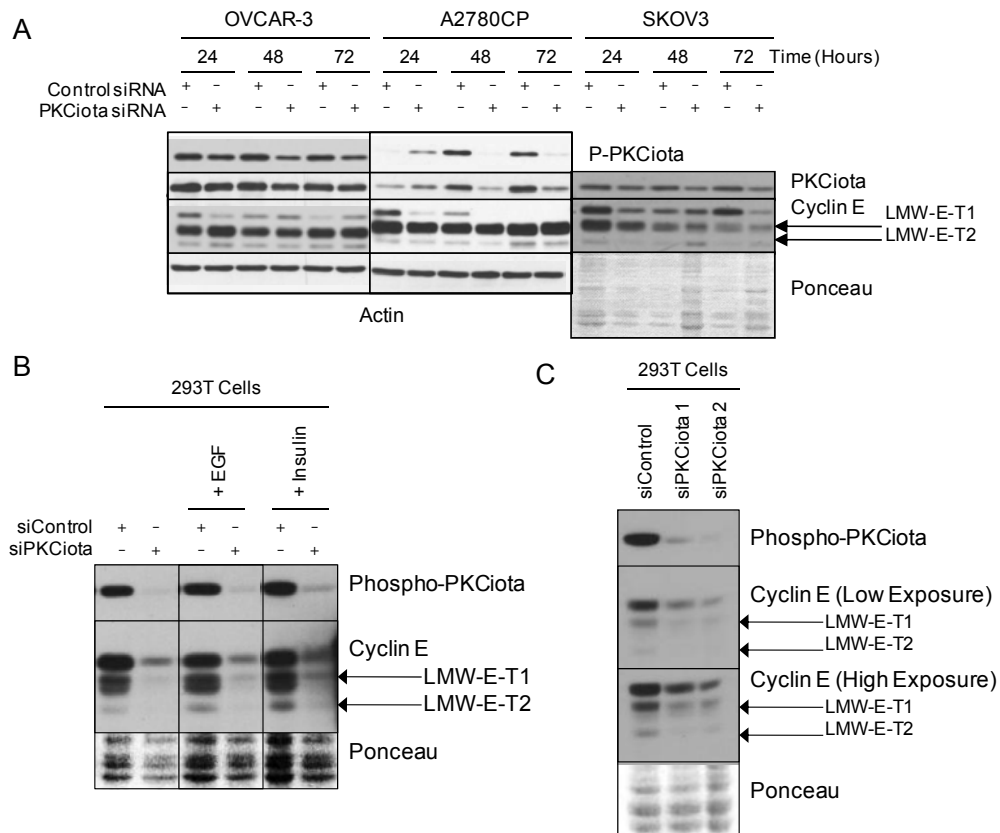


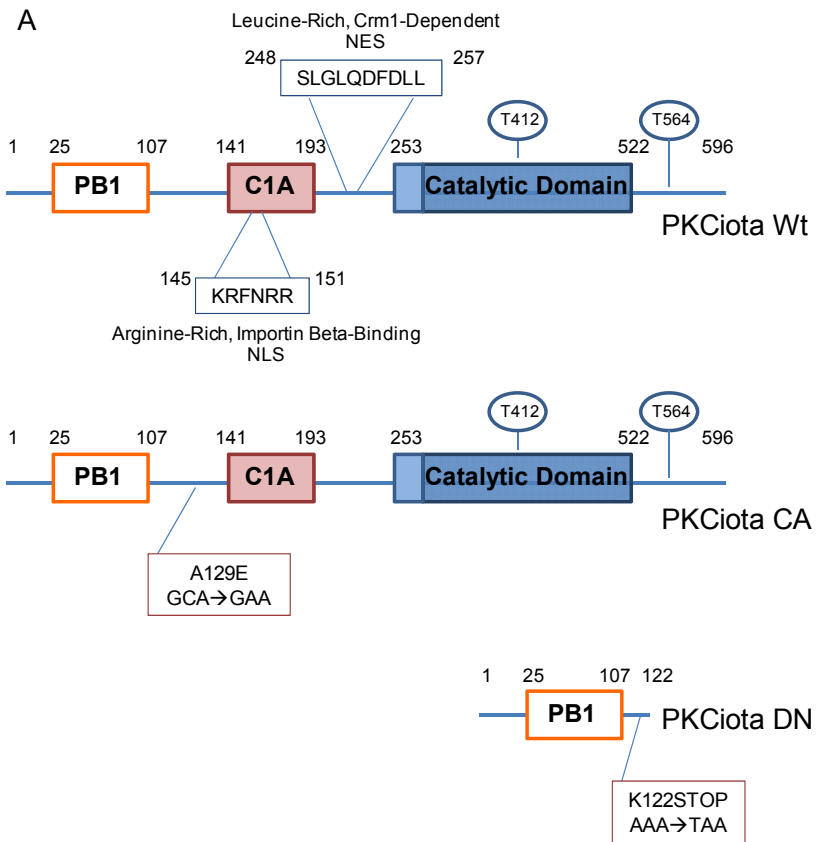
Figure 7: Transient Knock-down of PKC ϵ in Various Cell Lines

(A) Ovarian cancer cell lines: OVCAR3, A2780CP, and SKOV were all transfected with transient control (scramble) or PKC ϵ siRNA over 24, 48, and 72 hours. Western blot analysis using phospho-PKC ϵ , PKC ϵ , and cyclin E antibodies revealed that transient knock-down in these three cell lines would prove to be irregular and unrepeatable. Ponceau stain or actin were used as loading controls. (B) 293T cells were also transfected with transient control (scramble) or PKC ϵ siRNA for 72 hours. Western blot analysis using phospho-PKC ϵ and cyclin E antibodies indicated that transient PKC ϵ knockdown in 293T cells provided a highly reproducible model system for examination of the effect of PKC ϵ on cyclin E. Ponceau stain was used as a loading control. (C) Two distinct PKC ϵ siRNA oligos were used in conditions similar to (B) to determine the specificity of the effect of PKC ϵ knockdown on cyclin E.

Specifically, stable knockdown of PKCιota in IGROV cells and transient knockdown in OVCAR3 and 293T cells resulted in a decrease in cyclin EL and LMW-E levels. This connection between these two important oncogenic proteins raises several questions: does overexpression of PKCιota result in a corresponding increase in cyclin E levels and how is PKCιota regulating cyclin E?

PKCιota Overexpression Corresponds with Increases in Cyclin Levels

Upon knockdown of PKCιota, either stably or transiently, cyclin EL and LMW-E levels decrease at the same ratio suggesting that the regulation of cyclin E by PKCιota is most likely through regulation of cyclin EL. Cyclin EL in turn modulates the changes in LMW-E without affecting the LMW-E-generating machinery. To examine the regulation of cyclin E by PKCιota, PKCιota was overexpressed in 293T and IGROV cells, and the effect on cyclin E levels were examined by Western blot analysis. In order to examine the effect of PKCιota overexpression on cyclin E, first constitutively active (CA) and dominant negative (DN) mutant constructs were generated by site-directed mutagenesis to the PKCιota wild-type (Wt) construct as previously described (Jamieson, 1999; Regala, 2005). pCMV6-XL5-PKCιota cDNA, which was purchased from Origene, was subject to site-directed mutagenesis (Figure 8a). A mutation of PKCιota (A129E/A120E) results in a conformational change of PKCιota into its active or open conformation, thus creating a constitutively active (CA) PKCιota mutant (Jamieson, 1999). Another mutation of PKCιota (K122STOP/K113STOP) creates a truncated PKCιota, which only contains the PB1 domain, and acts as a dominant negative (DN) PKCιota (Regala, 2005). Because PKCιota was determined to have 9 additional amino acids at its N-terminus, the mutations are labeled differently depending on the time of their publication. After site-directed mutagenesis using the primers identified in Figure 8a, ten CA and ten DN constructs were sent for sequencing (Figure 8b). Of these 20 constructs, shown in Figure 8b to contain the PKCιota insert, three CA and three DN constructs were determined to contain the required mutations. Of the three mutated constructs, PKCιota CA clone 3a and PKCιota DN clone 3a were chosen for amplification to be used in further experimentation (Figure 8b and S4). Figure S4 shows the sequencing data that confirms the necessary mutation in PKCιota CA 3a and PKCιota DN 3a compared to the control PKCιota wildtype (Wt) sequence. In 293T cells, overexpression of PKCιota Wt and CA, but not PKCιota DN, resulted in an increase in cyclin E levels (Figure 8c). Particularly, the increase in cyclin EL was consistent with the hypothesis that PKCιota is regulating cyclin E through its expression rather than its processing by elastase/elafin.



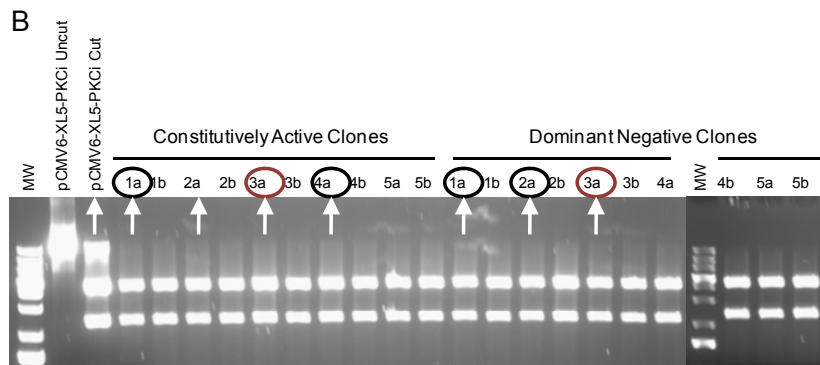
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CA Antisense: 5'-GCT TTC TCC AGC GGC GTT CAC CTC TAC GG-3'

DN Sense: 5'-GCC TTG TCC AGG AGA AGA TTA ATC CAT CTA CCG-3'

DN Antisense: 5'-CGG TAG ATG GAT TAA TCT TCT CCT GGA CAA GGC-3'



KEY:



= Sent For Sequencing



= Positive for Mutation



= Positive for Mutation and Amplified for Future Use

C

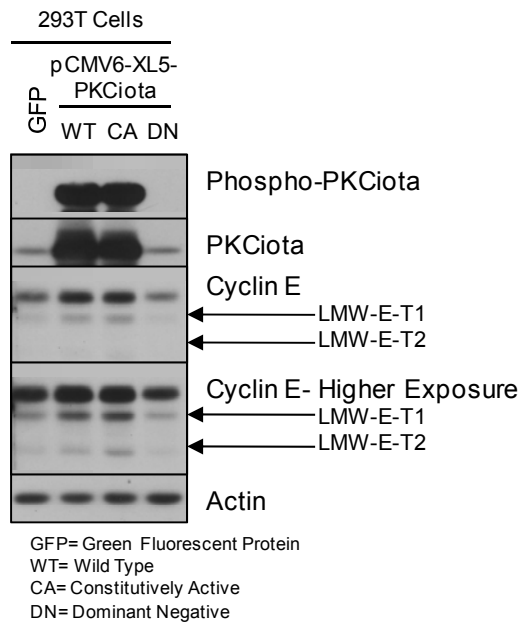


Figure 8: Generation of PKCiota Wt, CA, and DN

(A) PKCiota Wt (wildtype) in pCMV6-XL5 vector was subject to site-directed mutagenesis, using the indicated primers to make PKCiota CA (constitutively active) and PKCiota DN (dominant negative). Mutations necessary are indicated in the figure. (B) Upon site-directed mutagenesis, colonies were picked and sent for sequencing. The black circles indicate positive for mutations and red circles indicate that was the clone used in future experiments (CA3a and DN3a). (C) Transient transfection of these constructs into 293T cells indicate the ability to use these constructs in other experiments. Western blot analysis was performed using phospho-PKCiota, PKCiota, cyclin E, and actin (loading control).

Overexpression of PKC α , in 293T cells, results in a concomitant increase in cyclin E levels. However, the level of PKC α overexpression is much higher than physiologically relevant in 293T cells due to the extremely high transfection efficiency of these cells. To overcome this potential problem, PKC α would need to be overexpressed in another cell line, preferably an ovarian cancer cell line. IGROV cells were chosen due to the ability of PKC α knockdown in these cells to lead to a decrease in cyclin E levels. Since IGROV cells do not transfect well, we co-transfected the different PKC α vector with GFP and sorted the cells to increase the population of positively transfected cells in the sample population. For these experiments, IGROV cells were co-transfected with GFP and pcDNA3.1-PKC α Wt or CA. The sorted cells were either harvested immediately for Western blot analysis or re-plated and selected with G418 (Figure 9b). The pcDNA3.1+ vector would need to be used instead of the pCMV6-XL5, because the latter vector does not contain a mammalian selection marker. PKC α Wt, CA, and DN constructs were subcloned from pCMV6-XL5 to pcDNA3.1+ for the purpose of generation of stable clones (see Supplemental Figure S5 for details).

Cyclin E levels increased in 293T cells when transfected with PKC α (either Wt or CA). For IGROV cells, they were co-transfected with the pcDNA3.1-PKC α Wt and CA and GFP and sorted cells were subjected to G418 selection and single clones were picked and subjected to western blot analysis with an antibody against PKC α (Figure 9c). The results revealed that the level of PKC α in the 'potential positive' clones (lanes 3-9) was only modestly higher than the control GFP/pcDNA3.1+ vector clones (lanes 1 and 2), and no trend was observed in the levels of cyclin E whether or not PKC α was overexpressed. However, in the GFP+/PKC α sorted transient PKC α -expressing cells, both PKC α Wt (lane 3) and PKC α CA (lane 4) resulted in a subsequent increase in cyclin E (cyclin EL and LMW-E) expression, as well as an increase in cyclin D1, CDK2, and p53 levels and a decrease in p27 levels (Figure 9d). The levels of cyclin E were quantitated by densitometric analysis (Figure 9e). The increase in transient PKC α expression resulted in a corresponding increase in full length and LMW-E expression. Thus, in IGROV and 293T cells, transient PKC α expression resulted in an increase in overall cyclin E expression. Because increased cyclin EL levels resulted from increased PKC α expression, this led to the hypothesis that cyclin E was being directly regulated by PKC α rather than through changes in the cleavage machinery of cyclin E to generate LMW-E. However, if cyclin E was being directly regulated by PKC α , what is the mechanism for this regulation? Cyclin E is likely regulated transcriptionally by PKC α , thus transcription would be the first mechanism of cyclin E regulation examined.

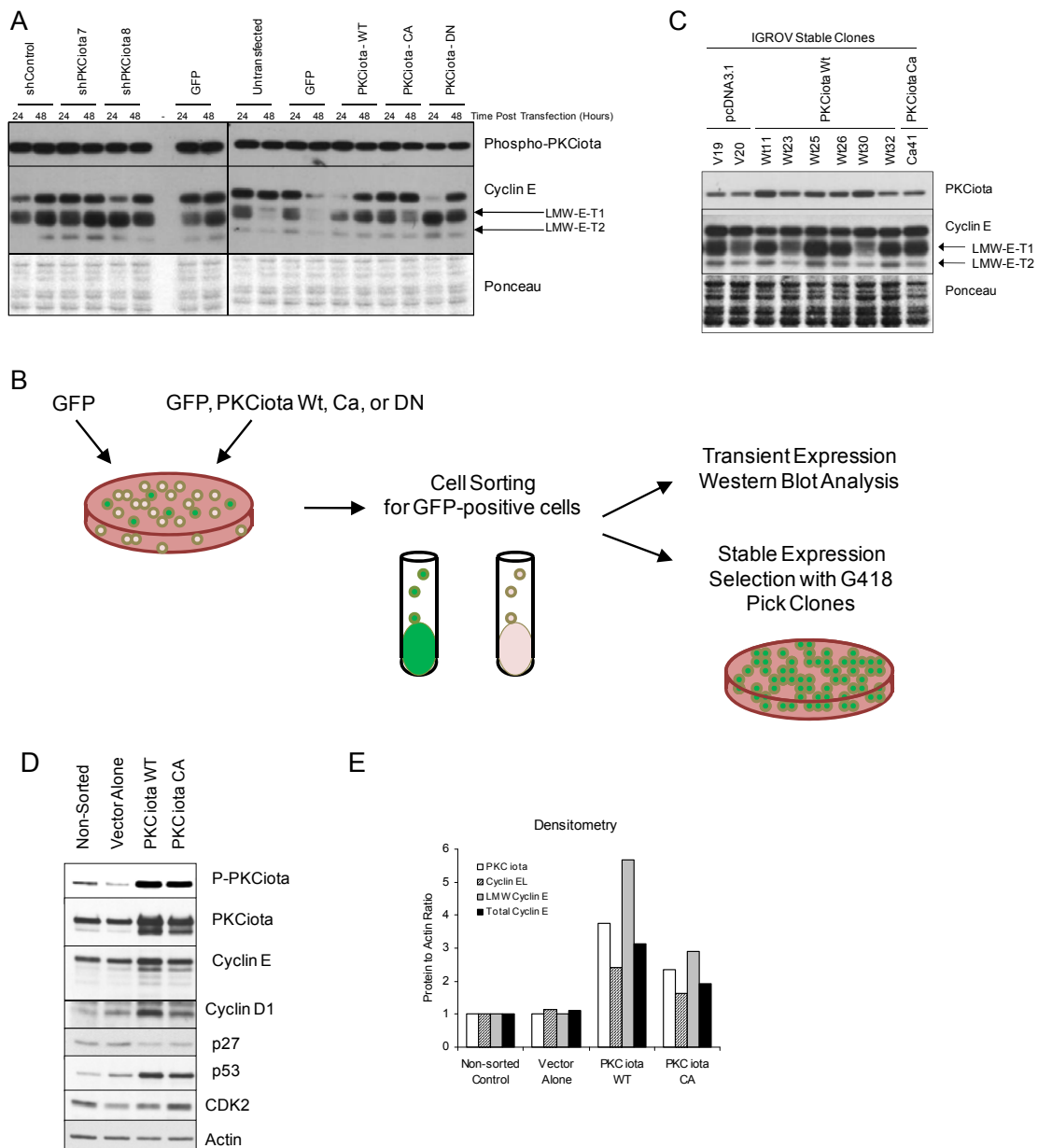


Figure 9: Overexpression of PKCιota in IGROV Cells. (A) IGROV ovarian cancer cells were transfected with shControl, shPKCιota (constructs 7 or 8), PKCιota (Wt-wildtype, Ca-constitutively active, of Dn-dominant negative), and GFP (negative control) and harvested at 24 and 48 hours post transfection for analysis by Western blot. Antibodies to phospho-PKCιota and cyclin E reveal that no changes in the activity of PKCιota (and therefore PKCιota levels) were observed. Ponceau stain was used as a loading control. (B) Schematic of cell sorting that occurred to overcome the lack of effect observed in (A). (C) Final result of the cell sorting/ selection with G418 clone isolation process that resulted in changes in PKCιota levels, but no change in cyclin E. Western blotting was performed as above. (D) Final result of the transient cell sorting that was run on Western blot as above immediately following sorting. Antibodies to phospho-PKCιota, PKCιota, cyclin E, cyclin D1, CDK2, and p53 reveal that as PKCιota levels are increased, so are phospho-PKCιota, p53, CDK2, and the cyclins. The p27 antibody however indicated a decrease in p27 in response to PKCιota overexpression. This change in cyclin E in response to PKCιota was quantitated using densitometry (E). Actin was used as the loading control. Site-directed mutagenesis of PKCιota Wt into PKCιota Ca and DN. Subcloning PKCιota into a vector with G418 resistance (pcDNA3.1+) was necessary for this figure. See the next figure for generation of these necessary constructs.

The Regulation of Cyclin EL by PKC α is Not at the level of Transcription

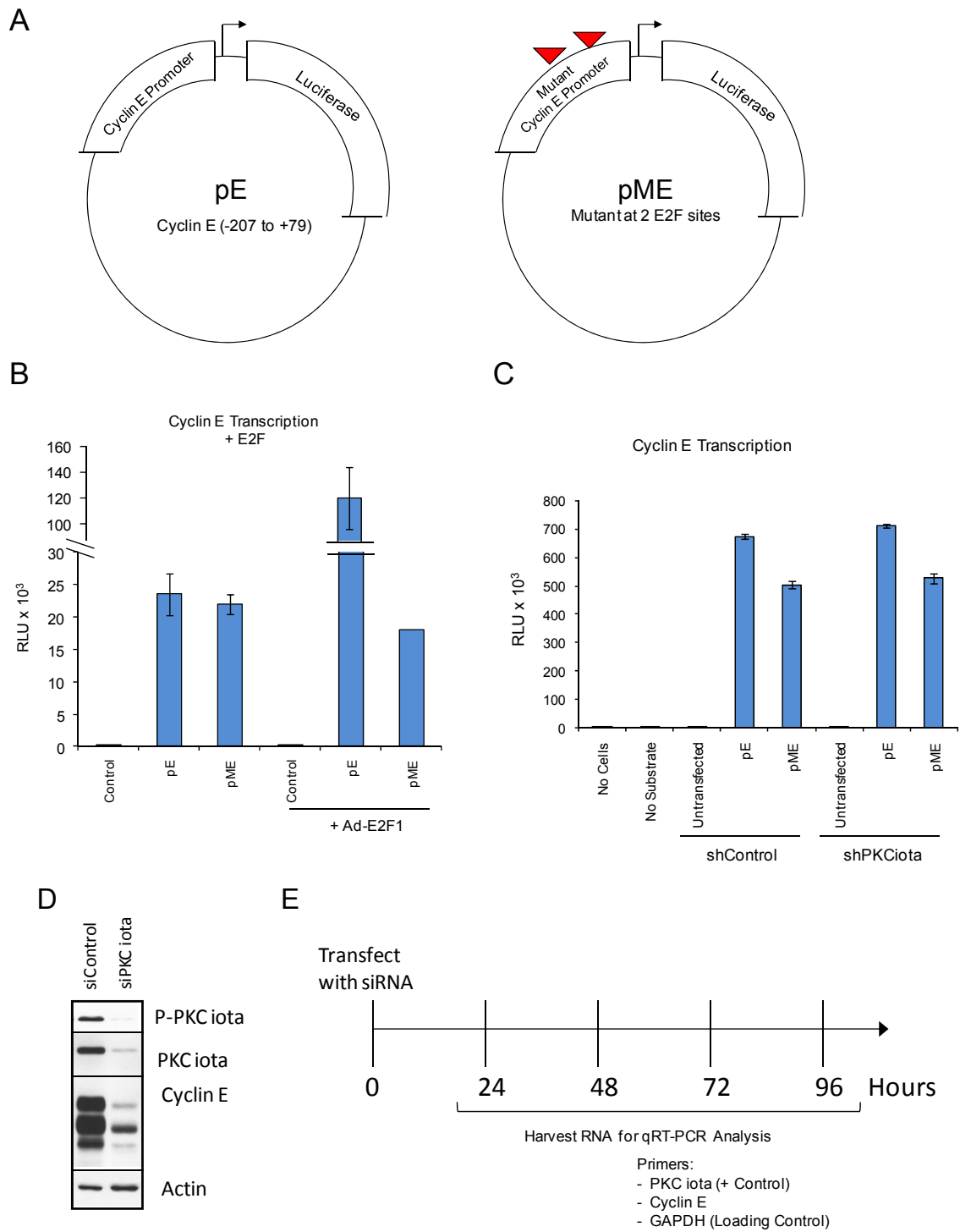
PKC α is able to up-regulate cyclin D1 protein levels, and this regulation is through a transcriptional increase in cyclin D1 (Hellbert, 2000; Mwanjewe, 2001). Therefore, because cyclin D1 and cyclin E are both upregulated in response to PKC α , we hypothesized that the regulation of cyclin E by PKC α could be transcriptional. To test the effect of PKC α on cyclin E transcription, we used a cyclin E promoter-luciferase gene construct and compared the difference between shControl IGROVs and shPKC α stably silenced IGROV cells for their ability to transcriptionally activate cyclin E.

Transcriptional regulation of cyclin E occurs in response to mitogenic factors and involves the transcription factor E2F1 (Ohtani, 1995; Botz, 1996). The regulation of cyclin E by E2F1 was determined by use of two cyclin E reporter constructs, one with the cyclin E promoter (-207 to +79; termed pE) and one with the cyclin E promoter with two mutations at E2F1 binding sites (termed pME) (Ohtani, 1995) (Figure 10a). To ensure that cyclin E transcriptional activity can be modulated by its own activator, we also included Ad-E2F1 as a positive control for these studies (Figure 10b). In the control lane (no luciferase construct), no luciferase activity was observed. However, pE and to a lesser extent pME exhibited luciferase activity in IGROV cells. In the presence of Ad-E2F1, pE luciferase activity increased 6-fold while pME luciferase activity remained unaffected. Thus, the pE and pME constructs provide a feasible system for the investigation of transcriptional regulation of cyclin E by PKC α . Next we used IGROV cells stably expressing either shControl or shPKC α to determine if downregulation of PKC α modulates the transcriptional activity of cyclin E as measured by the pE (and pME) cyclin E luciferase reporter construct (Figure 10c). The results revealed that no difference in cyclin E transcription was observed between the IGROV shControl and shPKC α cells. Thus, the regulation of cyclin E by PKC α is likely through another mechanism rather than transcription.

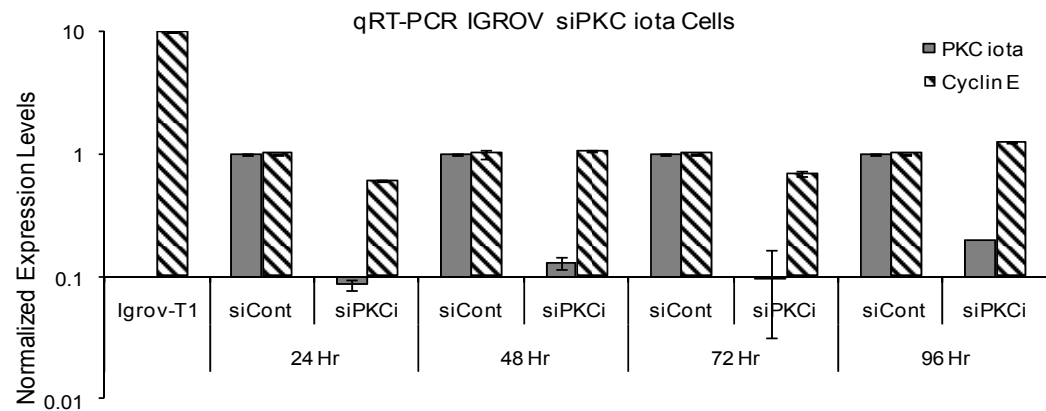
To confirm the finding that cyclin E was not transcriptionally regulated by PKC α , qRT-PCR was also used to measure cyclin E mRNA levels under both stable and transient knockdown of cyclin E in IGROV cells. While stable knockdown of PKC α in IGROVs using shRNA to PKC α has been documented (Figures 5 and 6), PKC α would need to be transiently silenced in IGROV cells to determine the immediate effect on cyclin E transcription (Figure 10d). Upon the transient silencing of PKC α with siRNA, mRNA was harvested at 24, 48, 72, and 96 hours post transfection and subjected to qRT-PCR analysis of cyclin E transcription (see Figure 10e for a time table). While the PKC α transcript decreased upon siPKC α transfection, the cyclin E levels remained unchanged at all 4 time points (Figure

10f). To test the validity of the cyclin E primer, IGROV cells infected with Ad-Cyclin E-T1 (IGROV-T1) were used as a control. No change was observed in cyclin E levels at 24, 48, 72, and 96 hours post siPKC ϵ transfection, thus validating the results of the cyclin E reporter gene study.

The cyclin E reporter gene assay (Figure 10 a-c) was performed using IGROV stable shPKC ϵ clones and resulted in no change in cyclin E transcription. To examine the cyclin E mRNA levels in the IGROV shPKC ϵ clones, qRT-PCR of cyclin E (and PKC ϵ as a control) was performed (Figure 10). While PKC ϵ mRNA levels were substantially decreased in the shPKC ϵ clones compared to the shControl clones (right panel), no pattern of cyclin E mRNA down-regulation was observed in these clones (left panel). The levels of cyclin E mRNA did vary from clone to clone, however, this was unlikely to be due to PKC ϵ stable knockdown due to the lack of a pattern among clones. Thus, based on the data observed using a cyclin E reported construct and qRT-PCR, we conclude that the regulation of cyclin E by PKC ϵ is non-transcriptional in IGROV cells.



F



G

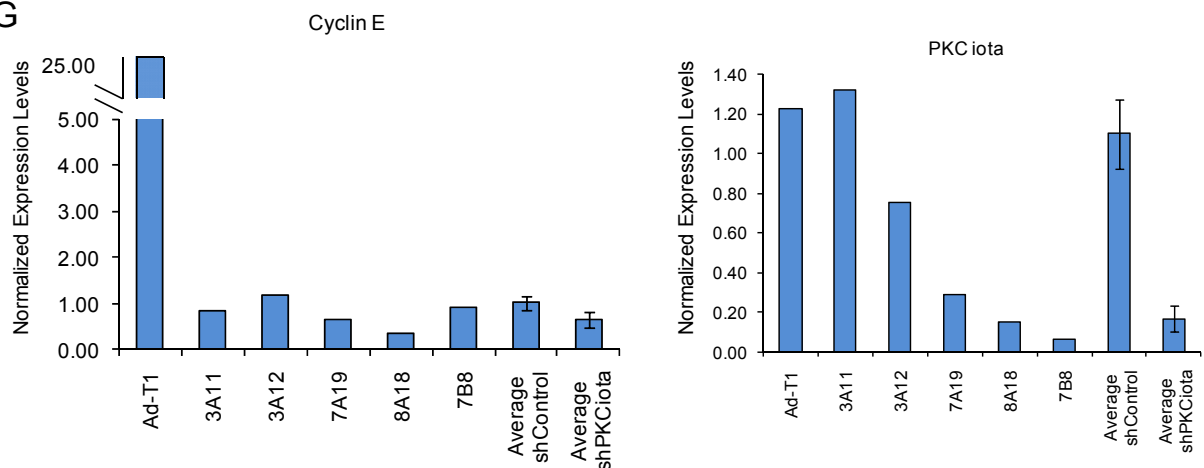


Figure 10: Regulation of Cyclin E by PKC*iota* is Non-Transcriptional

(A) Schematic representation of the pE and pME luciferase reporter constructs used to determine cyclin E promoter activity. pE contains the cyclin E promoter sites -207 to +79. pME contains the same bases as pE, but with mutation at two putative E2F-binding sites (TGTCCTCGC at position +7 to TGTCATGC and changed TTCCGCGC at position -16 to TTCCGATC). These promoter sites were published in: OHTANI, K., DEGREGORI, NEVINS, J. PNAS. Vol. 92, pp. 12146-12150, December 1995. (B) Luciferase Assay demonstrating the validity of these two constructs in IGROV cells. E2F adenovirus increased pE activity while not affecting pME activity. (C) Luciferase assay comparing cyclin E transcriptional upregulation in the shControl IGROV cells versus the shPKC*iota* stably silenced clones. No difference was detected between these two groups. (D) Western blot analysis was performed to confirm knockdown of PKC*iota* in siPKC*iota* samples. Cyclin E protein levels decrease upon silencing of PKC*iota*. Actin was used as a loading control. (E) Timeline of qRT-PCR experiment in (F). Upon transient knockdown of PKC*iota* for 24, 48, 72, and 96 hours, RNA was harvested at each time point and used for analysis of cyclin E mRNA levels. (F) qRT-PCR of IGROV samples listed in part (E). While PKC*iota* RNA expression levels decreased, cyclin E remained unchanged. IGROV infected with cyclin E-T1 (IGROV-T1) was used as a control for the cyclin E primers. GAPDH was used as a loading control, thus all samples were normalized to GAPDH of that sample. (G) qRT-PCR of IGROV stably knocked down PKC*iota* samples. Ad-T1 is positive control for the cyclin E promoter. 3A11 and 3A12 are shControl IGROV cell lines, while 7A19, 8A18, and 7B8 are shPKC*iota* IGROV cell lines. Similar to part (F), PKC*iota* RNA expression levels decreased, while cyclin E remained unchanged. Expression levels were normalized as in part (F).

Cyclin E Stability is Regulated by PKC α

In addition to the transcriptional regulation of cyclin E, cyclin E is also regulated by degradation. As a dividing cell passes through S-phase, cyclin E levels decrease due to ubiquitin-mediated degradation. More specifically, cyclin E gets phosphorylated by either CDK2 or GSK3 at specific sites (at T62, S372, T380, and S384) and is then able to bind FBW7 (F-box and WD-40 domain protein 7), which transports cyclin E to the SCF (Skp1-Cullin1-F-box) ubiquitin ligase complex for degradation (Koepp, 2001; Moberg, 2001; Strohmaier, 2001; Welcker, 2003). Cyclin E levels decrease in response to knockdown of PKC α , which here and based on our lack of transcriptional regulation, we hypothesize to be through increased degradation as opposed to decreased transcription. To examine the regulation of cyclin E stability by PKC α , we overexpressed exogenous cyclin E (through adenovirus infection) in stably silenced IGROV shPKC α cells and quantitatively measured the levels of cyclin E. Because cyclin E transcriptional regulation would then be under the CMV promoter instead of the cyclin E promoter, we can conclude that any changes in cyclin E levels are likely post-translational (such as ubiquitin-mediated degradation). In order to determine if changes in cyclin E levels were a result of proteasomal degradation, MG132 (a proteasome inhibitor) was used to block degradation. If cyclin E is not degraded in the presence of MG132, even in response to PKC α knockdown, then the regulation of cyclin E by PKC α is likely through the degradation of cyclin E.

To determine the effect of PKC α knockdown on exogenous cyclin E expression, IGROV shPKC α cells were infected with either Ad-LacZ, Ad-Cyclin EL, Ad-LMW-E-T1, or Ad-LMW-E-T2 and the resulting levels of cyclin E were examined by Western blot analysis (Figure 11a). Although the two shControl and the two shPKC α cell lines were infected simultaneously with Ad-cyclin at the same MOIs, the expression level of cyclin EL, LMW-E-T1, and LMW-E-T2 were lower in the cells with stable knockdown of PKC α compared to the control cells. The exogenous cyclin E was under the control of the CMV promoter and thus could not be regulated at the same transcriptional level as endogenous cyclin E (which was also decreased in the shPKC α clones). The levels of endogenous cyclin E (Ad-LacZ) and exogenous cyclin E (Ad-cyclin EL, LMW-E-T1, and LMW-E-T2) were quantitated by densitometric analysis (Figure 11b). While the levels of cyclin E decrease in all conditions, only cyclin EL and LMW-E-T2 were shown to be significant ($p = 0.028$ and $p = 0.019$, respectively). LMW-E-T1 was also decreased in the shPKC α clones compared to the shControl clones. However, this decrease in LMW-E-T1 expression was not significant. The lack of significance of the LMW-E-

T1 decrease could be due to the extremely large amount of LMW-E-T1 that was expressed. The levels of FBW-7 and the components of the SCF complex could lead to a potential limiting threshold effect in the degradation of cyclin E. While the decreased exogenous cyclin E protein levels in the shPKCιota clones compared to the shControl clones implies regulation via degradation, the direct effect of cyclin E degradation in response to PKCιota knockdown would need to be investigated further.

While PKCιota knockdown results in a decrease in both endogenous and exogenous cyclin E, the direct effect of PKCιota knockdown on the proteasome needs to be addressed. To investigate the proteasomal regulation of cyclin E by PKCιota, the proteasome inhibitor, MG132, was used in the presence of both stable (IGROV cells) and transient (293T cells) PKCιota knockdown (Figure 11c). Silencing PKCιota was able to cause a decrease in cyclin E as seen in lane 3 (IGROV shPKCιota) and lane 4 (293T siPKCιota) compared to the controls, lanes 1 and 5 respectively. However, upon addition of 5 μ M MG132, cyclin E failed to be degraded, implying PKCιota regulation of cyclin E is through regulation of proteasomal degradation. The total cyclin E and the PKCιota (control) levels of these lanes were quantitated by densitometric analysis (Figure 11d). In the IGROV stable PKCιota knockdown cells (left panel), addition of MG132 resulted in a partial return of cyclin E as compared to the 293T cells (right panel), which exhibited a complete return of cyclin E in response to MG132. This could be easily explained by the stable versus transient nature of the two model systems. While the 293T cells have only functioned without PKCιota and as a result cyclin E for a matter of days, these cells are likely unable to select for cells that survive through upregulation of alternative pathways. Conversely, the stable IGROV shRNA cells have been passaged three times (at multiple days each) without PKCιota (and thus cyclin E) and have potentially been able to up-regulate PKCιota/cyclin E- independent survival pathways-through secondary oncogenic events. Because of the possible selection in IGROV stable shPKCιota clones, cyclin E could likely not be up-regulated at the same rate as the pre-shRNA cells, accounting for the lack of complete return of cyclin E levels in IGROV shPKCιota cells in response to MG132. Therefore, due to the ability of PKCιota knockdown to cause a decrease in exogenous cyclin E and the ability of MG132 to block cyclin E degradation, we conclude that regulation of cyclin E by PKCιota knockdown in both IGROV and 293T cells is post-translational, specifically this regulation is at the level of proteasomal degradation. Because cyclin E degradation is mediated by the F-box protein, FBW7, the effect of PKCιota knockdown on this protein needs to also be addressed.

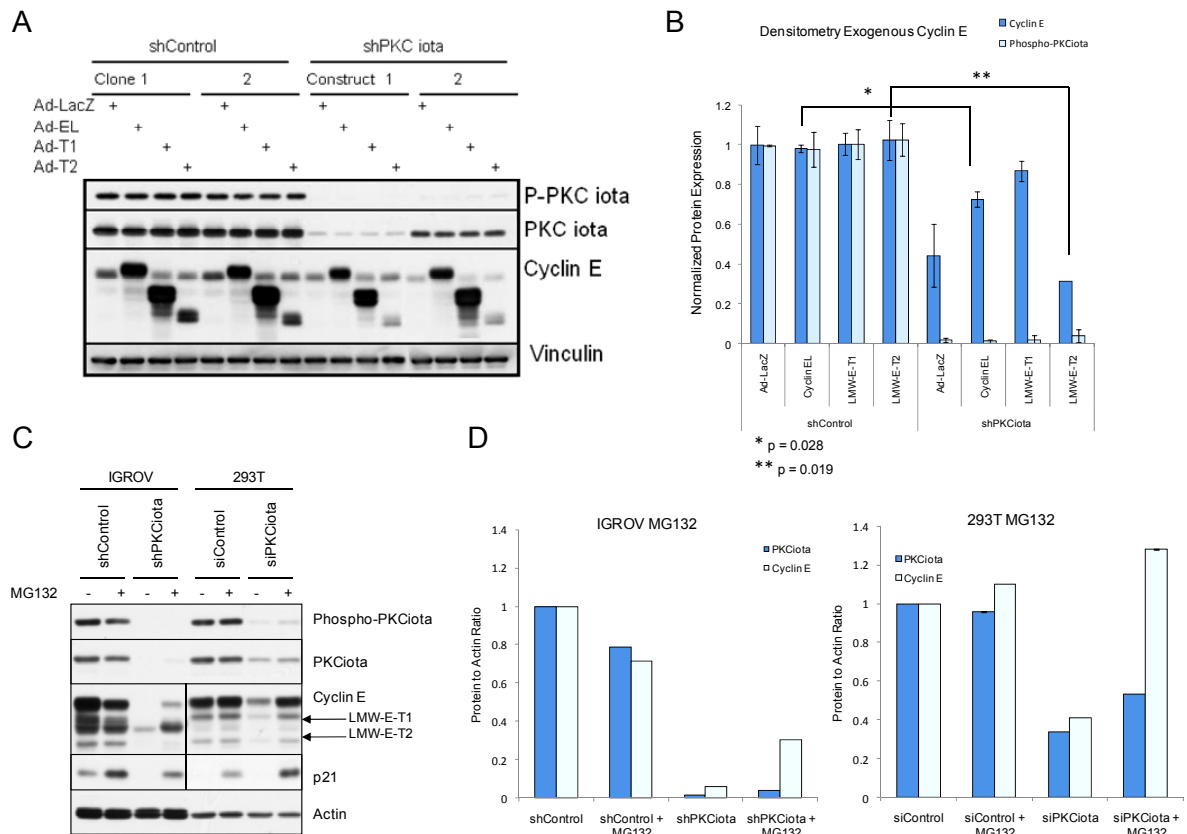


Figure 11: PKCiota Knockdown Affects Cyclin E Stability

(A) IGROV stably silenced shControl and shPKCiota clones were infected with Ad-LacZ (control), Ad-Cyclin EL, Ad-LMW-E-T1, and Ad-LMW-E-T2. shControl clones 1 (3A11) and 2 (3A12) and shPKCiota clones 1(7A19) and 2 (8A18) were infected for 48 hours with adenovirus, and lysates were subjected to Western blot analysis using phospho-PKC iota, PKCiota, cyclin E (HE-12), and vinculin (loading control). When PKCiota levels decreased, so did exogenous cyclin EL, LMW-E-T1, and LMW-E-T2. The levels of cyclin E and phospho-PKC iota (control) were quantitated by densitometric analysis (B). The decrease in cyclin EL and LMW-E-T2 were statistically significant between the average shControl and the average shPKCiota expression levels ($p = 0.028$ and $p = 0.019$, respectively). (C) In both IGROV stable shPKCiota cells and 293T transient siPKCiota cells, MG132 at least partially blocked degradation of cyclin E (full length and LMW-E). Western blot analysis was performed using phospho-PKC iota, PKCiota, cyclin E, p21 (control for MG132 response), and actin (loading control). Although all samples were run on the same gel, the exposure time for cyclin E and p21 Western blots were much longer in the 293T cells than the IGROV cells due to the innately low endogenous levels of these two proteins in 293T cells. With both stable (IGROVs) and transient (293Ts) PKCiota knockdown, addition of MG132 blocked the degradation of cyclin E, which was quantitated by densitometric analysis (D).

Because cyclin E protein levels decrease in response to PKC α knockdown, we hypothesized that FBW7 expression would increase, which could account for the decrease in cyclin E. To determine the effect of PKC α knockdown on FBW7 expression, IGROV cells transiently silenced with siRNA against PKC α were examined for changes in FBW7 expression (both total and individual isoforms) by qRT-PCR analysis. IGROV cells were transfected with either no siRNA, siControl (scramble), or siPKC α for 48 hours and RNA was harvested for qRT-PCR analysis (Figure 12a and 12b). Positive (PKC α) and negative (cyclin E and p53) controls (Figure 12a) as well as FBW7 α , β , and γ isoforms (Figure 12b) were examined. In response to PKC α knockdown, PKC α and FBW7 levels appeared to decrease while the negative controls (cyclin E and p53) remained unchanged. Thus, although we hypothesized that FBW7 levels would increase, we found that they actually decreased by 48 hours post PKC α knockdown. This decrease in FBW7 could be due to an initial increase in FBW7 levels resulting in a negative feedback loop. To test the levels of FBW7 expression as a function of time, we examined FBW7 (both isoforms and total) at 24, 48, 72, and 96 hours (Figure 12c and 12d). While FBW7 individual isoforms (Figure 12c) and total FBW7 (Figure 12d) mRNA levels varied, no significant trend was observed. Thus, changes in FBW7 mRNA levels are likely not the cause of PKC α knockdown-induced cyclin E degradation. However, because changes are not seen at the mRNA level, does not mean that there are not changes at the level of protein or localization that could be influencing the degradation of cyclin E in this circumstance.

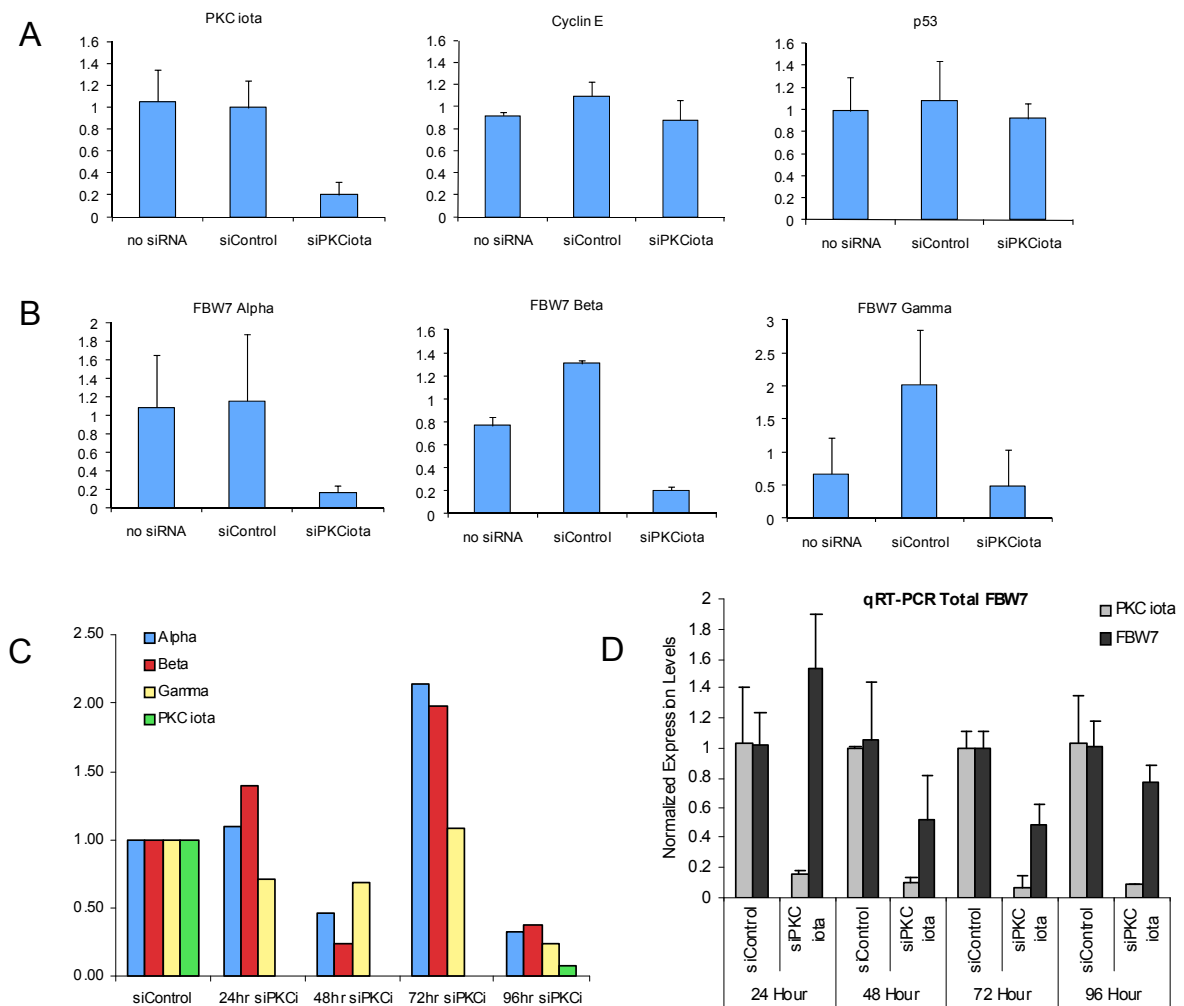


Figure 12: PKCιota Knockdown Does Not Result in Increased FBW7 Expression

(A,B) qRT-PCR was performed on IGROV cells with transiently silenced PKCιota (at 48 hours). No siRNA and siControl (scramble) were used as controls for the siPKCιota RNA sample. (A) Primers to PKCιota (positive control) and p53 and cyclin E (negative controls) were used to determine the ability to use these RNA samples for examination of FBW7 expression. (B) Primers to FBW7 alpha, beta, and gamma were used to examine the mRNA expression levels of these three transcripts at 48 hours. FBW7 alpha, beta, and gamma all appeared to have decreased expression at 48 hours. (C) qRT-PCR of FBW7 alpha, beta, and gamma in siControl and siPKCιota in IGROV cells over a 24 to 96 hour time frame. No trend in FBW7 alpha, beta, and gamma appeared. (D) qRT-PCR of FBW7-Total in siControl and siPKCιota in IGROV cells over a 24 to 96 hour time frame. No apparent or consistent trend in FBW7-Total was observed in response to PKCιota downregulation

RPPA Analysis of Ectopic PKC α Reveals Potential Pathways for Cyclin E Regulation

PKC α regulates the stability of cyclin E, both full length and LMW-E. However, this regulation is not through the transcriptional regulation of FBW7. Thus, potential pathways that would affect both PKC α and cyclin E would need to be identified. To identify several pathways simultaneously we used an RPPA (Reverse Phase Protein Array) assay with 293T cells transiently expressing PKC α and IGROV stably silenced shRNA samples. In utilizing a protein expression-based array such as the RPPA assay we are able to examine hundreds of protein expression levels in a high-throughput manner (Tibes, 2006). RPPA analysis was performed on the IGROV shRNA clones (first panel: 7 control [rows 1-7] and 7 shPKC α [rows 8-14]) as well as transiently expressed PKC α wildtype (Wt), constitutively active (CA), and dominant negative (DN) in 293T cells (second panel) (Figure 13a-293T and S6a-IGROV). To validate the change in PKC α protein levels in the two RPPA sets (293Ts and IGROVs), Western blot analysis was used (Figure 13b and S6b, respectively). Because of the increased passage of IGROV shRNA cells, cyclin E levels returned in the shPKC α clones, rendering these clones as potentially useless (Figure S6b). However, transient expression of PKC α both Wt and CA but not DN in 293T cells resulted in an increase in cyclin E (both cyclin EL and LMW-E levels) (Figure 13b). These 293T PKC α -transfected cell lysates yielded promising hits in their RPPA analysis (Figure 13c,d). PKC α overexpression resulted in a number of increased and decreased proteins within the PI3K pathway. In Figure 13c, the antibodies in bold print indicate a greater possibility of a valid hit. However, these antibodies would still need to be validated by Western blot analysis. Figure S6c shows the graphs of the potential increased and decreased protein levels in response to PKC α overexpression in 293T cells. The black titles indicate an increase in expression, while the red titles indicate a decrease in expression. Of note, MEK/phospho-MEK and the PI3K pathway returned several positive hits in the RPPA analysis of PKC α -overexpressing 293T cells (Figure 13d). All proteins that are highlighted in green increased in response to PKC α Wt and CA expression, while proteins highlighted in red decreased. As indicated by the affected proteins, translation (decreased S6 and increased GSK3) and apoptosis (decreased Caspase 9) should be inhibited, while anchorage-independent growth (increased cyclin E) and proliferation (increased c-myc, STAT3, and cyclin E) should be increased. Thus, MEK and PI3K pathways, which are known factors that influence or are influenced by PKC α , will be examined in cyclin E regulation.

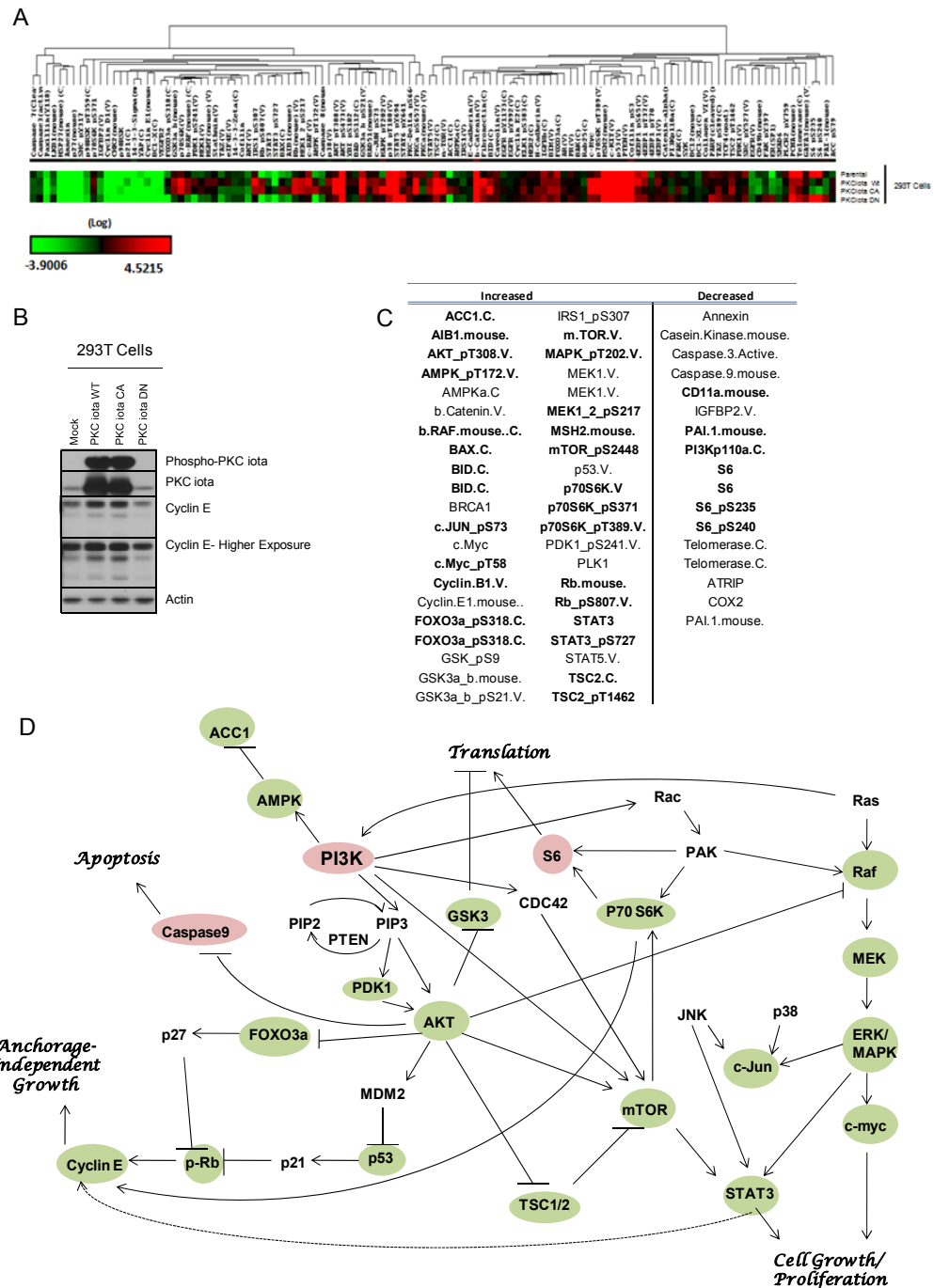


Figure 13: RPPA Analysis of 293T Cells with PKCiot Overexpression

(A) Reverse Phase Protein Array (RPPA) heatmap for 293T samples analyzed. 293T samples transiently transfected with PKCiot wildtype (Wt), constitutively active (CA), or dominant negative (DN) were analyzed. (B) Western blot analysis depicting the 293T transiently transfected cells used in the RPPA assay in part (A). Antibodies against phospho-PKCiot, PKCiot and cyclin E were used, while actin was used as a loading control. (C) List of proteins whose expression levels either increased or decreased in the 293T-PKCiot Wt and CA samples but not the control or PKCiot DN samples. Bold letters indicate higher possibility of a true effect. (D) Schematic representation of the PI3K pathway. Green circles represent potential upregulated proteins (or phosphorylation events, see part (C) for details) in the PKCiot Wt and CA samples of the RPPA assay, while the red circles represent potential downregulated proteins.

Regulation of Cyclin E by PKC α is MEK-Independent but PI3K-Dependent

The RPPA assay of PKC α overexpression in 293T cells revealed several potential factors that could provide the mechanism of cyclin E inhibition as highlighted in the pathway that RPPA proteins target (Figure 13d). One of the downstream targets identified in the RPPA screen was MEK. Of note, PKC α has been shown to activate MEK1/2 through the: PKC α → Rac1 → Pak → MEK1/2 → ERK1/2 pathway (resulting in transformation) (Zhang, 2004; Regala, 2005; Regala, 2008; Justilien, 2009; Scotti, 2010). However, regulation of MEK has also been shown to be PKC α -independent (Kampfer, 1998; Hellbert, 2000). Similarly, in IGROV cells, PKC α knockdown did not reveal any relevant pattern of phospho-ERK expression (data not shown). However, Ras has been shown to regulate cyclin E degradation in an FBW7 and MEK-dependent, but PI3K independent manner in HELA and NIH3T3 cells with ectopic expression of cyclin E/CDK2, FBW7, RasV12, and MEK (Minella, 2005). To examine the effect of MEK on cyclin E expression, the MEK inhibitor, U0126, was used on IGROV cells, and the resulting levels of cyclin E were analyzed by Western blot analysis. Because MEK is likely to be downstream of PKC α , cyclin E levels should decrease without further modulating PKC α or phospho-PKC α . To examine the effect of the MEK inhibitor, U0126, on cyclin E expression, IGROV parental cells were serum starved for 18 hours followed by addition of serum and U0126 in the presence or absence of EGF (Figure 14a). While cyclin E levels decreased in the presence of U0126, so did the levels of both total and phospho-PKC α . Figure 14b gives the quantitative densitometric analysis of Figure 14a. Because the levels of PKC α were altered in response to MEK inhibition, we were unable to conclude that PKC α knockdown was MEK-dependent. One possible explanation is that inhibition of MEK could possibly be causing the decrease in cyclin E through the decrease in PKC α . Also, because PKC α knockdown resulted in no obvious pattern of phospho-ERK expression, we conclude that MEK is likely not the PKC α effector molecule responsible for cyclin E regulation.

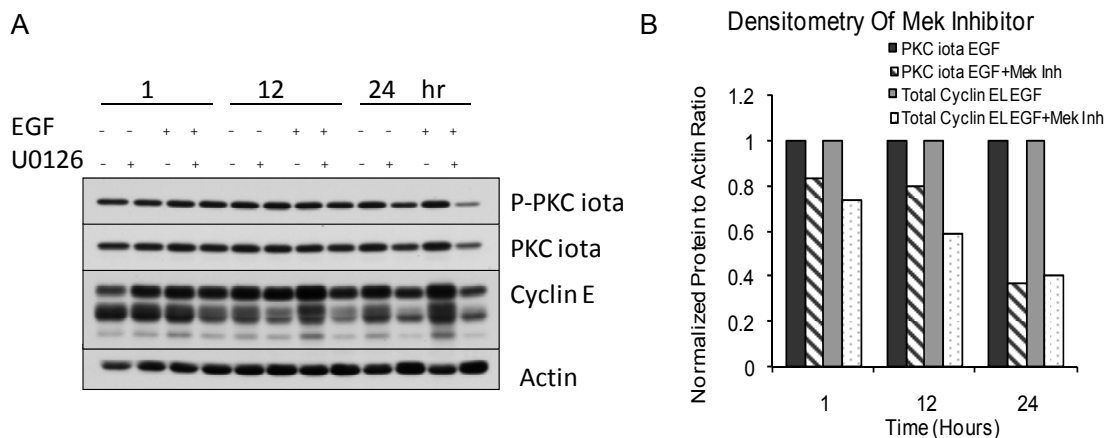


Figure 14: Inhibition of MEK Decreases both Cyclin E and PKC ι Protein Levels
 (A) IGROV cells were treated with the MEK inhibitor, U0126, for 1, 12, and 24 hours in the presence or absence of EGF. Western blot analysis was performed using antibodies against phospho-PKC ι , PKC ι , cyclin E, and actin (loading control). Both PKC ι and cyclin E levels were quantitated against actin levels using densitometric analysis (B). Expression levels of PKC ι and total cyclin E were normalized to the untreated control expression level of each group. Both PKC ι and total cyclin E levels decreased in response to U0126 regardless of the presence of EGF.

PKC α is also downstream of PI3K-PDK1 (Chou, 1998; Akimoto, 1998; Standaert, 1999; Kanoh, 2003). It is possible that PI3K inhibition would result in a decrease in phospho-PKC α levels, leading to a decrease in cyclin E expression. To examine the effect of PI3K inhibitors on cyclin E expression, the inhibitors GDC-0941 and PI-103 were used to treat 293T parental cells followed by Western blot analysis. The PI3K inhibitors, GDC-0941 and PI-103, were used at increasing doses to determine their effect on phospho-PKC α compared to PKC α (Figure 15a). While the active form of PKC α (phospho-PKC α) decreased in response to PI3K inhibitors, steady-state levels of PKC α remained unaffected. Thus, PKC α signaling is inhibited by the inhibition of PKC α . To test the effect of PI3K inhibition on cyclin E levels, these two PI3K inhibitors were used to treat 293T cells based on the concentrations tested in Figure 15a (Figure 15b). Both PI3K inhibitors resulted in decreased phospho-PKC α and cyclin E (cyclin EL and LMW-E) levels. This decrease in cyclin E was quantitated by densitometric analysis (Figure 15c). Both inhibitors resulted in considerable decreases in cyclin E expression. Thus, we conclude that inhibition of PI3K results in decreased phospho-PKC α leading to decreased cyclin E expression.

Therefore, we have shown that PKC α is upstream of cyclin E. More specifically, PKC α knockdown results in decreased cyclin E stability as opposed to decreased transcriptional regulation. The regulation of cyclin E by PKC α is downstream of PI3K (Figure 16). Thus, PI3K or PKC α inhibitors could clinically provide a feasible target for inhibition of several oncogenic pathways simultaneously.

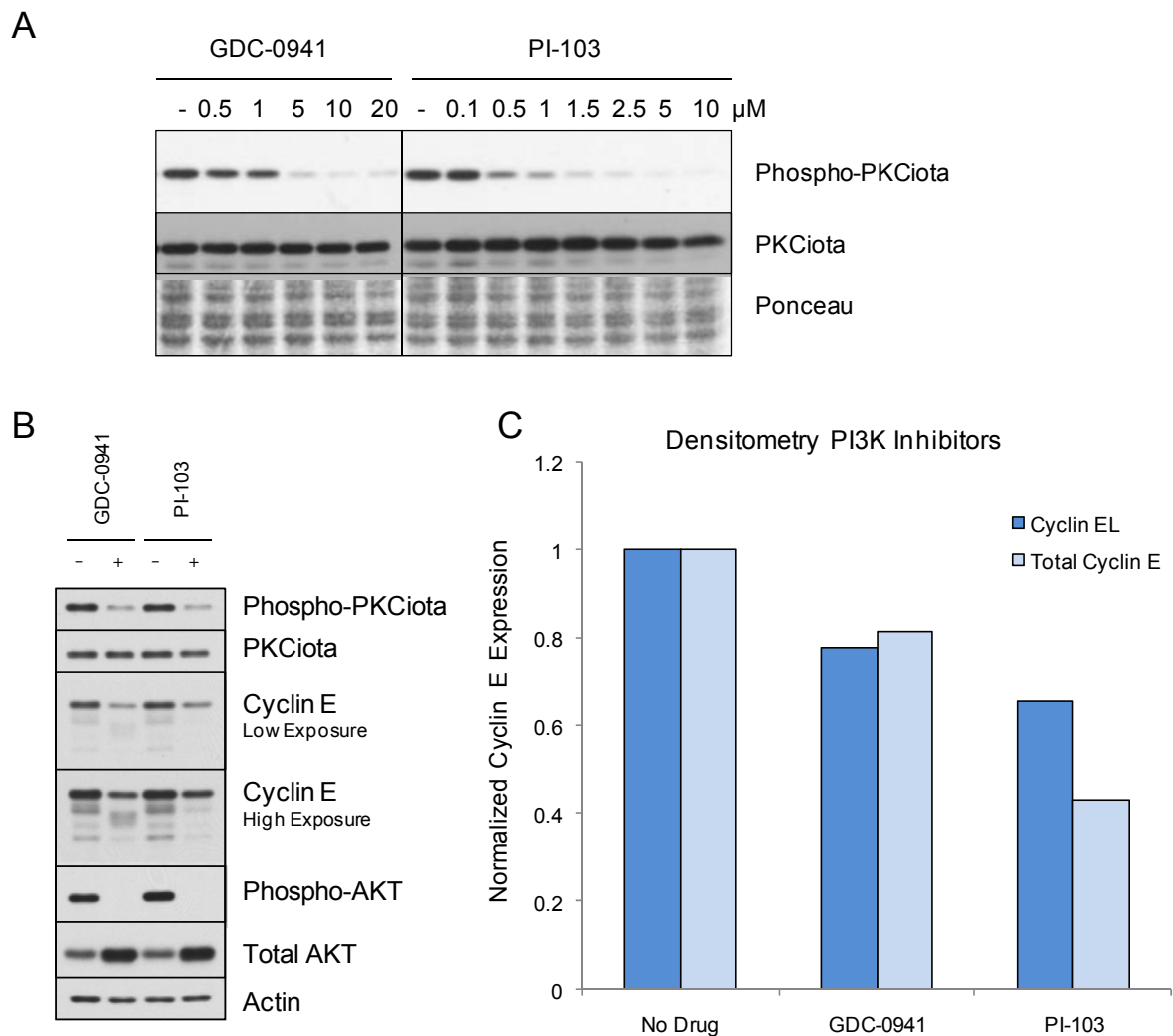


Figure 15: Inhibition of PI3K Decreases Cyclin E Expression

(A) 293T cells were exposed to PI3K inhibitors, GDC-0941 and PI-103, at increasing concentrations. Western blot analysis was used to examine changes in phospho-PKCiota and PKCiota levels. Ponceau stain was used as a loading control. (B) Based on the concentration of GDC-0941 and PI-103 from part (A), these two PI3K inhibitors were used on 293T cells and cyclin E expression was measured by Western blot analysis. Antibodies to phospho-AKT and AKT were used to validate the effectiveness of the GDC-0941 and PI-103. Actin was used as the loading control. The cyclin E (both cyclin EL and total cyclin E) levels were quantitated by densitometric analysis using the untreated counterpart as the control (C). Drug-treated sample values were normalized to the control untreated sample then normalized to actin levels. Error bars represent standard error.

CHAPTER 2: DISCUSSION

In this chapter, we demonstrated that PKC α can regulate cyclin E expression, both the full length form (cyclin EL) and the low molecular weight isoforms (LMW-E), in several model systems through both overexpression and targeted knockdown of PKC α . However, changes in cyclin E (both cyclin EL and LMW-E) in several cell lines under both transient and stable conditions were unable to alter the expression or activation of PKC α . Thus, no feedback loop between cyclin E and PKC α exists. PKC α and cyclin E are both amplified and overexpressed in epithelial ovarian cancer and are designated as oncogenes based on their ability to drive ovarian cancer tumorigenesis (Bast, 2009). These two oncogenes had also previously been shown to correlate in human ovarian cancer, and a direct effect of PKC α on both cyclin E and proliferation had been demonstrated in an *in vivo* drosophila eye model (Eder, 2005).

While PKC α is able to transcriptionally regulate cyclin D1 (Hellbert, 2000; Kampfer, 2001), we have shown that cyclin E is regulated in a different manner by PKC α than cyclin D1. We found that PKC α regulates cyclin E stability instead of its synthesis. Of note, in 1995, Minella et al found that cyclin E stability was regulated by Ras and was dependent on MEK (Minella, 2005). Using an RPPA assay, which measures protein expression, we identified MEK as being upregulated in response to PKC α overexpression, even though the increase was moderate at best. Using a MEK-inhibitor (U0126), we did indeed decrease cyclin E levels. However, U0126-treatment also resulted in decreased phospho-PKC α and total PKC α levels, providing another role for MEK. One new role for MEK, that my study has provided, is the possibility that it can modulate cyclin E's protein levels directly. This seemed plausible, due to the findings of Minella et al that degradation of cyclin E occurred through MEK (Minella, 2005). However, in the Minella study, the MEK inhibitor was only used with exogenous expression of cyclin E and CDK2 along with exogenous expression of RasV12, FBW7, MEK, or a combination of these proteins. Alternatively, based on the results from my study, it is possible that the effect of the MEK inhibitor on PKC α lead to an indirect effect on cyclin E through a decrease in PKC α protein levels. Either way, the effect of U0126 on cyclin E was nowhere near as pronounced as the effect that direct downregulation of PKC α had on cyclin E levels. Also, by examining phospho-ERK (which is phosphorylated in response to MEK) by Western blot analysis in IGROV PKC α knockdown cells, no pattern in phospho-ERK

expression was identified. Collectively, these findings led us to conclude that regulation of cyclin E by PKC α is likely not through MEK.

In the publication by Minella et al, the PI3K inhibitor, LY294002 was used to demonstrate that cyclin E regulation by Ras was not in the PI3K pathway (Minella, 2005). Similarly, when we used the same approach and treated cells with LY294002, we found no effect on cyclin E levels as measured by Western blot analysis (data not shown). However, using two alternative PI3K inhibitors, GDC-0941 and PI-103, we found that only the phospho-form of PKC α changed (total PKC α remained unchanged) in response to PI3K inhibition, but at higher concentrations, cyclin E levels also decreased. Thus, the PI3K pathway has implications in the regulation of cyclin E through PKC α .

The PI3K catalytic subunit gene, *PIK3CA*, is located on chromosome 3q26.3. This loci is important because, in addition to housing the PKC α gene (*PRKCI*), this loci is also the site of the most frequently amplified genomic amplicon in ovarian cancer (Suzuki, 2000). While PKC α is overexpressed in nearly 80% of epithelial ovarian cancer and mislocalized in 100% of serous epithelial ovarian cancer, the PI3K pathway is overexpressed in 70% of epithelial ovarian cancers (Bast, 2009; Eder, 2005; Bast, 2008). This activation of the PI3K pathway is not only through the genomic amplification of *PIK3CA*, but also through other mutations, including activating mutations within *PIK3CA* and inactivating mutations in *PTEN*, the tumor suppressor gene which inhibits PI3K activity (Bast, 2009). Interestingly, in xenograft mouse models, PI3K inhibitors are able to inhibit ovarian cancer proliferation, and these inhibitors are entering Phase I and II clinical trials (Hu, 2002; Raynaud, 2007). Thus, at least part of the potential mechanism of PI3K inhibitors *in vivo* could be through inhibition of PKC α resulting in decreased cyclin E levels, and thus less uncontrolled tumor cell proliferation. In the next chapter, we will be discussing the vital role of cyclin E in PKC α -induced transformation, which could also be part of the mechanism of PI3K inhibitor-induced growth arrest in mouse xenograft models.

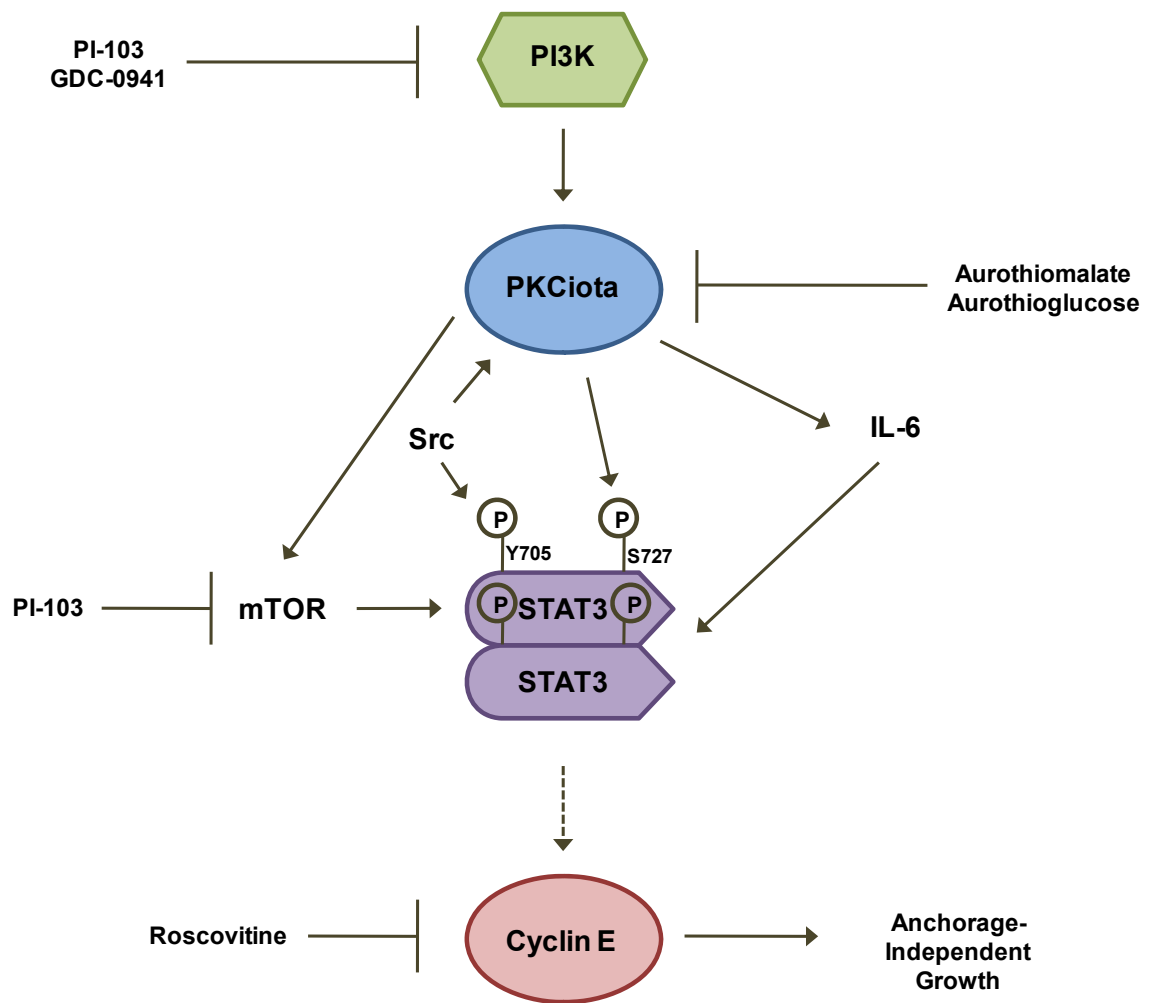


Figure 16: PKCιota Regulation of Cyclin E through PI3K

Schematic representation of the regulation of cyclin E by PI3K, possibly through mTOR and the phosphorylation of STAT3. PI-103 and GDC-0941 are PI3K inhibitors (PI-103 has off target effects on mTOR). Roscovitine is a CDK2-inhibitor.

As mentioned, the inhibition of PKC α phosphorylation by the two PI3K inhibitors (in Figure 15) was at low concentrations, while the effect on cyclin E was not until the higher concentrations. In fact, with GDC-0941, only the highest used concentration of this drug (20 μ M) resulted in decreases in cyclin E levels. PI-103 however, exhibited a dose-dependent decrease in cyclin E levels starting at 1.5 μ M and increasing until the final used concentration of 10 μ M. PI-103, while it is a PI3K inhibitor, also inhibits mTOR at similar IC₅₀ values to those of PI3K. mTOR is also downstream of PI3K, and according to our RPPA assay with overexpressed PKC α , mTOR levels are increased 3-fold in 293T cells overexpressing PKC α wildtype (Wt) and constitutively active (CA) but not dominant negative (DN) mutant constructs (Figure 13d and 16). Furthermore, the MEK inhibitor used in Figure 14, U0126, has the ability to decrease mTOR signaling, which could potentially account for the changes in cyclin E levels seen upon U0126 treatment (Fukazawa, 2000). Thus, mTOR could potentially be part of the mechanism of regulation of cyclin E by PI3K/PKC α (Figure 16).

STAT3 is another potential effector molecule linking PI3K, PKC α , and cyclin E. STAT3, which is upregulated by both IL6 and mTOR (Zhou, 2007; Fujitani, 1994), was also identified in our RPPA screen as being upregulated by 2-fold in PKC α Wt and CA-overexpressing cells. Phospho-STAT3 (Ser727) was found to be increased by 3-fold in PKC α Wt cells and nearly 6-fold in PKC α CA cells. In addition to tyrosine phosphorylation at Tyr705 (by Src or TYK2), phosphorylation of Ser727 is also required for the transcriptional activation by STAT3 (Wen, 1995). Activation of STAT3 by PKC α is nothing novel. Two recent studies have shown that PKC α can cause STAT3 activation/ phosphorylation. (1) In human chondrocytes, PKC α contributes to inflammatory joint disease through the pathway: IL1 \rightarrow PKC α \rightarrow STAT3 phosphorylation (Litherland, 2010). (2) In prostate cancer cells, PKC α caused IL6/STAT3 induction resulting in an autocrine response loop. Specifically, PKC α knockdown by RNAi resulted in suppression of both STAT3 phosphorylation as well as IL6 expression (Ishiguro, 2009). Thus, it is highly possible that PKC α could regulate cyclin E through a PI3K \rightarrow PKC α \rightarrow mTOR/STAT3 \rightarrow cyclin E pathway (Figure 16).

In addition to STAT3 being downstream of PKC α , STAT3 has also been demonstrated to be upstream of cyclin E regulation, providing the potential link between PKC α and cyclin E. STAT3 has been shown to lead to the G1/S transition at least in part due to its regulation of cyclin E levels/ activity. As for cyclin E/CDK2 activity, when STAT3 is downregulated, it can result in decreased cyclin E activity through upregulation of the CKIs,

p21 and p27 (Fukada, 1998). In our study, upon PKC α knockdown, we observed a decrease in p27 levels, which can result in decreased cyclin E activity. Several studies have shown cyclin E levels decrease in response to STAT3 inhibition. (1) Cyclin E levels decreased in response to mutant dominant negative STAT3 expression (STAT3D) (Odajima, 2000). (2) In another study, siRNA to STAT3 resulted in decreased cyclin E levels in pancreatic cancer cells (Bharadwaj, 2008). (3) Similarly, in HCT116 and SW480 cells, upon addition of anti-sense STAT3, CDKs 2/4 and 6, and cyclins D1 and E levels all decreased (Ma, 2003). In our own study, we found that upon PKC α knockdown, CDKs 2 and 4 as well as cyclins D1 and E decreased (Figures 5 and 6). While we did not examine the effect of STAT3 knockdown on cyclin E levels, the literature would suggest that decreased STAT3 phosphorylation by PKC α knockdown could be the reason for decreased cyclin E levels. Thus, inhibition of STAT3 by PKC α knockdown could potentially account for several of the effects we observed on the cell cycle regulators, including the effect we observed in the decrease of cyclin E levels.

Therefore, in this chapter, we have demonstrated that PI3K, PKC α , and cyclin E, most likely through mTOR/ STAT3 activation, provide a novel ovarian cancer pathway to be targeted therapeutically. In serous epithelial ovarian cancer, because PKC α is always mislocalized or overexpressed (Eder, 2005), ovarian cancer patients with this cancer subtype would be most likely to benefit from PKC α inhibition. For non-serous epithelial ovarian cancer, screening these patients based on amplification of chromosome 3q26.3, the loci for the genes that encode PI3K and PKC α , would be a means for identifying the subpopulation of patients with non-serous epithelial ovarian cancer who would be most likely to benefit from PI3K or PKC α -targeted therapies via this novel cancer pathway.

CHAPTER 3:

The Functional Relevance of the PKC α / Cyclin E Pathway

CHAPTER 3: INTRODUCTION

Ovarian cancer is the leading cause of cancer-related death among the gynecologic malignancies (CF&F, 2010). While little progress has been made in the way of targeted therapeutics for ovarian cancer treatment, several potential targets have been identified, including cyclin E and PKC α , which are both genomically amplified and overexpressed in ovarian cancer (Courjal, 1996; Marone, 1998; Eder, 2005; Bast, 2009). Because we have shown that PKC α regulates cyclin E levels (Chapter 2), we examined which important functions of PKC α require cyclin E. Also, because cyclin E is an essential regulator of the cell cycle, we examined the effect of PKC α on the cell cycle. Identification of the PKC α -cyclin E functions, allows for stratification of ovarian cancer patients into those who would be most likely to benefit from PKC α -targeted therapies and those who would not.

PKC α , an atypical member of the protein kinase C (PKC) family of serine/threonine kinases, is a bona fide oncogene in ovarian cancer (Zhang, 2006). PKC α is involved in many diverse functions, such as proliferation, migration, transformation, and establishment and maintenance of apical-basal polarity (Weichert, 2003; Noda, 2001; Coghlan, 2000; Eder, 2005). The mechanism by which PKC α overexpression is able to disrupt epithelial cell polarity is through upregulation of cyclin E, which in turn leads to increased proliferation (Eder, 2005). PKC α and cyclin E expression correlate in high grade serous epithelial tumors, which exhibit a poor prognosis (Eder, 2005). Thus, it seems feasible that the other functions of PKC α could potentially require cyclin E.

Cyclin E, through binding to its catalytic binding partner CDK2, is essential for the G1 to S-phase transition (Ohtsubo, 1993; Ohtsubo, 1995; Resnitzky, 1994). Improper regulation of cyclin E can result in increased proliferation and transformation, which can lead to increased tumorigenesis (Resnitzky, 1994; Fang, 1996; Kang, 1996). In ovarian cancer, cyclin E is genomically amplified and overexpressed (Courjal, 1996; Marone, 1998) and can be cleaved post-translationally into hyperactive low molecular weight isoforms (LMW-E) (Bedrosian, 2004). LMW-E can lead to increased tumorigenesis through increased proliferation, increased cyclin E-dependent CDK2 kinase activity, resistance to p21/p27, and induction of genomic instability (Bedrosian, 2004; Wingate, 2005; Porter, 2001; Akli, 2004; Corin, 2006; Wingate, 2003; Bagheri-Yarmand, 2010; Bagheri-Yarmand, 2010). However, PKC α knockdown results in decreased cyclin E stability (both full length cyclin E and LMW-E) (Chapter 2).

In this chapter, we examined the functional relationship between PKC α and cyclin E in serous epithelial ovarian cancer. We found that PKC α knockdown results in a decrease

number of proliferating cells through a G1 arrest as opposed to increased cell death. In 3D-matrigel, downregulation of PKC ϵ in IGROV serous ovarian cancer cells resulted in a return to an apical-basal polarized-like acinus structure, complete with upregulation of E-cadherin and $\alpha 6$ integrin. However, this result was likely cyclin E-independent, due to the inability of endogenous cyclin E reversion to rescue the phenotype. We found that decreases in cyclin E and cyclin E-associated kinase activity were both necessary and sufficient in causing a PKC ϵ -knockdown-mediated decrease in anchorage-independent growth. Lastly, we found that PKC ϵ knockdown-induced decreased migration was independent of cyclin E. Summarized, either PKC ϵ -or cyclin E- targeted therapies could provide a means of inhibiting anchorage-independent growth in serous epithelial ovarian tumors.

CHAPTER 3: METHODS

Methods are in the order of appearance in this chapter.

Tissue Culture/ Generation of Stable Clones. See Chapter 2

MTT Assay.

Using a 96-well plate (tissue culture-treated, flat-bottomed plate- Costar 3595), 5,000 cells/200 μ L of cell suspension was added to each well (Day 1). After 24hrs post plating (Day 1), cells were treated with drug (either ATM-aurothiomalate, ATG-aurothioglucose, or Oncrasin-1) at varying concentrations. This drug plus induction was incubated at 37°C for 3 days (until Day 5). At day 5, the cells were immediately harvested by addition of 50 μ L of 2.5mg/mL MTT reagent (Sigma M-5655- Methylthiazolyldiphenyl-tetrazolium bromide) in serum-free media per well. The plates were then sealed in foil and incubated at 37°C for 4 hours. Post-incubation, these 96-well plates were then aspirated, and 100 μ L solubilization solution (0.04N HCL, 1%SDS, in isopropyl alcohol) was added to each well. Plates were then lightly agitated at room temperature for 2-3 hours. After solubilization occurred, these plates were read at 590nm by the Perkin Elmer- Wallac- Victor3 1420 multilabel counter. Results were analyzed using Microsoft Excel.

Western Blot Analysis. See Chapter 2

Primary Antibodies. For a complete list of antibodies used, see Chapter 2, plus this antibody: E-cadherin (BD Transduction Labs 1:250 in Blotto)

Growth Curves/ Doubling Time Analysis.

All cell lines analyzed, see results for details, were plated in triplicate at 25,000 cells per well (6-well plates-Costar) on day 0. Cell numbers were counted every 48 hours for eight days using a Coulter counter. Cell numbers were averaged and analyzed in Microsoft Excel. Doubling times were determined using the results of the growth curves according to the formula $DB = (0.301 \times \Delta T) / (\log_{10} [N/N_0])$.

FACS Cell Cycle Analysis.

Cells were plated in duplicate at 0.5×10^6 cells per plate on day 1. At day 2, fresh media was added. At day 3, cells were trypsinized and washed with cold PBS two times. Cell pellet was resuspended in 1.5 mL cold PBS by vortexing while PBS was added drop wise. 3.5 mL of ice

cold 200 proof EtOH was then added drop wise. Cells were then fixed overnight at 4°C. The PBS:EtOH solution was removed and fixed cells were washed with cold PBS twice. Cell pellet was then resuspended to an approximate concentration 1×10^6 cells/ mL in PPR (40 mL PBTB; 400 μ L 1 mg/mL PI- propidium iodide; 80 μ L of 10 mg/mL RNAase A). PBTB (0.5% Tween 20 and 0.5% BSA in PBS). Cell:PPR mixture was wrapped in foil and incubated at 37°C for 1 hour before being analyzed by a flow cytometer (BD Facscalibur) in the MD Anderson core facility. Readouts were analyzed by Microsoft Excel.

Apoptosis Analysis.

SubG1 Population. SubG1 population was determined by use of FACS samples- above. When the cell cycle distribution was determined by flow cytometry, the subG1 fraction was noted and analyzed using Microsoft Excel.

TUNEL Assay. All samples were prepared/ fixed as with the FACS analysis- see above. The percent TUNEL positive cells were established using a TUNEL assay kit (APO-BRDU Kit- BD Pharmingen) as per the manufacturer's instruction. Positive and negative controls were provided in the kit. Percent positive TUNEL cells were determined using a flow cytometer (BD Facscalibur) in the MD Anderson core facility. Readouts were analyzed by Microsoft Excel.

3D Culture- Plating Cells in Matrigel.

Growth Factor Reduced (GFR) Matrigel (BD Biosciences Cat # 356231) was thawed on ice for one hour and plated evenly at 40 μ L per chamber on 8-chamber slides (Nunc) being careful not to cause air bubbles. Slides were then incubated at 37°C for 15 minutes to allow for polymerization. Pre-trypsinized cells were rinsed one time with fresh media before being plated at 2500 cells per chamber in 400 μ L of media and 2% matrigel. Media used was Alpha Complete Media (HyQ MEM alpha-modification cell culture medium (HyClone) which was supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, non-essential amino acids, 2 mM L-glutamine, sodium pyruvate, hydrocortisone, and 10 μ g/mL Cipro). Media (plus 2% matrigel) was changed every 4 days for 14 days.

3D Culture- Staining/ Confocal Microscopy.

All solutions were filter sterilized before use in staining.

Fixation: Media (with 2% matrigel) was aspirated from chamber slides. Residual matrigel/cells were rinsed with cold sterile PBS three times (5 minutes each wash) and fixed with 4% paraformaldehyde (4% PFA in PBS- freshly prepared) for ten minutes at room temperature.

Slides were then washed with cold sterile PBS three times (5 minutes each).

Permeabilization: Methanol (100%) was then added to the slides for 10 minutes. PBS containing 0.5% Triton X-100 was added to the slides for 10 minutes at room temperature.

Slides were then washed with cold sterile PBS three times (5 minutes each).

Blocking Solution: Add blocking solution (3% bovine serum albumin- BSA and 1% horse serum in PBS) and incubate 1 to 2 hours at room temperature. Slides were then washed with cold sterile PBS three times (5 minutes each).

Primary Antibody: Primary antibody (1:1000 to 1:5000 in blocking buffer) was added to the slides and incubated overnight at 4°C. To prevent contamination, slides were wrapped in parafilm before 4°C incubation. Slides were then washed with cold sterile PBS three times (5 minutes each). *Primary Antibodies Used:* Primary antibodies used were PKC α (BD Transduction Laboratories- 610176), cyclin E (HE-12, Santa Cruz Biotechnology), Phospho-PKC α (Abcam: Phospho T555 + T563)

Secondary Antibody: Fluorescent conjugated secondary antibody (1:2000- Alexa-conjugated; Molecular Probes) was added to the slides and incubated at room temperature in the dark for 1 hour. Slides were then washed with cold sterile PBS three times (5 minutes each). Slides were then stained with DAPI (4',6-diamidino-2-phenylindole) at 0.5 ng/mL (5 μ L in 2 mL of PBS) for 15 minutes in the dark at room temperature. Slides were then washed with cold sterile PBS three times (5 minutes each). The chambers were then removed from the slides and a cover slip was mounted with mounting solution (Dako). After the cover slip was allowed to dry (approximately 15 minutes) slides were sealed with nail hardener and placed in the cold room until able to be read. Confocal microscopy was performed using the Olympus DSU spinning disc confocal fluorescent microscope.

Soft Agar Colony Formation.

Base Agar. Make 2.4% Noble agar (Difco) in autoclaved water. Heat to melt agar in the microwave. Combine pre-warmed (37°C) complete media with 2.4% Noble agar at an 80:20 ratio. Immediately add 1 mL/ per plate (35mm plates- gridded). Allow to cool while preparing the top agar and cells.

Top Agar. Combine 5.8 mL pre-warmed completely media with 1mL 2.4% Noble agar (from above) and 1 mL of 1×10^5 cells/mL. Immediately add 1mL of the cells: agar mixture on top of the base agar plates. Incubate at 37°C/ 6.5% CO₂ for 14 days.

Analysis. Count gridded cells completely both horizontally and vertically. Analyzed cell counts using Microsoft Excel.

Infection with Ad-Cyclin E. See Chapter 2.

Wound Heal Assay.

In 6-well plates, cells were cultured to 90% confluency. Cells were then serum starved overnight. Using a black marker, a reference line was drawn on the bottom of each well. Using a sterile p200 pipet tip, 3 scratches were made perpendicular to the reference line. Cells were then rinsed and 1.5 mL fresh complete media was added. Pictures were taken at 10X zoom on an inverted microscope at 0 hours and then every 6-12 hours depending on the experiment. Fresh media was added before each picture. The same scratch was measured for each condition. Pictures were printed and measured with a ruler. The distance of wound closure at each time point was documented and total numbers were analyzed using Microsoft Excel.

CHAPTER 3: RESULTS

PKC ϵ Alters Proliferation through Cyclin E and Changes in Cell Cycle Distribution

PKC ϵ has been implicated in the proliferation of ovarian cancer (Weichert, 2003). In a drosophila *in vivo* model, overexpression of PKC ϵ resulted in increased proliferation as determined by BrdU incorporation, most likely due to a subsequent increase in the cell cycle protein, cyclin E. Also, in tissue microarrays, PKC ϵ exhibited a significant positive correlation with cyclin E ($p = 0.01$) and proliferation as determined by Ki67 levels ($p = 0.02$) (Eder, 2005).

In Chapter 2, we demonstrated that knockdown of PKC ϵ caused a decrease in cyclin E protein levels. Because PKC ϵ is involved in cyclin E regulation, we expect proliferation to decrease in response to PKC ϵ inhibition in ovarian cancer cells. To test the need for cyclin E inhibition as the mechanism of decreased proliferation by inhibition of PKC ϵ , we used the PKC ϵ inhibitors, aurothiomalate (ATM), aurothioglucose (ATG), and oncrasin, in IGROV and OVCAR3 cells. IGROV cells were chosen because stable knockdown of PKC ϵ resulted in decreased cyclin E levels (Chapter 2: Figure 7 and 8). Likewise, OVCAR3 cells were chosen because stable knockdown of PKC ϵ did *not* result in alterations of cyclin E levels (Chapter 2: Figure 9c). The PKC ϵ inhibitors, ATG and ATM, did not alter the rate of proliferation in the OVCAR3 cells (Figures 1a and 1b, respectively). This result was not surprising, given the inability of PKC ϵ knockdown to change cyclin E levels. In OVCAR3 cells, ATG and ATM did not alter the expression of phospho-PKC ϵ , PKC ϵ , or cyclin E (Figure 1c and 1d). While PKC ϵ inhibition in OVCAR3 cells did not alter the proliferation rate, in IGROV cells, both ATM and the Ras/PKC ϵ inhibitor oncrasin (Guo, 2008; Guo, 2009) were able to decrease proliferation (Figure 1e and 1f). However, phospho-PKC ϵ and cyclin E levels did not decrease in response to these two inhibitors (Figure 1g). Thus, PKC ϵ inhibition results in a decrease in proliferation in IGROV cells but not OVCAR3 cells. This decrease in proliferation in IGROV cells is seen without detectable changes in phospho-PKC ϵ or cyclin E. The lack of changes in protein levels in IGROV cells treated with ATM and oncrasin can be explained by the ability of the proliferating cells to upregulate either alternative pathways or alter regulation within the PKC ϵ /cyclin E pathway, in response to PKC ϵ inhibition. This effect was observed in IGROV cells previously, when PKC ϵ was stably silenced, after several passages, PKC ϵ remained silenced but cyclin E protein returned (Chapter 2; Figure 18b). Therefore, inhibition of PKC ϵ by small molecule inhibitors in IGROV cells resulted in a decrease in proliferation. However, as with any small molecule inhibitors, non-specific events

can occur. To rule out the possibility of the decrease in proliferation being non-specific, we would need to examine the direct effect of PKC ϵ knockdown on proliferation.

PKC ϵ inhibition by ATM and oncrasin resulted in a decrease in proliferation as determined by MTT assay. In order to exclude the possibility of non-specific targets mediating this effect on IGROV cell proliferation, we need to specifically target PKC ϵ and examine the effect on proliferation. To investigate the role of PKC ϵ in proliferation, we stably silenced PKC ϵ using shRNA and subjected these cells to growth curves in order to measure changes in doubling times. Also, to rule out the possibility of cell death being the cause of a decrease in cell number, TUNEL and FACS (subG1) analysis were performed to determine the percentage of apoptotic cells. In IGROV cells, when PKC ϵ was stably silenced, over time the number of cells declined compared to the control (Figure 2a-all clones and 2b-averages of clones). In fact, after eight days, the number of cells decreased from 3×10^6 cells to 2×10^6 , a 33% decrease upon PKC ϵ knockdown. This change in the rate of growth corresponded with a significant increase in doubling time ($p = 0.043$) (Figure 2c). Because the number of cells could be skewed by an apoptotic effect, the possibility of PKC ϵ knockdown-induced apoptosis needed to be excluded. No change was observed in the percentage of apoptotic cells when PKC ϵ was silenced (Figure 2f). Using both TUNEL and subG1 FACS analysis, no change was observed in the number of apoptotic cells in the shPKC ϵ clones compared to the shControl clones. Thus PKC ϵ is able to decrease proliferation in IGROV cells independent of cell death. This raises the question, do changes in PKC ϵ alter cell cycle distribution?

When PKC ϵ is stably silenced in IGROV cells, the proliferation rate decreases unaided by cell death. In chapter 2, we saw that the cell cycle proteins, CDK2/4 and cyclins D1/E decreased and p27 increased in response to PKC ϵ knockdown (Chapter 2; Figures 7a, 8). Given these two points, we hypothesized that changes in cell cycle proteins as a result of PKC ϵ knockdown would translate to a change in cell cycle distribution. To test this hypothesis, we used FACS analysis with PI staining to determine the percentage of cells (IGROV shPKC ϵ versus shControl clones) in the different phases of the cell cycle. Upon PKC ϵ knockdown, the percentage of cells present in the G1 phase increases with a corresponding decrease in the S-phase fraction (Figures 2d and 2e). The G2-phase fraction remains unaffected. Thus, PKC ϵ knockdown results in an apparent G1 arrest, which could account for the decrease in the proliferation rate. While proliferation in 2D cultures is an important first step, the need for more *in vivo*-like studies is essential for physiological

relevance. Thus, the effect of PKC ι knockdown in 3D cultures is vital, especially given PKC ι 's vital role in establishment and maintenance of apical-basal polarity.

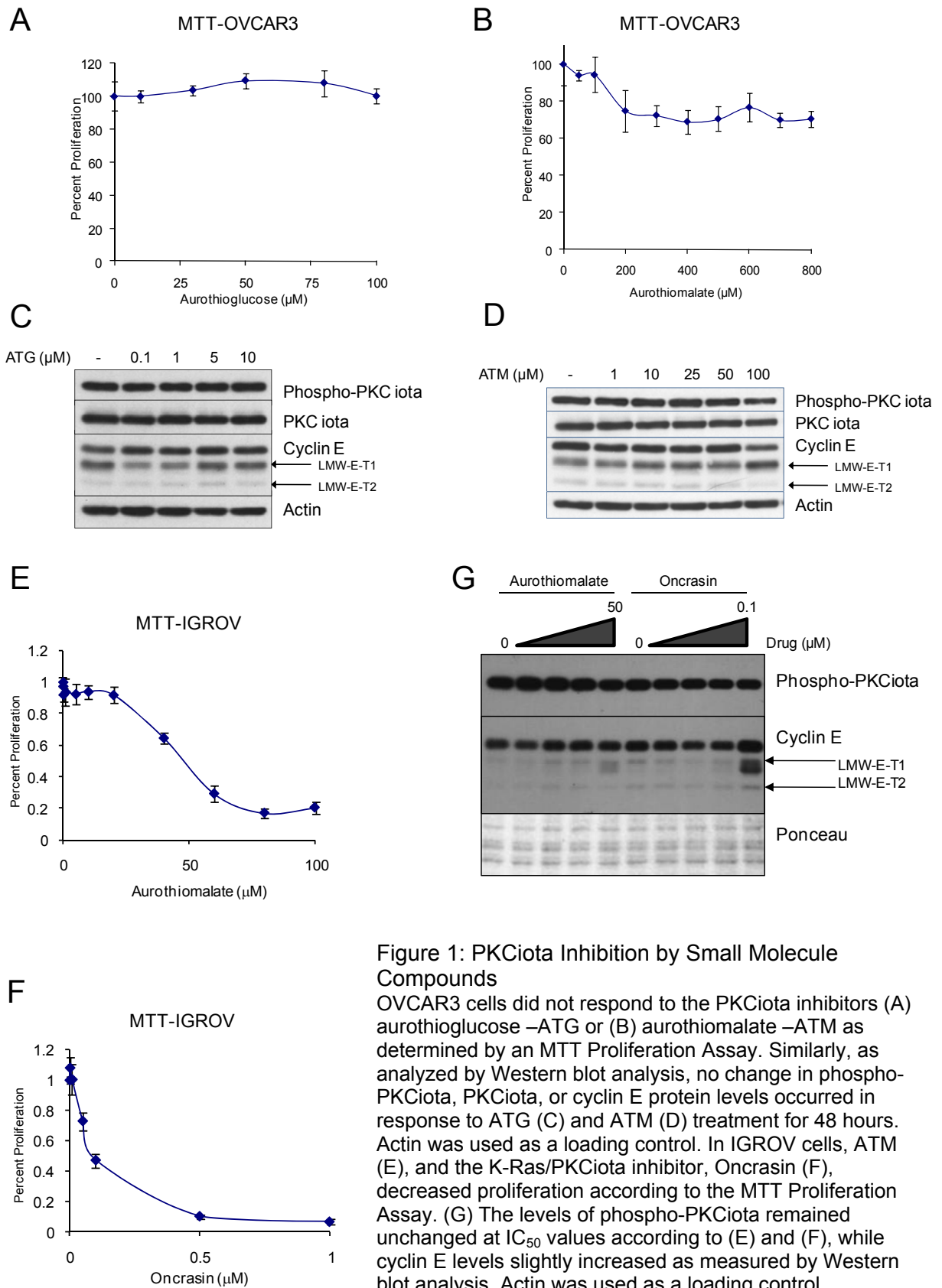


Figure 1: PKCiota Inhibition by Small Molecule Compounds

OVCAR3 cells did not respond to the PKCiota inhibitors (A) aurothioglucose –ATG or (B) aurothiomalate –ATM as determined by an MTT Proliferation Assay. Similarly, as analyzed by Western blot analysis, no change in phospho-PKCiota, PKCiota, or cyclin E protein levels occurred in response to ATG (C) and ATM (D) treatment for 48 hours. Actin was used as a loading control. In IGROV cells, ATM (E), and the K-Ras/PKC_{iota} inhibitor, Oncrasin (F), decreased proliferation according to the MTT Proliferation Assay. (G) The levels of phospho-PKCiota remained unchanged at IC₅₀ values according to (E) and (F), while cyclin E levels slightly increased as measured by Western blot analysis. Actin was used as a loading control.

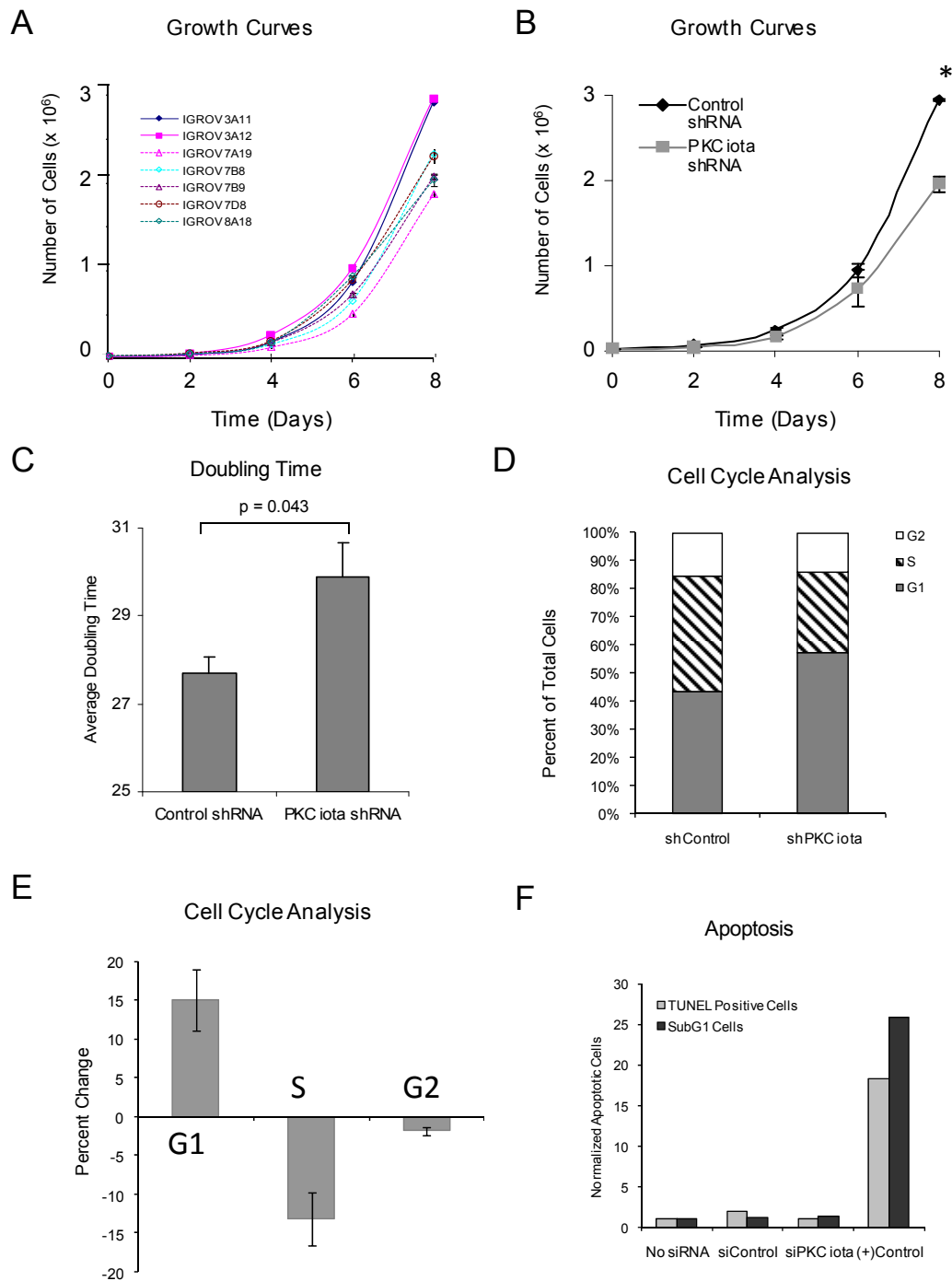


Figure 2: PKC ι ota Knockdown Leads to Decreased Proliferation Through Alterations in the Cell Cycle
 (A) Growth curves showing IGROV shControl clones (3A11, 3A12) and shPKC ι ota clones (7A19, 7B8, 7B9, 7D8, and 8A18). (B) Growth curves with the averages of the IGROV shControl and shPKC ι ota clones from (A). * $p = 0.01$. (C) Averages of the doubling times of the IGROV shControl and shPKC ι ota clones from part (A). $p = 0.043$. (D) FACS cell cycle analysis of IGROV shControl and shPKC ι ota clones. Changes in the phases of the cell cycle were measured and graphed with standard deviation (E). The G1 fraction increased, the S fraction decreased, and the G2 phase remained unchanged. (F) Apoptosis in the IGROV transient siRNA of PKC ι ota (versus control) was measured by both quantitation of the SubG1 fraction (by FACS) as well as TUNEL analysis. BD Biosciences 'positive control' cells were used as the positive control.

In 3D Matrigel PKC ϵ Expression is Indicative of a More Aggressive Phenotype

PKC ϵ , a component of the PKC ϵ /PAR-6/PAR-3 polarity complex, is mislocalized or overexpressed in serous epithelial ovarian cancer (Eder, 2005). We hypothesized that PKC ϵ knockdown could revert the tumor cells in 3D matrigel (*in vivo*-like culture) to a pre-tumorigenic like structure. To test this hypothesis, we examined cell lines with different PKC ϵ expression levels in both 2D and 3D culture conditions. Then, we used IGROV serous epithelial cells with stably silenced PKC ϵ to examine the role of PKC ϵ in the structure/ size of 3D acini. Using cells with low (HeyA8; 59M) or high (OVCAR3; IGROV) PKC ϵ / cyclin E levels as determined by Western blot analysis in Chapter 2 (Chapter 2; Figure 1b and 1c), we plated these cells in 3D matrigel to examine the resulting phenotype (Figure 3a). In 3D matrigel, ovarian cancer cells with low PKC ϵ /cyclin E levels formed either a blanket layer of cells on top of the matrigel (HeyA8) or very small/ spherical acini (59M) versus the cells with high PKC ϵ / cyclin E levels (OVCAR3 and IGROV), which formed large asymmetric acini, possibly indicative of a more tumorigenic phenotype. Quantitation analysis of the acini revealed that the averages of the diameter of the acini were significantly larger in the OVCAR3 and IGROV cell lines than the 59M cell line ($p = 0.0007$ and $p = 1.8 \times 10^{-11}$, respectively) (Figure 3b). Similarly, the percent of asymmetric acini was significantly higher for the OVCAR3 and IGROV cell lines than the 59M cells ($p = 0.00006$ and $p = 0.004$, respectively). However, the number of acini was not significantly different between these cell lines. This lack of difference in the number of acini is likely due to the large, clumped, globular structures that are observed in the OVCAR3 and IGROV cell lines, making these structures difficult to quantitate. However, in 2D culture conditions the low PKC ϵ / cyclin E cells grew with a more spread blanket-like phenotype compared to the high PKC ϵ / cyclin E cells, which grew in tight clusters. The expression levels did not seem to change between the 2D and 3D conditions as determined by Western blot analysis (Figure 3c). Thus, we conclude that high expression levels of PKC ϵ / cyclin E could potentially be an indicator of larger, asymmetric 3D acini. However, all cell lines are heterogeneous and no two are identical, thus protein manipulation would need to be performed in a single cell line to validate the effect of PKC ϵ on the aggressive phenotype in 3D culture.

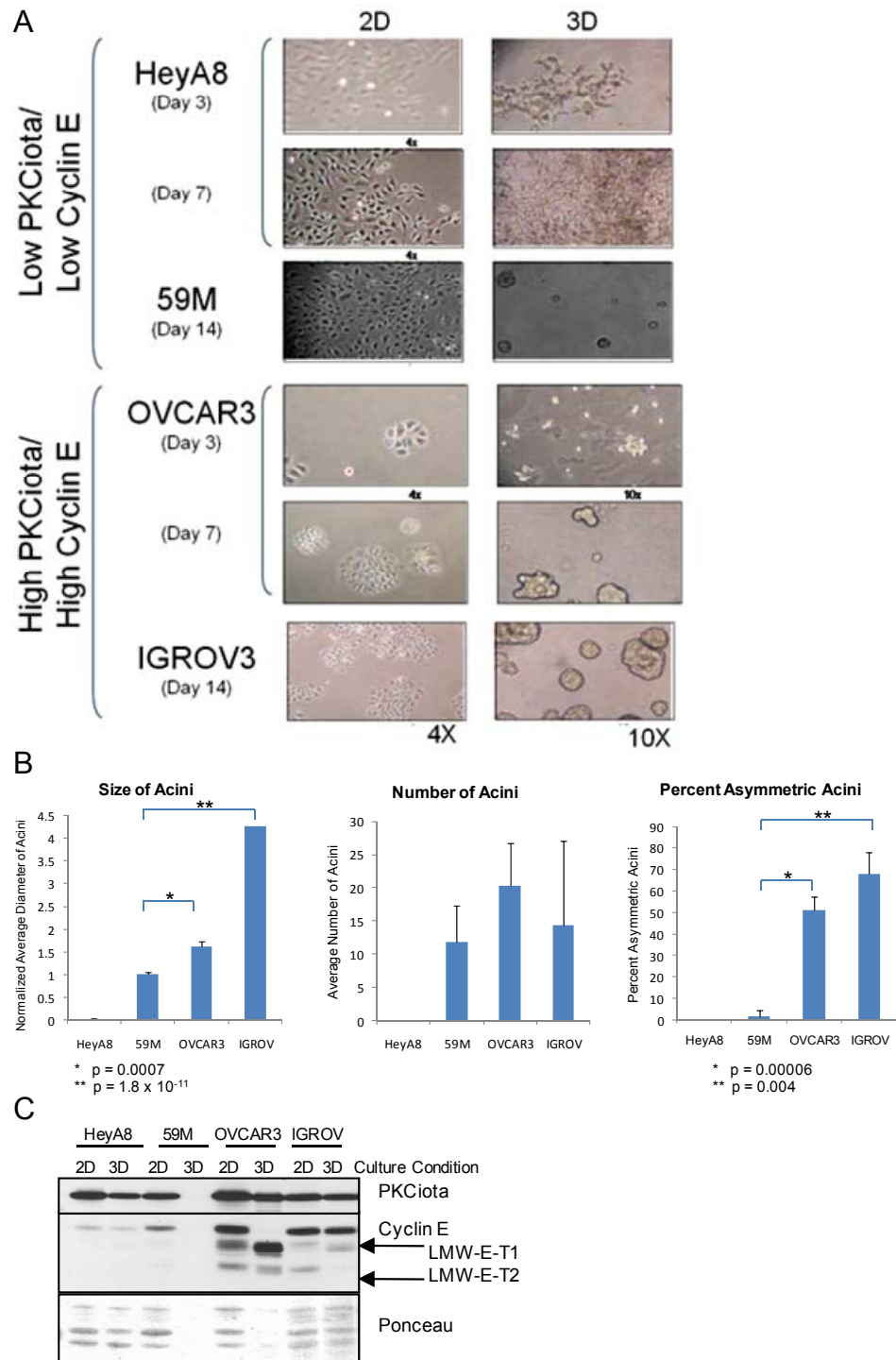
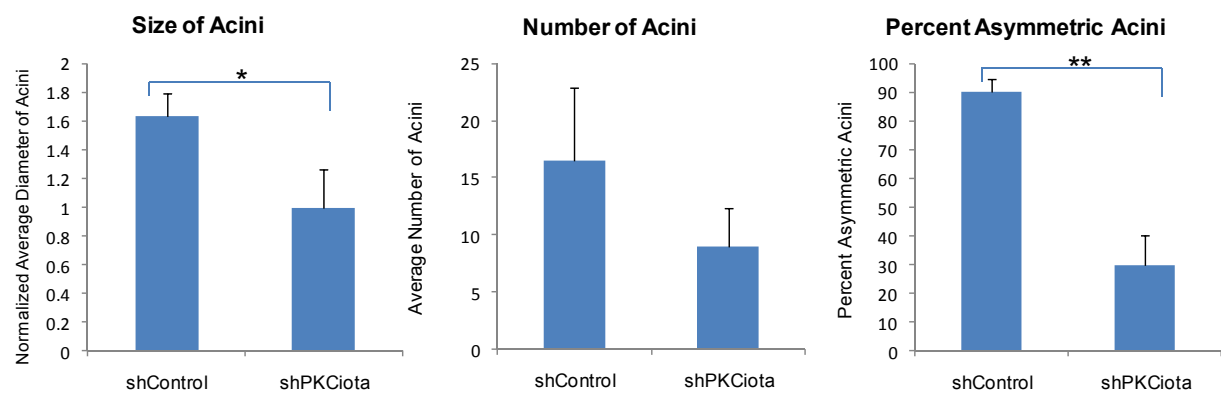
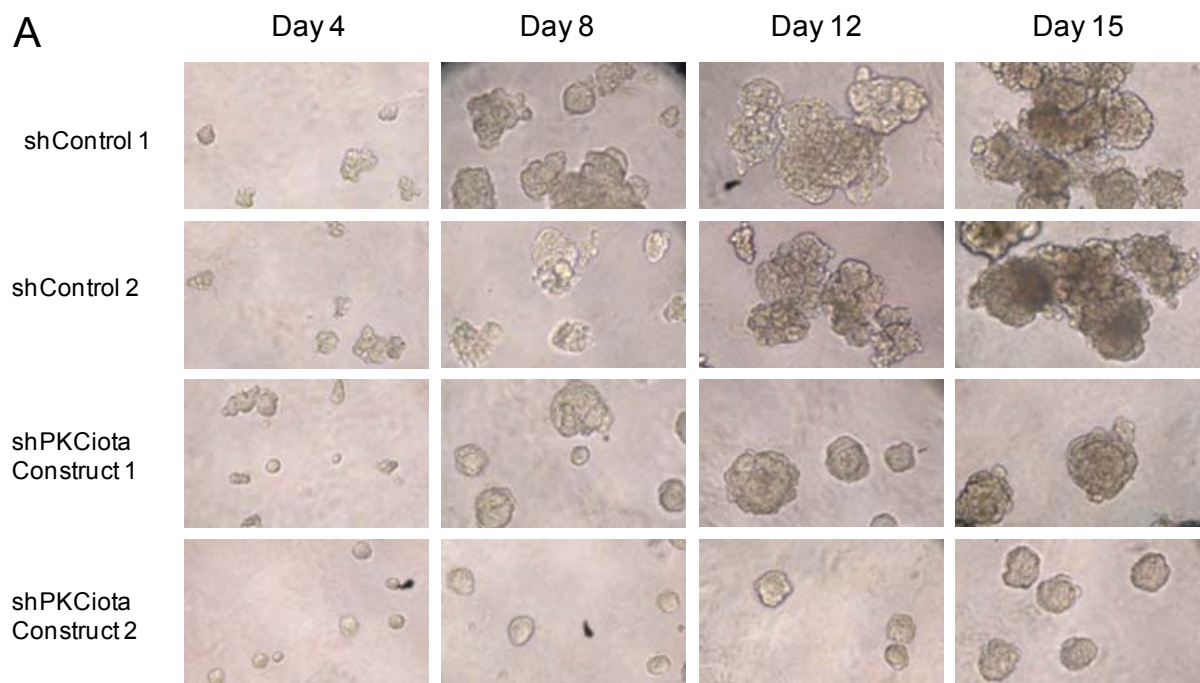


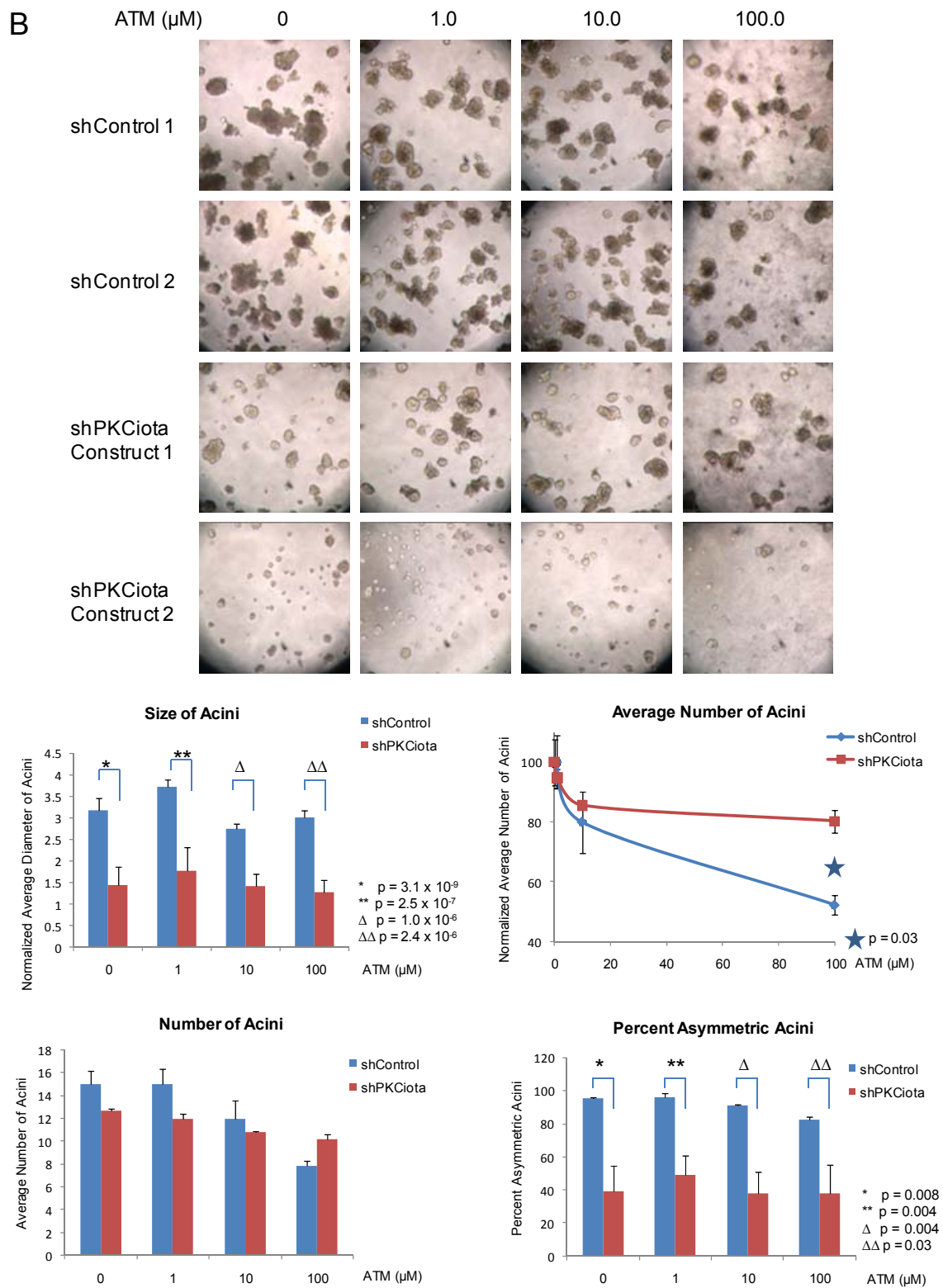
Figure 3: Differential Expression of PKC α / Cyclin E in 3D Matrigel

(A) Cell lines with low PKC α / cyclin E expression (HEYA8 and 59M) versus those with high PKC α / cyclin E (OVCAR3 and IGROV) were plated and photographed over time in 2D versus 3D matrigel culture. Images are 4X on left panels (2D) and 10X on the right (3D). (B) Quantitation and statistical analysis of the size (diameter), average number of acini per section, and percent asymmetry of the acini from part (A). The HeyA8 cell line does not form acini. (C) Expression of PKC α and cyclin E were examined in these two conditions in all four cell lines by Western blot analysis with antibodies against PKC α and cyclin E; actin was used as a loading control.

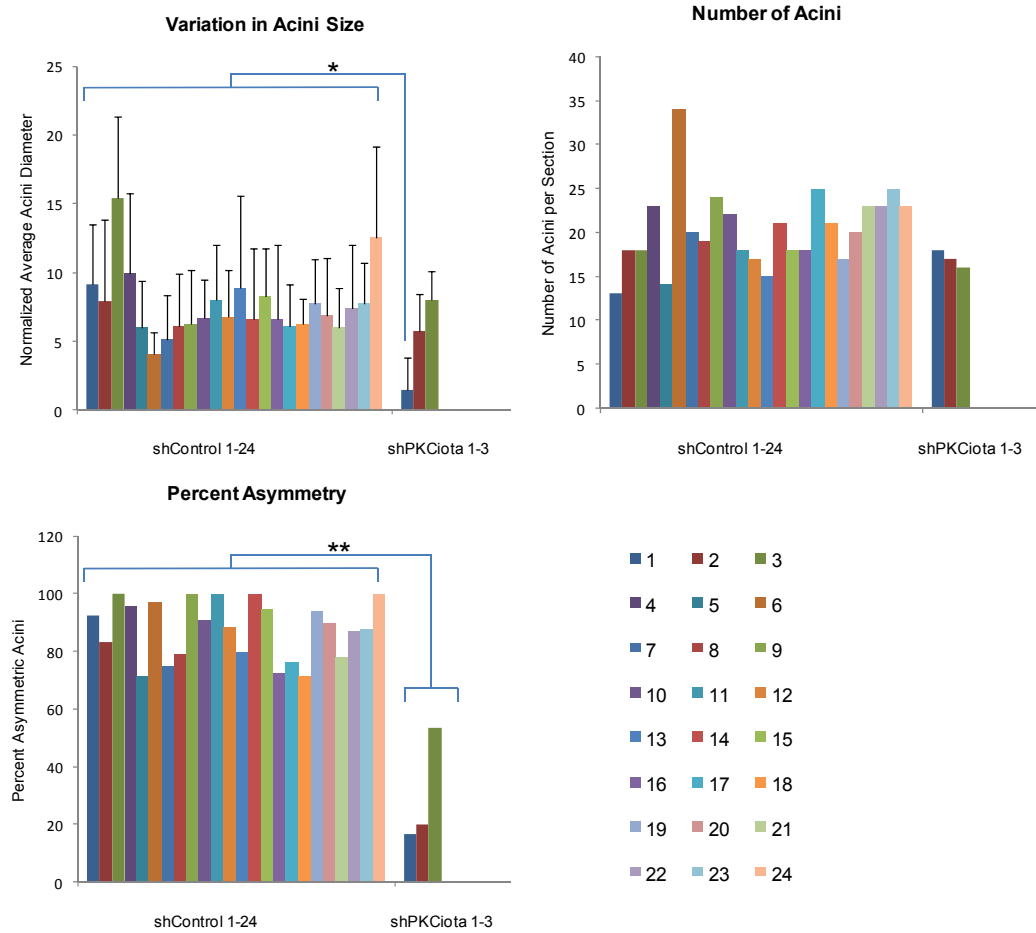
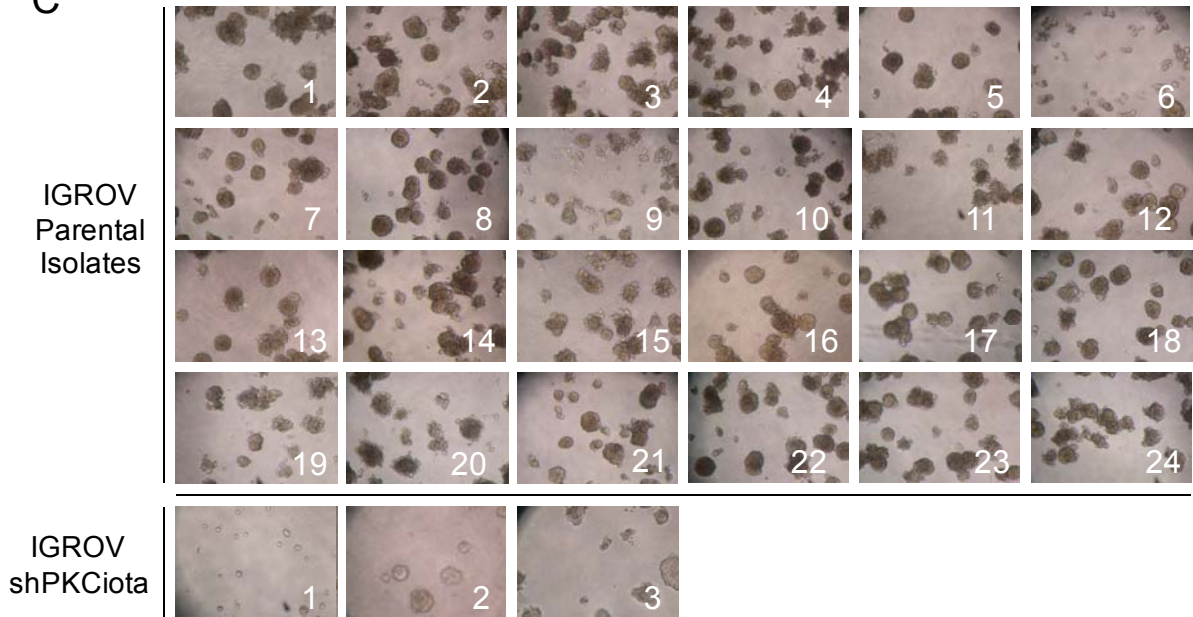
While PKC α levels seem to correlate with a more aggressive 3D culture phenotype (a greater number of acini, that are larger and asymmetric in morphology), we needed to know if this correlation could be directly linked to PKC α . To address this question, we used IGROV PKC α stably silenced clones or the PKC α inhibitor, ATM to examine the resulting acini morphology in 3D matrigel. When PKC α was stably silenced in IGROV cells, the size and morphology of the acini appeared more normal-like, exhibiting apical-basal-like polarity with a cleared lumen (Figure 4a and 5a). In the shPKC α clones, the acini were significantly smaller ($p = 0.00003$) and were more symmetrical ($p = 0.002$) than the shControl clones at Day 15. However, while the number of acini structures appeared to be lower, these results were not significant ($p = 0.08$). The use of the PKC α inhibitor, ATM (100 μ M) resulted in a significant decrease in the number of acini in the shControl clones ($IC_{50} = 100 \mu$ M) versus the shPKC α clones (80% inhibition at 100 μ M), which show resistance to ATM ($p = 0.03$) (Figure 4b). While ATM did not cause a significant decrease in the size or asymmetry of the acini in the shControl or shPKC α clones, significant decreases at each ATM concentration were observed between the shControl and shPKC α clones. Therefore, only the number of acini (not the size or symmetry) was affected by ATM-treatment, and this effect was increased in the shControl clones as opposed to the shPKC α clones, which no longer overexpress PKC α . This affect validates the find that ATM, through PKC α inhibition, leads to a 30% greater decrease in the number of acini in a 3D matrigel system. In order to exclude the possibility that the phenotype observed in Figure 4a,b was simply clonal variation, we clonally isolated IGROV parental cells into 24 isolates and cultured these cells in 2D (Figure 4d) and 3D (Figure 4c) conditions. In 3D conditions (Figure 4c), several clones (i.e. 5, 6, 7, and 13) appeared either smaller or polarized-like, but this phenotype did not correspond to the PKC α /cyclin E levels as determined by Western blot analysis (Figure 4d), which also varied extensively. The average diameter was significantly smaller in the shPKC α clone 1 than all the shControl isolates ($p < 0.005$ when compared to any individual shControl isolate). However, shPKC α clones 2 and 3 did not differ significantly in size. Also according to the quantitation, the number of colonies does not differ between the shControl and shPKC α clones, but the average of the percent asymmetry of the shPKC α is significantly smaller than the average percent asymmetry of the shControl clones ($p = 0.04$). Thus, the decrease in the tumorigenic-like phenotype in 3D matrigel in the IGROV shPKC α clones when compared to the shControl clones could potentially be due to clonal variation. Thus, another 3D approach would need to be utilized to determine the role of PKC α in a 3D system. Similarly, different

markers of polarity would need to be examined in both 2D and 3D culture conditions in several IGROV shPKC α clones, instead of only two.





C



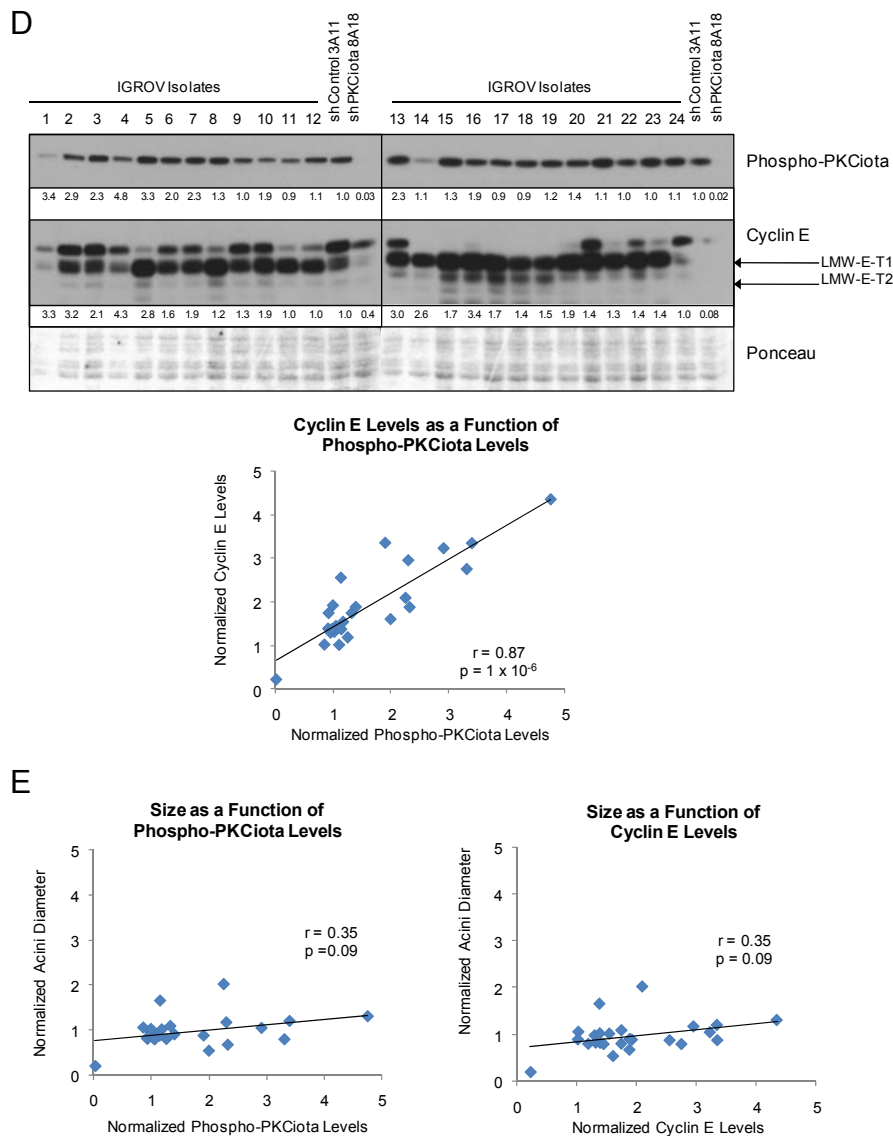
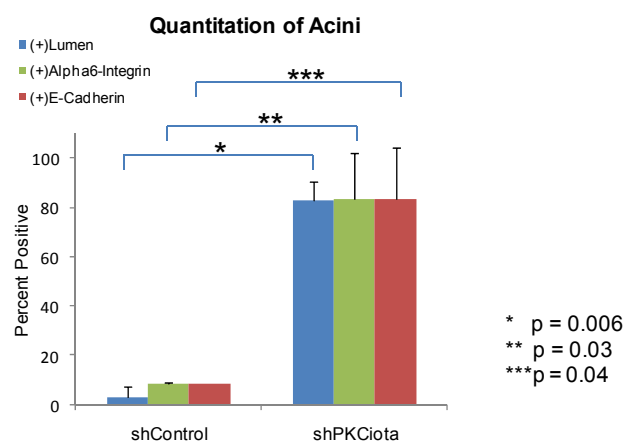
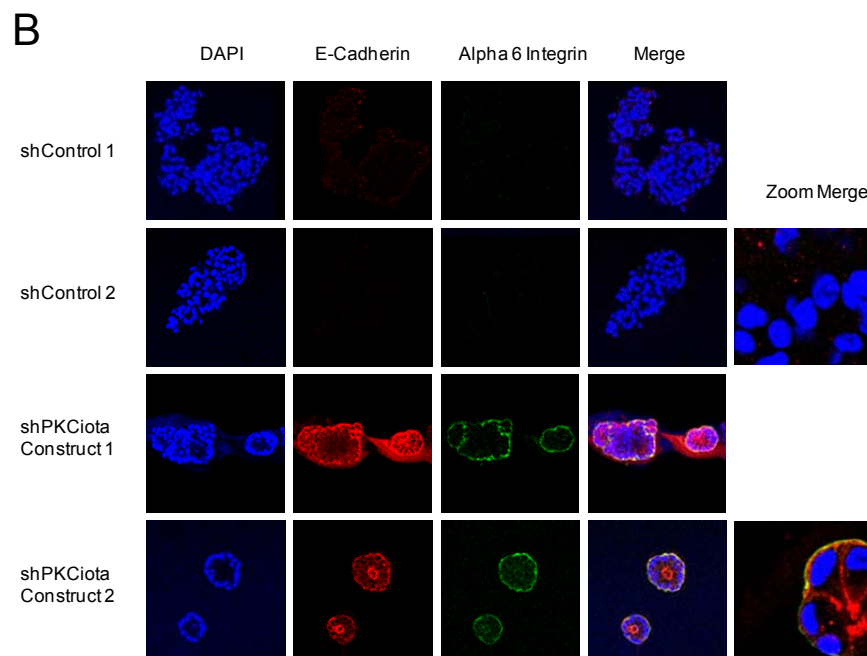
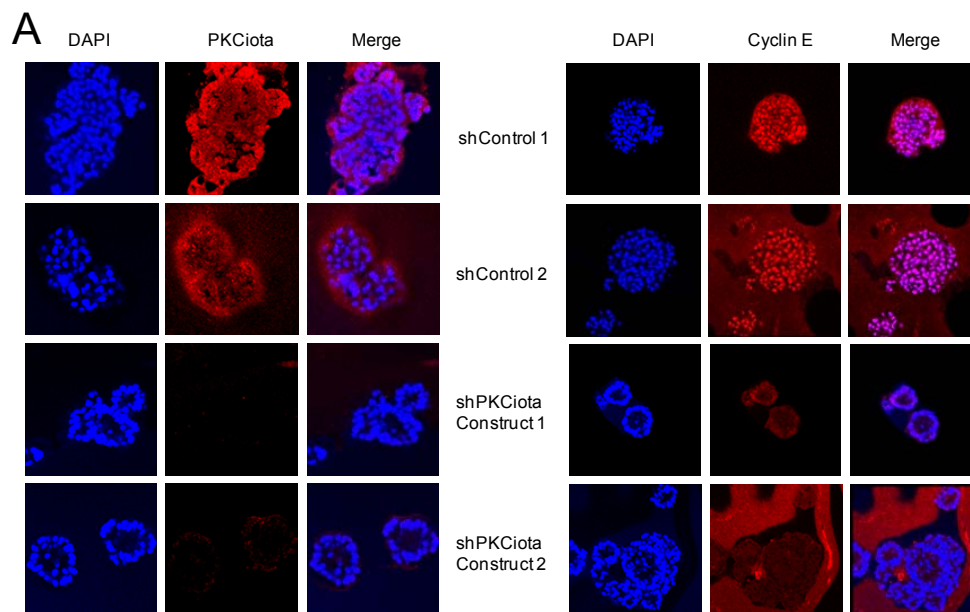


Figure 4: PKCiota Knockdown Results in Decreased Number / Size of 3D Matrigel Acini

(A) Two IGROV shControl and 2 shPKCiota clones were plated in matrigel and imaged at days 4, 8, 12, and 15. Images are at 10X zoom. Diameter (size), average number of acini per section (n), and the percent asymmetric acini for Day 15 were quantitated and plotted. n (shControl) = 66 and n (shPKCiota) = 36. *p = 0.00003 and **p = 0.002. (B) In IGROV cells from part (A), increasing concentrations of the PKCiota inhibitor, aurothiomalate (ATM) were used over a period of 2 weeks. Images are at 4X zoom. Dead cells (dirty looking matrigel) occurred at the highest concentration, mainly in the shControl clones. Acini size, number, and symmetry were quantitated and plotted at 0, 1, 10, and 100 micromolar concentrations of ATM. Significance for each plot was indicated to the right of its respective graph. If no p-value was indicated, the result for that graph was not statistically significant. N-values for 0 ATM, Control= 90, PKCiota= 76; 1 ATM, Control= 90; PKCiota= 72; 10 ATM, Control= 72, PKCiota= 65; 100 ATM, Control= 47, PKCiota= 50. (C) Clonal isolates of IGROV parental cells were plated in matrigel and imaged after 2 weeks. IGROV shPKCiota cells were used as a control. Acini were measured (diameter) and counted (total number and asymmetric number) and quantitated accordingly. * p-value is less than 0.005 for all 24 shControl isolates compared to the shPKCiota clone 1. shPKCiota clones 2 and 3 were not statistically significant. ** p-value is 0.04 comparing the average percent asymmetry of the shControl isolates to the average of the shPKCiota clones. (D) Western blot analysis of clonal isolates from part (C). Antibodies against phospho-PKCiota and cyclin E were examined. Ponceau stain was used as a loading control. Linear Regression of total cyclin E levels as a function of phospho-PKCiota levels was plotted, correlation coefficient (r) = 0.87, $p = 1 \times 10^{-6}$. (E) Acini diameter from part (C) was plotted as a function of phospho-PKCiota levels (panel 1) and cyclin E levels (panel 2) from part (D) densitometry. For phospho-PKCiota levels, $r = 0.35$, $p = 0.09$; for cyclin E, also = 0.35, $p = 0.09$.

In 3D culture, knockdown of PKC ϵ induced a return to normal-like acini, as indicated by a cleared lumen and restored apical-basal polarity. However, the possibility of this effect being clonal variation could not be excluded. Thus, we needed to next examine the downstream polarity effects in several IGROV shPKC ϵ clones. The hypothesis is that in 3D matrigel, PKC ϵ knockdown can cause a reversion to a polarized state through re-expression of polarity proteins, such as E-cadherin. To test this hypothesis, we examined several polarity markers, such as E-cadherin, α 6 integrin (a basal marker), and gm130 (an apical marker), in IGROV shPKC ϵ clones in 3D culture using immunofluorescence (IF) and in 2D using Western blot analysis. IGROV shPKC ϵ clones cultured in 3D matrigel exhibited a decrease in cyclin E levels (Figure 5a). This observation was consistent with previous finding that PKC ϵ knockdown resulted in decreased cyclin E levels in 2D culture conditions (Chapter 2). In these stable IGROV shPKC ϵ clones, expression levels of E-cadherin and α 6 integrin increased coupled with a cleared internal lumen (Figure 5b), indicating the possibility of PKC ϵ knockdown resulting in a reversion to polarity through E-cadherin. However, while PKC ϵ knockdown may result in changes in polarity, this result is independent of cyclin E. In IGROV cells that have been passaged multiple rounds, cyclin E levels can revert to a pre-PKC ϵ -silenced state (Figures 5c and 5d). However, this reversion of cyclin E expression did not alter E-cadherin levels. While E-cadherin was more highly expressed in shPKC ϵ clones, more of a correlation than a direct effect can be concluded due to the high expression of E-cadherin in shControl clone 3A3. Similarly, IGROV shPKC ϵ clone 7B20 (Figures 5c and 5d) reverted to upregulation of PKC ϵ levels, and this reversal, even over several passages did not seem to decrease the levels of E-cadherin, indicative of a potential clonal variation effect. Also, in IGROV cells that have not reverted to re-express cyclin E or PKC ϵ , E-cadherin levels also fluctuate between clones (Figure 5e). In OVCAR3 cells, which are resistant to PKC ϵ inhibition, the PKC ϵ expression pattern is similar to that of IGROV cells, with PKC ϵ being mislocalized to the basal-lateral membrane (Figure 5g). In OVCAR3 cells, like IGROV cells, E-cadherin levels fluctuate in clones independent of PKC ϵ knockdown (Figure 5f). In Chapter 2, in the RPPA analysis of the IGROV shRNA clones, E-cadherin levels changed. However, in the 293T cells transiently transfected with PKC ϵ Wt, CA, or DN, no change in E-cadherin was observed (Chapter 2; Figure 18 and data not shown). Thus, we conclude that the changes in E-cadherin expression and possibly the altered polarity in the IGROV shPKC ϵ clones is likely due to clonal variation. This raises the question, does PKC ϵ knockdown truly result in a decrease in the tumorigenic phenotype, and if so, is this through cyclin E?



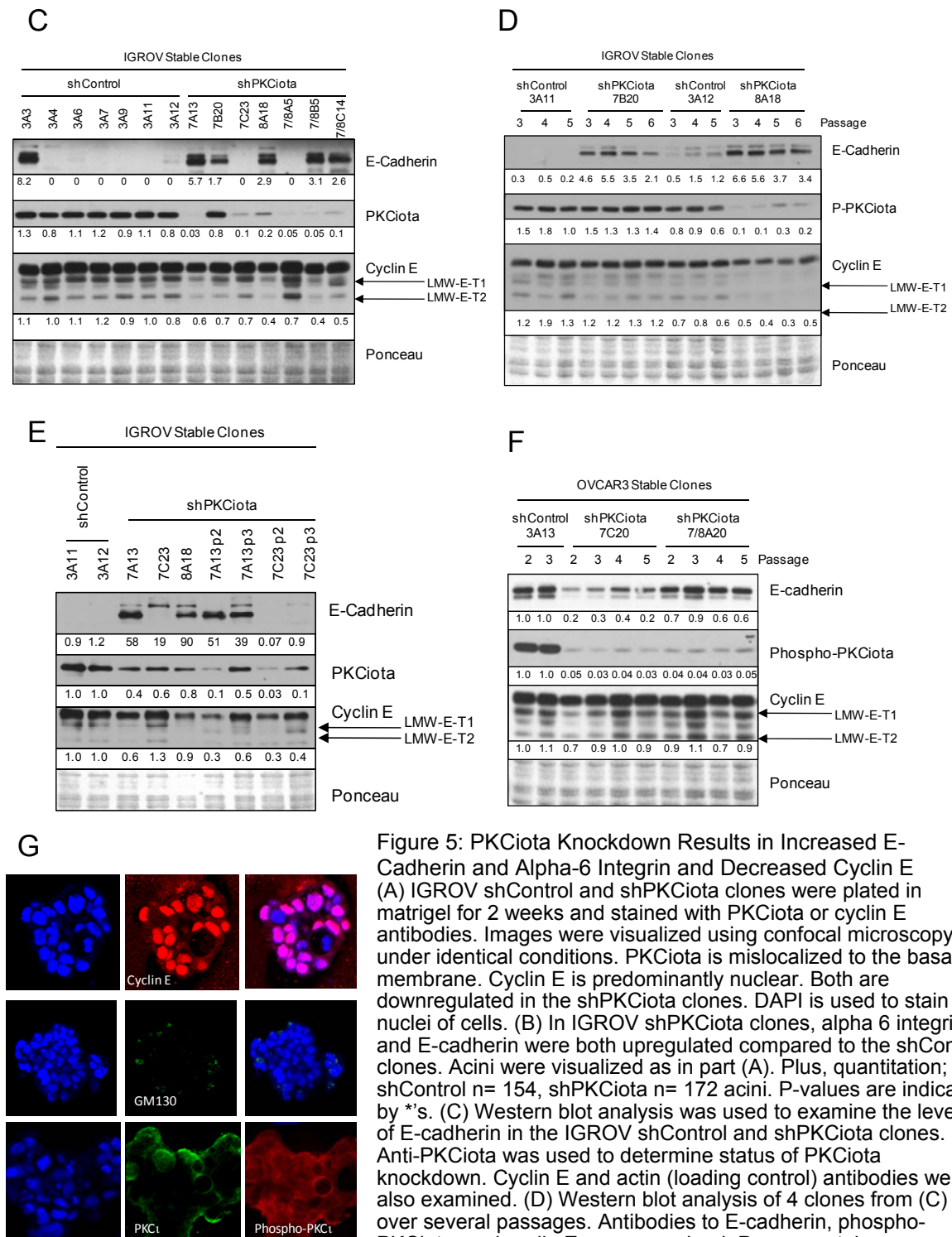


Figure 5: PKCiota Knockdown Results in Increased E-Cadherin and Alpha-6 Integrin and Decreased Cyclin E

(A) IGROV shControl and shPKCiota clones were plated in matrigel for 2 weeks and stained with PKCiota or cyclin E antibodies. Images were visualized using confocal microscopy under identical conditions. PKCiota is mislocalized to the basal membrane. Cyclin E is predominantly nuclear. Both are downregulated in the shPKCiota clones. DAPI is used to stain the nuclei of cells. (B) In IGROV shPKCiota clones, alpha 6 integrin and E-cadherin were both upregulated compared to the shControl clones. Acini were visualized as in part (A). Plus, quantitation; shControl n= 154, shPKCiota n= 172 acini. P-values are indicated by *'s. (C) Western blot analysis was used to examine the levels of E-cadherin in the IGROV shControl and shPKCiota clones. Anti-PKCiota was used to determine status of PKCiota knockdown. Cyclin E and actin (loading control) antibodies were also examined. (D) Western blot analysis of 4 clones from (C) over several passages. Antibodies to E-cadherin, phospho-PKCiota, and cyclin E were examined. Ponceau stain was used as the loading control. (E) Western blot analysis of several shPKCiota clones over 3 passages. Antibodies to E-cadherin, PKCiota, and cyclin E were examined. Ponceau stain was used as the loading control. (F) OVCAR3 cells with stably silenced PKCiota do not exhibit changes in cyclin E or E-cadherin levels as determined by Western blot analysis with indicated antibodies. Ponceau stain was the loading control. (G) Confocal microscopy shows that in 3D matrigel after 2 weeks, in OVCAR3 cells cyclin E is predominantly nuclear, and PKCiota/ phospho-PKCiota are mislocalized to the basal membrane of the acinus structure. GM130 was used as an apical marker, and DAPI was used to stain nuclei.

PKC α Alters Anchorage-Independent Growth by Way of Cyclin E

Because the matrigel assay exhibited so much variability, and matrigel is not the proper assay to measure anchorage-independent growth, we turned our focus to soft-agar, which is commonly used in transformation assays as an indicator of a cell's ability to grow independently of an extracellular matrix (such as matrigel). Cyclin E and PKC α individually have been implicated in the transformation stage of tumorigenesis through alterations in anchorage independent growth (Fang, 1996; Kang, 1996; Coghlan, 2000; Murray, 2004). In chapter 2, we found that PKC α regulates cyclin E levels. Therefore, we hypothesized that the effect of PKC α on anchorage-independent growth could be through cyclin E. To examine the effect of PKC α on anchorage-independent growth, we first used soft agar colony formation to compare cell lines with various levels of PKC α / cyclin E expression in the presence or absence of PKC α inhibition. Next, we used IGROV shPKC α clones to examine the effect on soft agar colony formation. When PKC α resulted in a decrease in the number of soft agar colonies, we re-introduced cyclin E (EL and LMW-E) using adenovirus to examine the possibility of cyclin E's ability to revert the shPKC α phenotype. In a previous publication, Regala et al had shown that NSCLC cell lines with higher PKC α expression resulted in fewer soft agar colonies in response to the PKC α inhibitor, ATM, than those cell lines which exhibited low levels of PKC α (Regala, 2008). In seven ovarian cancer cell lines and 293T cells, we examined the effect of PKC α expression on response to PKC α inhibition with ATM and ATG (Figure 6a). Cell lines with high PKC α / cyclin E levels as determined in Chapter 2; Figure 1 (OAW42, Hec1, IGROV, 293T) or with low PKC α / cyclin E levels (Hec7, EF027, A2780CP, SKOV3) did not differ in their response to PKC α in soft agar. Similarly, half of the high and half of the low PKC α expressing cell lines did not even grow in soft agar, thus no pattern was established based on PKC α expression alone. Upon examination of phospho-PKC α levels compared to the response to ATM in soft agar, the higher phospho-PKC α levels seemed to respond worse to ATM than those with lower phospho-PKC α expression, an opposite effect of the Regala study in NSCLC (Figure 6b and 6c). In order of highest PKC α expression OAW42 > IGROV > Hec7 > SKOV3, the highest expresser of phospho-PKC α (OAW42) responded the least to ATM when compared to the lowest expresser of phospho-PKC α (SKOV3). However, this study only consisted of four cell lines, so results are not substantial. To examine a direct effect of PKC α on soft agar colony formation, we used IGROV cells, which respond to ATM, but have high levels of PKC α and cyclin E, and we stably silenced PKC α to determine the effect on soft agar colony formation (Figure 6d-f). IGROV shPKC α clones exhibited stable knockdown of

PKC ϵ resulting in low levels of PKC ϵ (Figure 6d and Chapter 2). These clones were grown in soft agar for 14 days, and soft agar colonies were quantitated (Figure 6e and 6f). Figure 6f shows all clones tested in order to rule out the possibility of clonal variation. However, Figure 6e shows clones used in future experiments (shPKC ϵ clone 1 = clone 7A19, and clone 2 = 8A18). When PKC ϵ was silenced, fewer soft agar colonies were present. Specifically, silencing PKC ϵ resulted in a 2-fold (IGROV shPKC ϵ clone 7B9) up to an 8-fold decrease in the number of soft agar colonies ($p < 0.0005$ for all shControl versus shPKC ϵ comparisons). Thus, PKC ϵ was determined to be necessary for soft agar colonies formation, indicative of anchorage-independent growth, in IGROV serous epithelial ovarian cancer cells. However, this leaves the question, is the role of PKC ϵ in anchorage independent growth through its regulation of cyclin E?

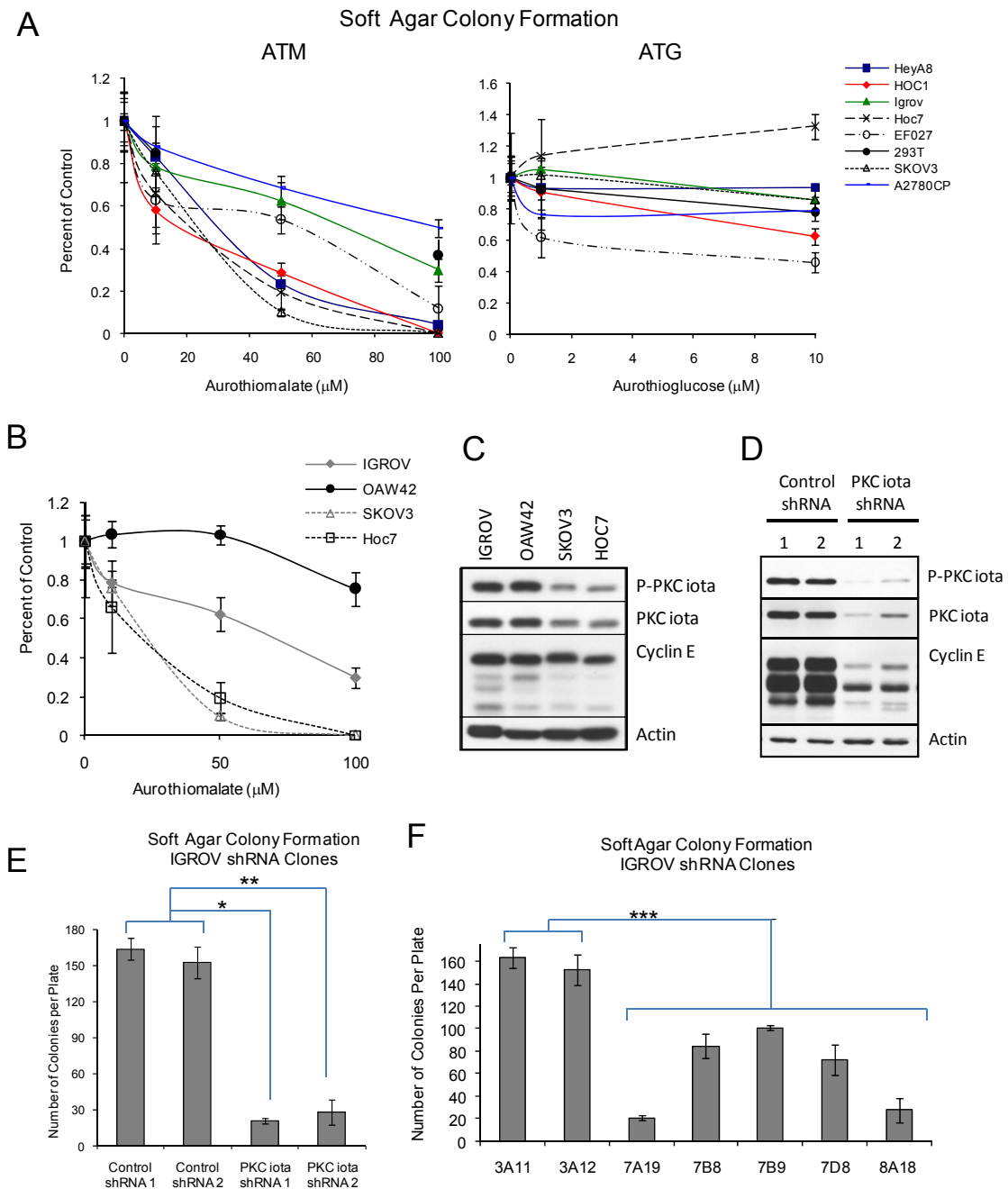


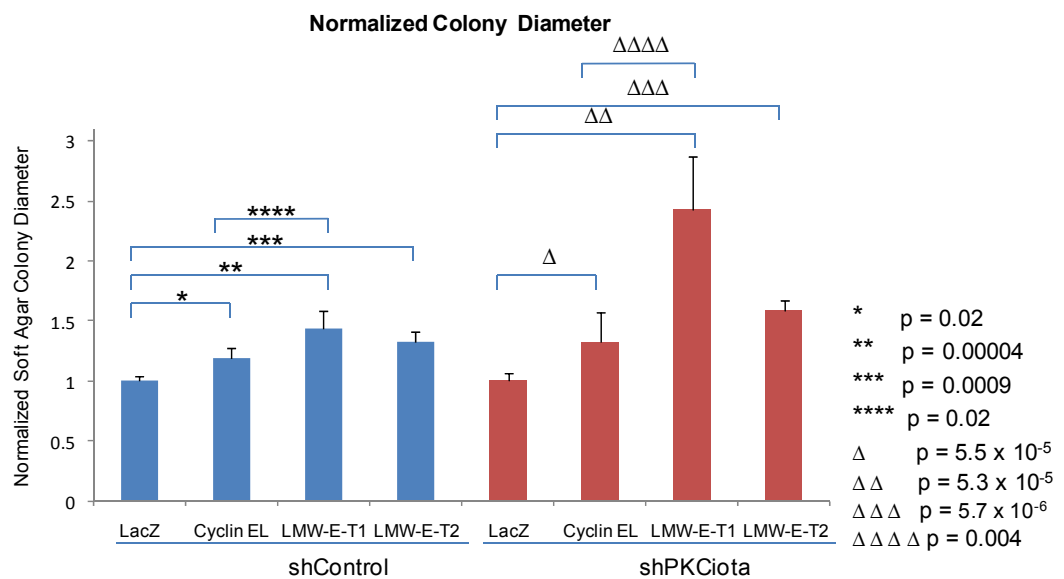
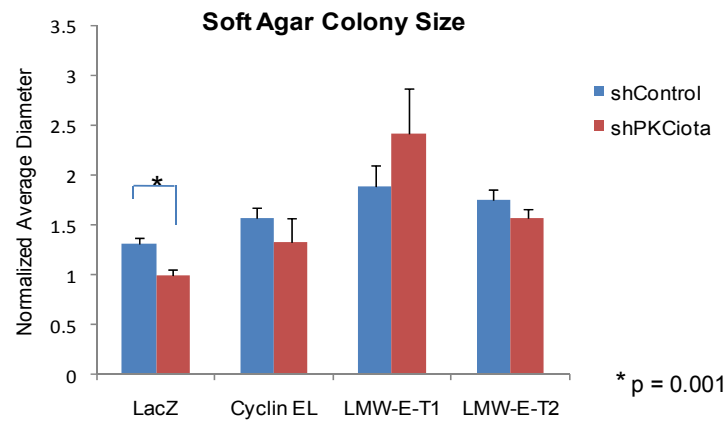
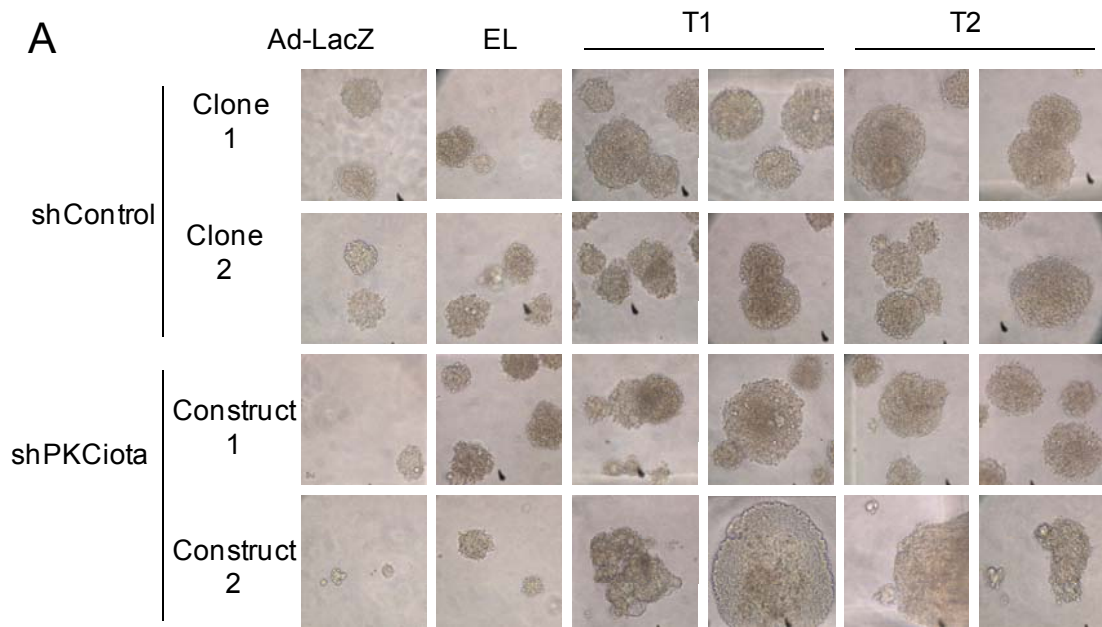
Figure 6: Altered PKC ι ota Expression Results in Changes in Anchorage-Independent Growth

(A) Soft agar colony formation was decreased in all 7 cell lines examined in response to the PKC ι ota inhibitor ATM, but not ATG. Concentrations of ATM and ATG were based on IC₅₀ values in a previous publication (Stallings-Mann 2006). Examination of 2 high PKC ι ota/ cyclin E (IGROV and OAW42) versus 2 low PKC ι ota/ cyclin E (SKOV3 and HOC7) cell lines by soft agar colony formation in response to ATM (B) and by Western blot analysis (C). Actin was used as a loading control. (D) Western blot analysis showing PKC ι ota knockdown in IGROV shControl versus shPKC ι ota clones. Anti-phospho-PKC ι ota, PKC ι ota, and cyclin E antibodies were used, and actin was used as a loading control. (E) Bar graph demonstrating a decrease in soft agar colony formation in IGROV shPKC ι ota clones. These clones were used in future experiments. * $p = 2.7 \times 10^{-7}$; ** $p = 4.4 \times 10^{-5}$ (F) Bar graph showing the decrease in soft agar colonies in all IGROV shPKC ι ota clones. *** $p < 0.0005$ for all individual comparisons.

Because cyclin E and PKC α have both been implicated in anchorage-independent growth, a hallmark of cancer, we hypothesized that cyclin E was required for PKC α -induced anchorage-independent growth. To test this hypothesis, we used IGROV shPKC α clones from Figure 6d,e and re-introduced cyclin E by use of adenovirus. Because cyclin E is regulated at the level of protein stability by PKC α (Chapter 2; Figure 16), cyclin E levels are slightly lower in the shPKC α clones compared to the shControl clones. However, because adenovirus infection results in such a high level of expression, the effect on cyclin E levels was not considered to be a variable. When cyclin E (EL or LMW-E) was re-expressed in shPKC α clones, the number of the soft agar colonies increased to pre-PKC α knockdown values from an averages of 75 colonies per plate (in shPKC α untreated and LacZ control) up to 150 colonies (a 2-fold increase), which was similar to the shControl numbers (Figure 7a and 7b). In shPKC α clones with re-expressed cyclin E, especially LMW-E, the number (2-fold) (Figure 7b) and size (Figure 7a, panels 2 and 3) of the colonies increased, possibly due to the increase in cyclin E-associated CDK2 activity. While the number of shPKC α colonies versus the number of shControl colonies were a 2-fold decrease (which was reverted upon re-expression of cyclin E, the change in diameter was not as pronounced as the change in colony numbers (Figure 7a; panel 2) However, in shControl clones with re-expressed cyclin E, the number did not change as appreciably (less than 25%), but the colonies were overall larger in size (Figure 7a and 7b). More specifically, in both the shControl and shPKC α conditions, the cyclin EL-overexpressing colonies were significantly larger than the LacZ-control colonies ($p_{\text{shControl}} = 0.02$ and $p_{\text{shPKC}\alpha} = 5.5 \times 10^{-5}$). Similarly, when compared to the LacZ control colonies, the LMW-E-T1 and LMW-E-T2- overexpressing colonies were larger in diameter (LMW-E-T1, $p_{\text{shControl}} = 0.00004$ and $p_{\text{shPKC}\alpha} = 5.3 \times 10^{-5}$; and LMW-E-T2, $p_{\text{shControl}} = 0.0009$ and $p_{\text{shPKC}\alpha} = 5.7 \times 10^{-6}$) (Figure 7a, panel 3). Interestingly, the LMW-E-T1 (but not LMW-E-T2)-overexpressing colonies were significantly larger than the cyclin EL-overexpressing colonies in both the shControl ($p = 0.02$) and the shPKC α ($p = 0.004$) cells (Figure 7a, panel 3). As indicated in figure 7b, the cyclin EL and LMW-E-T2-overexpressing cells exhibited a larger increase in colony number compared to the LMW-E-T1-expressing cells, while in Figure 7a (panel 3), the LMW-E-T1-expressing cells exhibited large colony diameter, implying cyclin EL and LMW-E-T2 may cause more colonies, while LMW-E-T1 leads to larger colonies. Overall, in regard to the number of colonies, a potential threshold effect, where overexpression of cyclin E could potentially be limited by the concentration of CDK2 present in the cells, is possibly not allowing greater soft agar colony formation in the shControl clones (Figure 7b). Quantitative analysis of the soft agar colony formation seen in part 7a is represented in Figure

7b. The reversion of the shPKC ι phenotype by re-introduction of cyclin E (EL, LMW-E-T1, and LMW-E-T2) was shown to be statistically significant ($p = 0.046$, 0.03 , and 0.007 , respectively). Western blot analysis of the 2D cultures of the cells used in this experiment are represented in Figure 7c, and indicate that cyclin EL and LMW-E adenovirus infection was successful for this experiment. Thus, we conclude that the role of PKC ι in anchorage-independent growth is through its regulation of cyclin E. However, we wanted to know if a decrease in cyclin E is necessary or sufficient to decrease the anchorage independent growth.

To more specifically examine the role of cyclin E in anchorage-independent growth, in IGROV cells, we needed to see the effect of decreased cyclin E in IGROV cells. Because cyclin E-CDK2 activity was shown to be necessary for cells to grow in suspension (Fang, 1996), we used a CDK2 inhibitor, Roscovitine, in IGROV shControl and shPKC ι clones and quantitated the number of soft agar colonies (Figure 7d). CDK2 inhibition was able to decrease the number of soft agar colonies formed. The shPKC ι clones, which have decreased cyclin E-CDK2 levels, are not as sensitive to Roscovitine as the shControl clones, which is expected due to lower levels of cyclin E and CDK2 (Chapter 2; Figure 8) in the shPKC ι clones. Thus, decrease in cyclin E levels/ activity is necessary and sufficient in decreasing the amount of anchorage-independent growth in IGROV serous epithelial ovarian cancer cells. This raised the question, is cyclin E necessary for other PKC ι functions, such as migration?



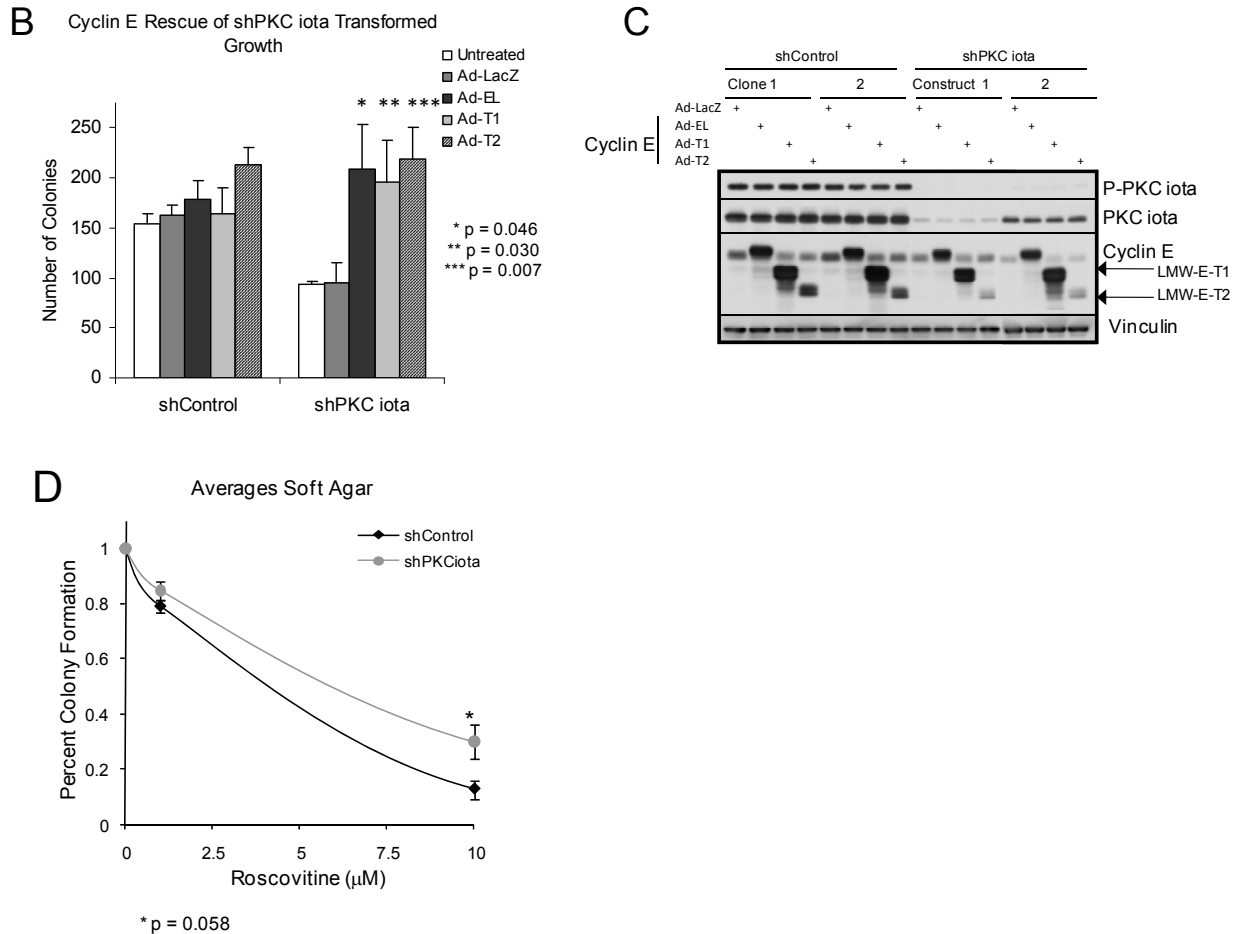


Figure 7: Exogenous Cyclin E Expression Reverted the PKC*iota* Knockdown Phenotype in Anchorage-Independent Growth

(A) Panel 1: Soft agar colonies. IGROV shControl and shPKC*iota* stable clones were infected with Ad-LacZ, Ad-Cyclin EL, Ad-LMW-E-T1 or -T2 and were plated in soft agar for only 10 days before being imaged. Images are at 10X zoom. Panel 2: Colony diameter was quantitated using 4 sections from each condition. The averages were normalized to the shPKC*iota* LacZ average. Statistical analysis was performed on all conditions, but only LacZ, shControl versus shPKC*iota* was significant ($p = 0.001$). $n = \text{LacZ-(shControl)} = 29$, $\text{shPKC}iota = 20$; Cyclic EL (shC = 72, shP = 57); LMW-E-T1 (shC = 54, shP = 37); LMW-E-T2 (shC = 64, shP = 68). Panel 3: Colony diameter was quantitated as above, and the averages were normalized to the LacZ average for each group (shControl versus shPKC*iota*). Statistical analysis was performed, and significant differences are presented above. (B) Bar graph showing the cell numbers from part (A) were quantitated and statistical analysis on several experiments was performed. $p_{EL} = 0.046$, $p_{T1} = 0.03$, and $p_{T2} = 0.007$. Exogenous cyclin E expression caused a reversion of the PKC*iota* knockdown phenotype on anchorage independent growth. (C) Western blot analysis confirming the infection of the IGROV stable shRNA clones with Ad-cyclin E. Phospho-PKC*iota* and PKC*iota* antibodies were used to confirm stable knockdown of PKC*iota*. Vinculin was used as a loading control. (D) Graph of IGROV shControl and shPKC*iota* soft agar colonies upon exposure to increased concentrations of the CDK inhibitor, Roscovitine. Roscovitine decreased soft agar colony formation in all shRNA cell lines, but to a greater extent in shControl cells.

The Role of PKC ϵ in Migration is Cyclin E-Independent

PKC ϵ plays a very important role in migration through its binding to Par-6 at the membrane ruffles of the leading edge of migrating cells (Noda, 2001). We have identified cyclin E as being necessary for PKC ϵ -induced anchorage independent growth. Therefore, we hypothesize that cyclin E is necessary for PKC ϵ -induced cell migration. To test this hypothesis, we used a wound healing/ scratch assay comparing IGROV shControl and shPKC ϵ clones in the presence or absence of ad-cyclin E expression. If cyclin E was necessary for PKC ϵ -induced migration, re-expression of cyclin E should be able to revert the shPKC ϵ phenotype to resemble that of the shControl phenotype. However, by 36 hours after the initial scratch, the shControl wounds had closed, while the shPKC ϵ wounds, regardless of cyclin E re-expression, remained open, indicating a deficiency in the rate of migration (Figure 8a). In the quantitation of these findings, we observed that the shControl-cyclin E clones resembled the shControl alone, and the shPKC ϵ -cyclin E clones resembled the shPKC ϵ alone clones (Figure 8b). The wound was 50% closed 24 hours sooner with the shControl cells than the shPKC ϵ cells (24 versus 48 hours to 50% closed), which was not altered upon re-expression of cyclin E. Thus, cyclin E was unable to revert the shPKC ϵ phenotype in migration, implying that the role of PKC ϵ in migration is cyclin E independent.

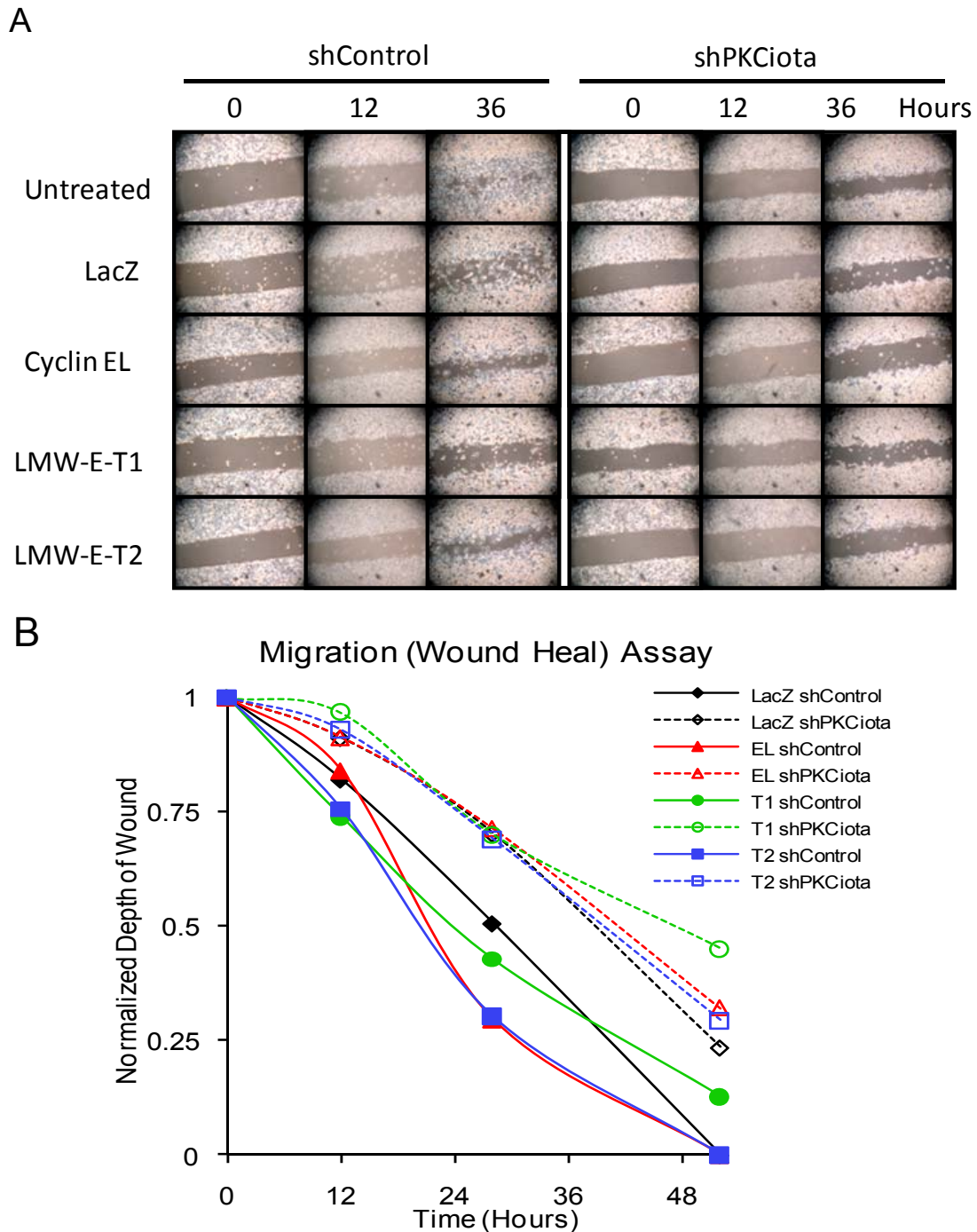


Figure 8: Exogenous Cyclin E Expression Can Not Revert the PKCiota Knockdown Phenotype in Migration

(A) Wound Healing (scratch) assay with IGROV shControl and shPKCiota clones. Clones have been infected with Ad-LacZ, Ad-Cyclin EL, Ad-LMW-E-T1 or -T2 for 48 hours. While several clones of each were examined, only one clone of each is represented here. shPKCiota clones exhibited delayed wound healing, and exogenous cyclin E expression did not revert this phenotype. (B) Graph representing the quantitation of part (A). Solid lines represent shControl clones, while dotted lines represent shPKCiota clones. shControl clones exhibit a closed wound by 48 hours, while shPKCiota clones exhibit delayed migration.

Thus, in this chapter, we have shown that PKC α can affect cell growth through alterations in the cell cycle distribution as opposed to changes in cell death. While PKC α knockdown in 3D matrigel resulted in a return to a smaller more polarized acinus state, the possibility of this effect being through clonal variation was not ruled out. However, in soft agar, cyclin E was shown to be both necessary and sufficient for PKC α -mediated anchorage-independent growth. Examination of the role of cyclin E in PKC α -mediated migration revealed that although cyclin E was necessary for anchorage-independent growth, cyclin E was dispensable for PKC α -mediated migration.

CHAPTER 3: DISCUSSION

In this chapter, we demonstrated that while cyclin E is necessary and sufficient for some of the functions of PKC α , others are cyclin E-independent. Similarly, we found that cyclin E-CDK2 activity is required for the cyclin E-dependent functions of PKC α . Because the genes that encode for PKC α and cyclin E are both amplified (Eder, 2005; Zhang, 2006; and Courjal, 1996; Marone, 1998, respectively) and overexpressed in epithelial ovarian cancer, these two oncogenic proteins provide one pathway to potentially be targeted in the treatment of ovarian cancer. In chapter 2, we demonstrated that PKC α knockdown can cause a decrease in cyclin E levels, and this decrease is through degradation rather than lack of expression of cyclin E. Thus, it is possible that targeting PKC α for treatment of ovarian cancer could result in a decrease in cyclin E, even in the rare circumstance that cyclin E protein levels are high due to genomic amplification.

PKC α has been found to be involved in several diverse cellular functions, such as establishment and maintenance of epithelial cell polarity, migration, protein trafficking, proliferation, transformation, invasion, differentiation, and survival (see Chapter 1). In a drosophila epithelial tissue model, Eder et al found that cyclin E was required for the establishment and maintenance of apical-basal polarity in epithelial cells (Eder, 2005). More specifically, cyclin E-associated kinase activity was required for polarity, because ectopic expression of the drosophila ortholog to p27, which inhibits cyclin E-associated kinase activity, reverted the non-polarized phenotype that resulted from overexpression of constitutively active PKC α . Thus, this begged the question, if cyclin E was necessary for a disruption in polarity by PKC α overexpression, which is a characteristic of serous epithelial ovarian cancer, what other functions of PKC α require cyclin E-associated kinase activity?

In IGROV serous epithelial cells, PKC α knockdown resulted in a decrease in cyclin E levels (and a decrease in other cell cycle proteins, such as CDK2/4 and cyclin D1) (Chapter 2). Because so many drivers of the cell cycle were decreased in response to stable PKC α knockdown, we wanted to examine the effect of PKC α knockdown on the rate of proliferation and cell cycle distribution. In IGROV cells, PKC α inhibition with the PKC α inhibitor aurothiomalate (ATM) resulted in a dose-dependent decrease in cell proliferation (IC_{50} = 45 μ M) (Figure 1e). Interestingly, in OVCAR3 cells, which did not result in a decrease in cyclin E upon stable PKC α knockdown (Chapter 2; Supplemental Figure S2), did not result in decreased proliferation in response to ATM (IC_{50} > 800 μ M) (Figure 1b). Thus, cyclin E is implicated as the downstream regulator of proliferation by PKC α .

In our study, we used the MTT assay, which measures proliferation, to determine the effect of PKC η inhibition on proliferation. However, the decrease in proliferation in response to PKC η inhibition could be due to either a lack of proliferation (induction of a quiescent state) or an increase in cell death. We found that PKC η knockdown resulted in an increase in the fraction of cells in the G1-phase of the cell cycle and a corresponding decrease in the S-phase fraction (Figure 2d,e). Because cell death could also cause a decrease in the number of proliferating cells, we examined the effect of PKC η knockdown on apoptosis. We observed no change in the percentage of apoptotic cells between stable PKC η knockdown and the control shRNA clones (Figure 2f). Thus, the decrease in proliferation observed by PKC η knockdown was likely due to an apparent G1-arrest of these cells, as opposed to cell death. The change in the G1 fraction in response to PKC η knockdown could be through downregulation of cyclin E. When cyclin E-CDK2 is inhibited, cells arrest in G1 (Tsai, 1993). Similarly, because proliferation did not change in the OVCAR3 cells, which do not downregulate cyclin E upon stable PKC η knockdown, this led us to infer that the change in proliferation in IGROV cells is though a decrease in cyclin E levels resulting in a G1 arrest leading to a decrease in proliferation.

PKC η also plays a role in polarity. As stated, in a drosophila epithelial tissue model, cyclin E activity was necessary to disrupt polarity in normal epithelium by way of exogenous constitutively active PKC η overexpression (Eder, 2005). We attempted the opposite experiment. In IGROV serous epithelial ovarian cancer cells, which exhibited overexpressed and mislocalized PKC η , we stably silenced PKC η with RNAi in an attempt to restore polarity (Figures 4a and 5a,b). At first glance, PKC η knockdown resulted in a restoration of apical-basal polarity, complete with a cleared lumen and increased expression of E-cadherin and alpha 6 integrin (a basal membrane marker) (Figure 5a,d). However, upon further investigation, we found that this effect could be due clonal variation in the IGROV shControl and shPKC η clones that occurred in nearly 50% of the shControl clones (based on acini size) and 15% (based on percent asymmetry). However, it should be noted that these clones were not examined by confocal microscopy for the presence of a hollow lumen or staining for polarity markers, such as alpha 6 integrin or GM-130. While the effect of PKC η knockdown on a reversion of a tumorigenic phenotype is highly likely, the best method to test this hypothesis would be through an inducible knockdown or Tet-off system. At the beginning of these experiments with IGROV cells, we did not realize that clonal variation would play such a significant role, but in retrospect, if repeated, one of these two systems would be utilized.

Thus, we directed our attention to the role of PKC α on anchorage-independent growth and migration, some of the earlier events in the tumorigenic process.

Both Cyclin E and PKC α individually have been demonstrated in anchorage-independent growth and transformation, one of the initial events in tumorigenesis (Fang, 1996; Kang, 1996; Coghlan, 2000; Murray, 2004). More specifically, Ras, which is infamous for its role in transformation, requires both cyclin E and PKC α in order to transform normal non-tumorigenic cells into invasive tumorigenic cells, which are anchorage-independent (Kang, 1996; Murray, 2004 respectively). Thus, we hypothesized that with PKC α and cyclin E having seemingly overlapping roles in transformation, PKC α requires cyclin E for transformation. Using the PKC α inhibitor ATM in IGROV cells, we found that PKC α inhibition resulted in a reduced number of soft agar colonies formed (Figure 6b). More specifically, we used IGROV cells with stable PKC α knockdown, and found that when PKC α was absent, the number of soft agar colonies decreased significantly (Figure 6e). Exogenous cyclin E expression was able to revert the PKC α knockdown phenotype, indicating that PKC α 's role in anchorage-independent growth was through cyclin E (Figure 6f). We also found that CDK2 inhibition could also result in a decrease in soft agar colonies, thus cyclin E through its binding to its catalytic partner, CDK2, was responsible for anchorage-independent growth in IGROV cells. This role of PKC α through cyclin E has implications in cancer therapy. If inhibition of PKC α could decrease cyclin E-induced transformation, one could infer that tumor volume would also be decreased.

In Chapter 2, we found that PKC α is regulating cyclin E via the PI3K pathway, and use of the PI3K inhibitor GDC-0941 reduced the levels of phospho-PKC α and cyclin E. In IGROV xenograft models, use of this PI3K inhibitor reduced the tumor growth (Raynaud, 2009). Thus, the reduction in tumorigenic growth of IGROV xenografts could potentially be through inhibition of the PKC α / cyclin E pathway. See Chapter 2 discussion for details about PI3K inhibition and the potential effects on the PKC α / cyclin E pathway.

Ras through PI3K leads to several aspects of transformation, including reorganization of the cytoskeleton (Rodriguez-Viciano, 1994). Similarly, PKC α was demonstrated to be necessary for Ras/PI3K- induced transformation by disassembly of actin fibers (Uberall, 1999). While cyclin E was not addressed directly in this paper, the same year, cyclin E overexpression was found to rescue the inhibition of Ras/Rho as measured by cytoskeleton re-arrangement (Ghosh, 1999). Thus, cyclin E and PKC α both have been implicated in cytoskeletal re-arrangement. Therefore, it is possible that Ras induces transformation through the PI3K \rightarrow PKC α \rightarrow cyclin E \rightarrow cytoskeletal rearrangement \rightarrow transformation pathway.

Lastly, because PKC ι plays an important role in migration, we attempted to establish cyclin E as a downstream regulator of PKC ι -induced migration. However, in IGROV cells, we found that PKC ι knockdown resulted in decreased migration, but expression of cyclin E, unlike with anchorage-independent growth, was unable to restore the phenotype. Thus, it is highly unlikely that cyclin E is involved in the migration function of PKC ι . Because PKC ι is co-localized with PAR-6 and Rac1 at the leading edge of migrating cells (Noda, 2001), the effect of PKC ι on migration is probably a more direct phosphorylation effect, as opposed to through signal transduction, as is the case with cyclin E and anchorage-independent growth.

CHAPTER 4:

Discussion/ Future Directions

PKC ι as a Potential Therapeutic Target in Ovarian Cancer

One in 72 women in the U.S. today will be diagnosed with ovarian cancer in their lifetime (NCI-SEER, 2011). This means that at some point in our lives, we all will likely know someone whose life is upset by this disease. However, also according to the NCI, the mortality rate due to ovarian cancer has not changed (less than 2%) since 1975. This lack of improvement in the diagnosis and treatment of ovarian cancer, demonstrates the current need for greater knowledge and understanding of ovarian cancer including in depth insight into the cancer pathways that drive ovarian cancer tumorigenesis.

Several oncogenes have been identified in ovarian cancer. Some oncogenes are more specific to ovarian cancer, such as Rab25, which is the most overexpressed oncogene (80-89%) in epithelial ovarian cancer, but serves as a tumor suppressor in other cancer types (i.e. breast and intestinal cancer) (Cheng, 2004; Cheng, 2006; Cheng, 2010; Nam, 2010). Other ovarian cancer oncogenes are the usual suspects in cancer initiation and progression, such as: Ras, PI3K, Myc, EGF, ErbB2, and AKT to name a few (Bast, 2009). A particular oncogene, PKC ι was first identified as an oncogene in lung cancer (NSCLC: non-small cell lung cancer) (Regala, 2005), but since 2005 has been designated as a *bona fide* oncogene in other cancer types, such as ovarian cancer and pancreatic cancer (Zhang, 2006 and Scotti, 2010 respectively). *PRKCI* (the gene that encodes for PKC ι) is the only gene in the PKC family of serine/threonine kinases that is designated as an oncogene in ovarian cancer (Zhang, 2006). While ten family members exist in the PKC family of kinases, PKC ι and PKC ζ , which often display overlapping functions, are the two members of the atypical branch of the PKC family. This distinction as 'atypical' is due to their lack of regulation by Ca²⁺, DAG, or phorbol esters, which regulate the other 8 PKC family members (Nishizuka, 1995). While PKC ι and PKC ζ have overlapping roles, only *PRKCI* due to either genomic amplification, overexpression, or protein mislocalization, has been designated as an oncogene in ovarian cancer. PKC ι activity is regulated through protein:protein interaction via a PB1 domain, providing a potential motif for drug design for the inhibition of PKC ι (Hirano, 2004). Also, understanding which PKC ι -binding partners exhibit which tumorigenic function is vital to the use of PKC ι as a therapeutic target.

PKC ι displays several diverse tumorigenic functions, such as cellular/tumor growth, proliferation, protein/lipid trafficking, glucose metabolism, transformation and anchorage-independent growth, survival, chemoresistance, migration, and invasion (Chapter 1). Because PKC ι is involved in so many steps of the tumorigenic process, PKC ι inhibition could potentially be useful in the clinic for ovarian cancer treatment regardless of tumor stage. In

fact, a small molecule inhibitor of the PKC α PB1 domain is currently in Phase I clinical trials for the treatment of pancreatic cancer and NSCLC (Mayo Clinic). If this PKC α inhibitor (ATM: aurothiomalate) proves to be successful in the treatment of these other types of cancer, ovarian cancer is likely the next type of cancer to be involved in clinical trials due to PKC α 's designation as an oncogene in ovarian cancer.

Based on the role of PKC α in ovarian cancer, I speculate that epithelial ovarian cancer patients will benefit tremendously from the use of ATM in the treatment of ovarian cancer. According to the NCI, more than half (62%) of the patients that are first diagnosed with ovarian cancer are already in the late stage: the cancer has already metastasized from the ovary to a distant site, making five year survival only 27.6% (NCI-SEER, 2011). While ovarian cancer is first "cured" by chemotherapeutic agents, the cancer often times will return, distributed throughout the peritoneum or peritoneal cavity, and is likely chemo-resistant. PKC α overexpression causes a tumor to potentially develop chemoresistance. While PKC α 's function specifically in ovarian cancer chemoresistance has not been explored, PKC α has already been implicated in chemoresistance in other tumor types, including lung cancer and leukemia (Jin, 2005; Jamieson, 1999; Lu, 2001). Similarly, PKC α has been implicated in cell survival, though upregulation of NF κ B, resulting in transcriptional upregulation of genes involved in the anti-apoptotic response (Chapter 1). Because PKC α inhibition could result in a decrease in cell survival due to a lack of induction of anti-apoptotic genes, PKC α inhibition in ovarian cancer could potentially cause these tumors to be sensitized to pro-apoptotic inducers, such as chemotherapy. Thus, due to the role of PKC α in cell survival and chemoresistance, PKC α appears to be a likely therapeutic target in an ovarian cancer model.

Similarly, PKC α has been shown to be involved in migration and invasion (Chapter 1). These two steps in metastasis obviously occur in metastatic ovarian cancer. However, it is likely that if ovarian cancer is first diagnosed at a later stage, PKC α inhibition could potentially at that point be too little too late. However, it seems reasonable to speculate that PKC α inhibition could result in inhibition of future migration and invasion.

Another important role of PKC α in tumorigenesis is in tumor growth. In two different NSCLC xenograft models (with A549 and A427 lung cancer cells), PKC α inhibitors ATM and ATG (aurothioglucose, a compound similar in function to ATM) resulted in a 4-fold decrease in tumor volume in both cases (Stallings-Mann, 2006; Regala, 2008). However, these two lung cancer models were chosen based on their high levels of PKC α expression/ activity. Thus, for ATM or ATG inhibition of ovarian cancer to be most successful, a comparable pre-

screening method needs to be employed in ovarian cancer similar to that in the lung cancer model. We found that PKCιota inhibition (by ATM and PKCιota RNAi to more specifically target PKCιota) in IGROV (a serous epithelial ovarian cancer cell line) resulted in a dose-dependent decrease in anchorage-independent growth (Chapter 3; Figure 1). In the lung cancer model, when lung cancer cell lines exhibited a decrease in anchorage-independent growth with the soft agar colony formation assay, these same cell lines when used in mouse xenograft models and were treated with the gold compounds (ATM/ATG) also resulted in a decrease in tumor volume. For example, with the H460 lung cancer cell line, that did not respond to ATM *in vitro* (number of soft agar colonies were not affected by treatment with ATM), when H460 cells were used in mouse xenografts, the resulting tumors did not decrease in volume in response to PKCιota inhibition (Regala, 2008). The ability of ATM to inhibit soft agar colony formation significantly correlated with the overexpression levels of PKCιota. Thus, it seems feasible that in ovarian cancer cells, prescreening of patient biopsies (by IHC or Western blot analysis to determine PKCιota protein levels) could be advantageous in determining a patient's potential response to PKCιota inhibitors. However, we have found in two epithelial ovarian cancer cell lines, both of which exhibit the highest levels of PKCιota and phospho-PKCιota out of 20 ovarian cancer cell lines (Chapter 2; Figure 1), upon treatment with ATM, the effect of this drug on proliferation was quite distinctive. While both cell lines should respond to ATM-treatment due to their high levels of PKCιota/phospho-PKCιota, only the IGROV cells exhibited a dose-dependent decrease in proliferation ($IC_{50} = 40$ micromolar as measured by MTT), while the OVCAR-3 cell line, even treated with up to 800 micromolar ATM, never reached an IC_{50} value (Chapter 3; Figure 1). Upon further examination, we found that knockdown of PKCιota resulted in a significant decrease in cyclin E levels in IGROV cells (Chapter 2; Figure 5) but not in OVCAR-3 cells (Supplemental Material). Thus, cyclin E is likely essential for ATM-responsiveness in regard to proliferation, making determination of PKCιota levels in ovarian cancer cells not on its own sufficient to determine a cell lines ability to respond to ATM, which was the case for lung cancer cell lines. A further method of addressing this issue would be to directly inhibit cyclin E-CDK2 activity in OVCAR-3 cells to see if direct inhibition of cyclin E-associated kinase activity could decrease the rate of proliferation in these cells. Perhaps one of the most important means of determining how critical cyclin E is in PKCιota-driven tumorigenesis, would be to examine both IGROV and OVCAR-3 in mouse xenograft models to determine the effect of PKCιota inhibition (ATM) on tumor growth. If the IGROV xenograft model responds to ATM, but the OVCAR-3 xenograft model does not respond, one could hypothesize that this difference in effects could be due to cyclin E inhibition in the IGROV

mouse model, which could be tested by the use of a CDK2-inhibitor. If PKC α is manifesting its effect through cyclin E, as I speculate is the case, the idea of combination treatment of both PKC α and CDK inhibitors should be explored. While PKC α inhibition would result in a decrease in cyclin E levels, other pathways could become compensatory leading to upregulation of cyclin E and hence the use of concurrent CDK inhibition alongside PKC α inhibition is justified. Because we already know that in IGROV cells, inhibition of PKC α results in a decrease of both cyclin E levels and proliferation, the IGROV cell line would make an excellent model system for studying the use of combination therapy of dual PKC α and cyclin E-associated kinase activity inhibition on survival analysis (*in vitro*). If the use of both PKC α and CDK inhibitors results in synergistic cell death, the use of these two inhibitors *in vivo* is warranted. However, in order for these inhibitors to move into clinical trials, they would also need to be used in combination with the standard of chemotherapy, which at MD Anderson Cancer Center is paclitaxel combined with a platinum-based drug (i.e. carboplatin or cisplatin). *In vivo* studies would need to be performed to optimize for the best order of treatment, chemotherapy versus targeted inhibition of PKC α /cyclin E. The use of both PKC α and cyclin E-associated kinase activity inhibitors could potentially sensitize the tumor-bearing mice to the effects of paclitaxel/carboplatin, allowing lower, less toxic concentrations of the chemotherapeutics to be needed to decrease overall tumor burden.

Mechanism of PKC α -Induced Tumorigenesis

PKC α and cyclin E expression correlate in serous epithelial ovarian cancer patient samples, and in non-serous epithelial ovarian cancer, overexpression of both cyclin E and PKC α resulted in a poor prognosis (Eder, 2005). These two oncogenes in ovarian cancer have individually been implicated in transformation and anchorage-independent growth and are both required for Ras-induced transformation (Haas, 1997; Murray, 2004), thus leading us to the hypothesis that cyclin E could potentially be downstream of PKC α in the transformation process. We began by showing that PKC α (phospho-PKC α) and cyclin E levels also significantly correlate in ovarian cancer cell lines (Chapter 2; Figure 1). Establishing this correlation was important to be able to study this relationship in an *in vitro* model. In order to establish the direction of regulation cyclin E \rightarrow PKC α versus PKC α \rightarrow cyclin E, we overexpressed cyclin E (full length or LMW-E) stably in two cell lines and transiently in a single cell line and found that no cyclin E isoform was capable of altering PKC α protein levels or activity (autophosphorylation of PKC α) (Chapter 2; Figure 3). Thus, not only did this rule out the cyclin E \rightarrow PKC α pathway, but this excluded the possibility of any type of positive feedback loop.

To examine the regulation of cyclin E by PKC α , we stably knocked down PKC α and examined the effect on cyclin E (Chapter 2; Figures 5 and 6- in Figure 7 PKC α was knocked down transiently) and conversely, we overexpressed PKC α to examine cyclin E protein levels (Chapter 2; Figures 8 and 9). Using these methods, we were able to establish the ability of PKC α to regulate cyclin E protein levels in several cell lines. Thus, we established PKC α as the upstream regulator in the PKC α /cyclin E relationship. We next wanted to address the question of which functions of PKC α would require cyclin E to be accomplished.

Because both PKC α and cyclin E are implicated in transformation and PKC α is able to regulate cyclin E, we next hypothesized that PKC α 's role in transformation was through its regulation of cyclin E. Using rescue/ reversion experiments, we were able to determine that cyclin E was required for PKC α -induced transformation (Chapter 3; Figures 6 and 7). Thus, the PKC α \rightarrow cyclin E \rightarrow transformation pathway provides a novel cancer pathway to potentially be targeted therapeutically. To further validate the role of cyclin E in transformation in the IGROV cell line, we used Roscovitine, a small molecule competitive inhibitor of CDK2 (the catalytic binding partner of cyclin E), and we found that CDK2-inhibition resulted in a dose-dependent decrease in the number of soft agar colonies (Chapter 3; Figure

7). Thus, cyclin E-associated kinase activity is necessary to accomplish PKC α 's effects on transformation.

However, we found that cyclin E was only involved in certain functions of PKC α . For example, PKC α is involved in migration through its interaction with PAR-6 and Rac1 at the membrane ruffles found at the leading edge of migrating cells (Noda, 2001; Xu, 2006). To examine the role of cyclin E in PKC α -induced migration, we stably knocked down PKC α and rescued with exogenous cyclin E (Chapter 3; Figure 8). However, unlike the result of this same experiment but with transformation as the readout instead of migration, we found that PKC α 's role in migration is cyclin E-independent. It seems likely that the role of PKC α in migration is quite direct, with PKC α and other migration proteins being localized at the leading edge, while the role of PKC α in transformation is through a more extensive signal transduction cascade.

Because PKC α exhibits both cyclin E-dependent and cyclin E-independent functions, one could speculate that PKC α as a therapeutic target would encompass more aspects of tumorigenesis than cyclin E-CDK2 inhibitors. However, cyclin E is also involved in many aspects of tumorigenesis that are not downstream of PKC α . For example, PKC α has never been linked to genomic instability, while cyclin E (both full length and LMW-E) has been implicated in centrosome amplification and genomic instability, which is central in tumor formation (Spruck, 1999; Bagheri-Yarmand, 2010; Bagheri-Yarmand, 2010). Because LMW-E exhibits a significant effect on the induction of genomic instability over the full length form (Akli, 2004; Nanos-Webb 2011 *in revision*), screening methods are still necessary to determine which targeted therapies to use. For example, in ovarian cancer, PKC α can be overexpressed and cyclin E can be cleaved into LMW-E forms. As stated, these two phenotypes often correlate in epithelial ovarian tumors of serous histology and in non-serous epithelial tumors, these two prognostic factors together result in a poor prognosis (Eder, 2005), thus the potential for dual targets through inhibition of both PKC α and cyclin E exists. Further studies would need to be done to determine the effect, whether synergistic, additive, or antagonistic, on combination treatments of PKC α and cyclin E inhibitors on cell death. Also, order of inhibition would need to be established.

Screening methods for patients with high PKC α /cyclin E expressing ovarian tumors

As mentioned above, PKC α and cyclin E are not always linked in their effects on tumorigenesis. Thus, IHC screening for both proteins (PKC α and cyclin E) needs to be performed on tumor tissue samples from the biopsies to determine if inhibitors to these proteins would be effective. With PKC α , either overexpression or mislocalization to the cytoplasm would likely be sufficient to warrant use of PKC α inhibitors in these patients, and with cyclin E, either overexpression or the presence of cytoplasmic cyclin E (LMW-E localizes to the cytoplasm (Delk 2009)) would likely be a good indicator of potential use of CDK2-inhibitors in these patients. While IHC is effective in identifying protein overexpression with specific proteins, genomic amplification can also occur in ovarian cancer in the case of both *PRKCI* and *CCNE1* (cyclin E), and genomic amplification of both of these genes individually is sufficient to result in poor prognosis (Eder, 2005; Zhang, 2006; Courjal, 1996; Marone, 1998). Thus, an alternative method of identifying ovarian cancer patients for potential sensitivity to PKC α and/or cyclin E inhibition could be through the examination of the gene copy number of these two genes. *PRKCI* (3q26) is amplified in 44% of epithelial ovarian cancer patient samples, while *CCNE1* (19q12) is amplified in 12-36% (Bast 2009). However, amplification of these genes correlated with poor survival, thus identification of these ovarian cancer patients that exhibit amplification at these loci could be beneficial in determining their response to targeted inhibition of the protein products of these genes.

As mentioned above, many ovarian cancer patients initially benefit from use of chemotherapy but then later develop metastases that are chemo-resistant. This chemo-resistant cancer is what is the actual cause of death for many ovarian cancer patients. I had mentioned above that PKC α is involved in chemoresistance, and whether this effect is through cyclin E is yet to be determined. However, in a PKC α -independent fashion, *CCNE1* amplification is involved in chemoresistance. In fact, Etemadmoghadam et al, using a high-resolution oligonucleotide microarray to measure gene copy number in 118 ovarian cancer tumor samples, found that *CCNE1* amplification was the largest predictor of chemoresistance in serous ovarian cancer (Etemadmoghadam, 2009). Thus, both *CCNE1* and *PRKCI* genomic amplification should be examined in patient samples to determine the course of action for the treatment of specific deregulated genes.

The next generation of ovarian cancer treatment

With so many biomarkers of various tumor types and so many inhibitors of these molecules, I believe the next generation of cancer therapy will be tailored to each patient. With arrays available today that can determine gene copy number, mRNA expression, or even protein or phospho-protein levels it seems feasible that a patient biopsy could be performed, and their samples could be arrayed to determine the course of treatment. While PKC α (*PRKCI*) and cyclin E (*CCNE1*) would be amplified or overexpressed in a tumor, it may be that other tumor-promoting pathways could also be overexpressed. Thus, personalized targeted treatments could potentially be custom made for each individual patient. However, knowledge of the commonly deregulated genes in ovarian cancer needs to be better established, but the increased levels of PKC α and cyclin E provide a piece of that puzzle. For example, because gene amplification of *CCNE1* leads to chemo-resistant tumors (Etemadmoghadam 2009), knowledge of *CCNE1* levels would allow an oncologist to examine these patients more frequently after they have undergone their treatment, and cyclin E-CDK2 inhibitors could potentially be included in their treatment regimen. Thus, an in-depth understanding of the genes/pathways commonly involved in ovarian tumorigenesis coupled to identifying an affordable array technique to classify these pathways in individual patients is what I believe to be the next generation of ovarian cancer treatment.

We have contributed to this cancer pathways analysis by identifying several protein expression levels that are up or down regulated upon exogenous PKC α expression (Chapter 2, Figure 13 and Supplemental Figure S6) using the reverse phase protein array (RPPA) assay as described in Chapter 2, which identifies changes in protein levels. Many of these proteins are involved in the PI3K pathway (Chapter 2, Figure 13). One of the proteins in the PI3K pathway that could potentially link the PKC α /cyclin E pathway is STAT3 (see Chapter 2: Discussion for details). However, others have yet to be linked at all with PI3K. While the RPPA analysis resulted in an excellent starting or reference point, further analysis would need to be performed in order to validate these findings, including transient silencing of endogenous PKC α and Western blot analysis. Also, in order to determine the mechanism of regulation, I would do qRT-PCR analysis.

Potential gaps/questions raised by this dissertation project

As with any research project, while this project answered many questions in regard to the regulation of cyclin E by PKC α , many gaps are present that would potentially be experimentally addressed. For example, all experiments in this dissertation project were performed *in vitro*. I believe one of the most important questions would be if IGROV (ATM-sensitive) and OVCAR3 (ATM-insensitive) cell lines (see Chapter 3; Figure 1) were injected into nude mice, would these cell lines exhibit similar effects *in vivo*? For example, *in vivo* inhibition of PKC α in IGROV xenografts result in a decrease in cyclin E levels/activity and would this be the reason for ATM-sensitivity (assuming that these cells would also be ATM-sensitive *in vivo*)? Also, would the OVCAR3 cell line still be ATM-insensitive *in vivo*? If OVCAR3 cells did become sensitive to ATM *in vivo* would it follow the same cyclin E-dependent mechanism, and if so, why was this mechanism present *in vivo* and not *in vitro* in this cell line?

Along the same line of thinking, another important question would be if overexpression of PKC α (for example in a PKC α -transgenic or inducible-PKC α *in vivo* mouse model) would be sufficient to induce cyclin E activity and cause tumor formation? Using these PKC α transgenic mice, one could examine the downstream effects in the tumor versus the normal tissue over a period of time to see if genomic instability is induced. Also, using this mouse model, we could examine the effect of PKC α overexpression on LMW-E generation, this could help us better understand if the generation of LMW-E is simply due to the increase in cyclin E in already transformed cells versus cells that would likely not have the cyclin E cleavage machinery (i.e. elastase) already established. We have shown that overexpression of cyclin E can cause an increase in elastase levels creating a feedback loop (Nanos-Webb 2011 *in revision*), but is this increase enough to generate the LMW-E isoforms in the PKC α transgenic model? Similarly, if these low forms of cyclin E are generated, is this sufficient to induce centrosome amplification and genomic instability? I think the most important question that one could ask, using these PKC α transgenic mice, would be if these mice do exhibit tumor formation, would cyclin E-CDK2 inhibitors be sufficient to decrease the tumor volume? Addressing this question would help us determine if PKC α is manifesting its effect on tumorigenesis solely through increases in cyclin E (and LMW-E).

Other questions that could arise due to this dissertation project are: what is the exact mechanism of PKC α 's regulation of cyclin E. We have shown that cyclin E stability is regulated by PKC α (Chapter 2; Figure 11). However, we did not see any changes in FBW7 mRNA expression (Chapter 2; Figure 12). We assume that because we used MG132 to block

the proteasome (Chapter 2; Figure 11), that the regulation of cyclin E by PKC α is proteasomal. In order to completely rule out FBW7 as the mechanism for this increased degradation, we would need to examine FBW7 protein levels or localization. However, limitations in the commercially-available anti-FBW7 antibodies have made this difficult to examine, but recently some antibodies, while weak, have emerged and FBW7 overexpression could now potentially be examined. Another possibility would be to examine the localization of FBW7 in response to alteration in PKC α levels using immunofluorescence.

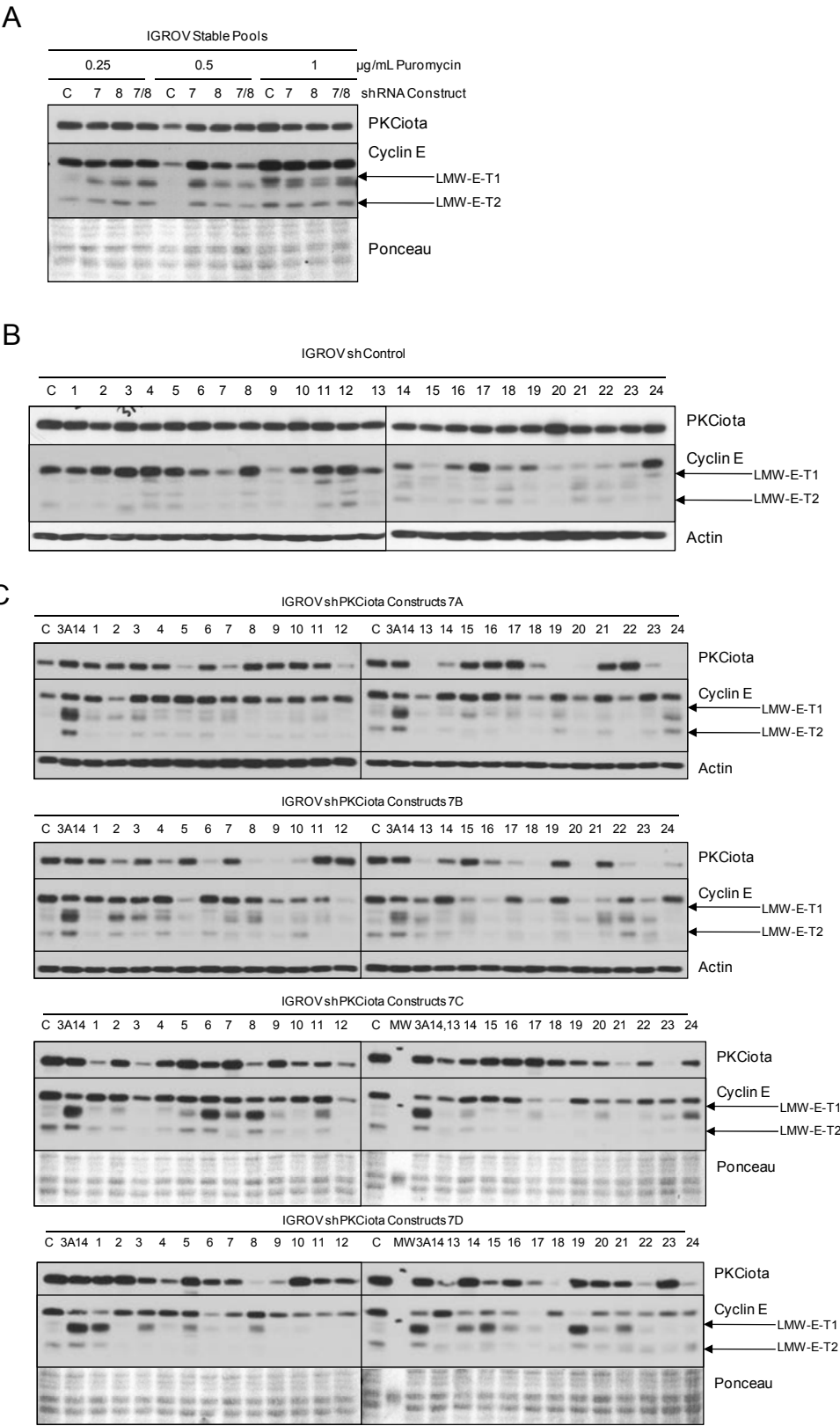
Other effector molecules in the PKC α /cyclin E pathway are yet to be validated. While the RPPA analysis of PKC α -overexpressing 293T cells resulted in a slew of PI3K pathway proteins being altered, whether these factors are all in the PKC α /cyclin E pathway is unknown (Chapter 2; Figure 13). Many of the proteins in the PI3K pathway have never been shown to affect cyclin E levels, thus an siRNA array of the PI3K family member would be useful in determining exactly which PI3K pathway proteins are involved in the PKC α /cyclin E pathway. By using an siRNA array, we would be able to individually examine which genes in the PI3K pathway, if not expressed, would lead to decreases in the levels of cyclin E. This would allow us to further understand exactly which genes within the PI3K pathway are responsible for the regulation of cyclin E. These genes could then be validated by either knocking down or overexpressing them and determining the result on cyclin E regulation. Based on the proteins involved, we would examine the effect of these proteins on mRNA expression and protein stability.

Another gap that exists in this dissertation is cyclin E's involvement with other PKC α functions. While we were able to show that cyclin E was necessary for PKC α -driven proliferation (Chapter 3; Figure 2) and transformation (Chapter 3; Figure 7), but cyclin E was not involved in PKC α -driven migration (Chapter 3; Figure 8), many other functions of PKC α exist in which cyclin E's role downstream of PKC α has not been addressed such as: (1) tumor growth, (2) glucose metabolism, (3) survival and chemoresistance, and (4) invasion. It seems quite feasible that many of these functions of PKC α may also require cyclin E. For example, current research suggests that (1) LMW-E plays a fundamental role in the *promotion of tumorigenesis* as determined by a cyclin E transgenic mouse model (Akli 2007; Akli, 2011), (2) recent data in our lab shows that cyclin E (full length as well as LMW-E) binds to ACLY, which is involved in *glucose metabolism* (Szymanski, dissertation in progress Keyomarsi lab), (3) *CCNE1* was recently identified as one of the key markers of *chemoresistance* in ovarian cancer (Etemadmoghadam 2009), and (4) LMW-E plays a vital role in *invasion* (Duong, dissertation in progress Keyomarsi lab). Thus, the role of cyclin E in

these functions of PKC α is completely feasible, and research could be performed to link these functions of PKC α to cyclin E. Much like we examined the role of cyclin E in PKC α -induced transformation and migration, the experiments to determine the role of cyclin E in these other cellular function could be performed by knockdown of PKC α followed by exogenous addition of cyclin E (EL or LMW-E) with different readouts. Instead of measuring soft agar colony formation or migration, we will examine the effect of cyclin E on the ability to revert the effects of PKC α knockdown on tumor formation, glucose transport/uptake, chemoresistance, and invasion.

SUPPLEMENTAL MATERIAL

Supplemental Figure S1.



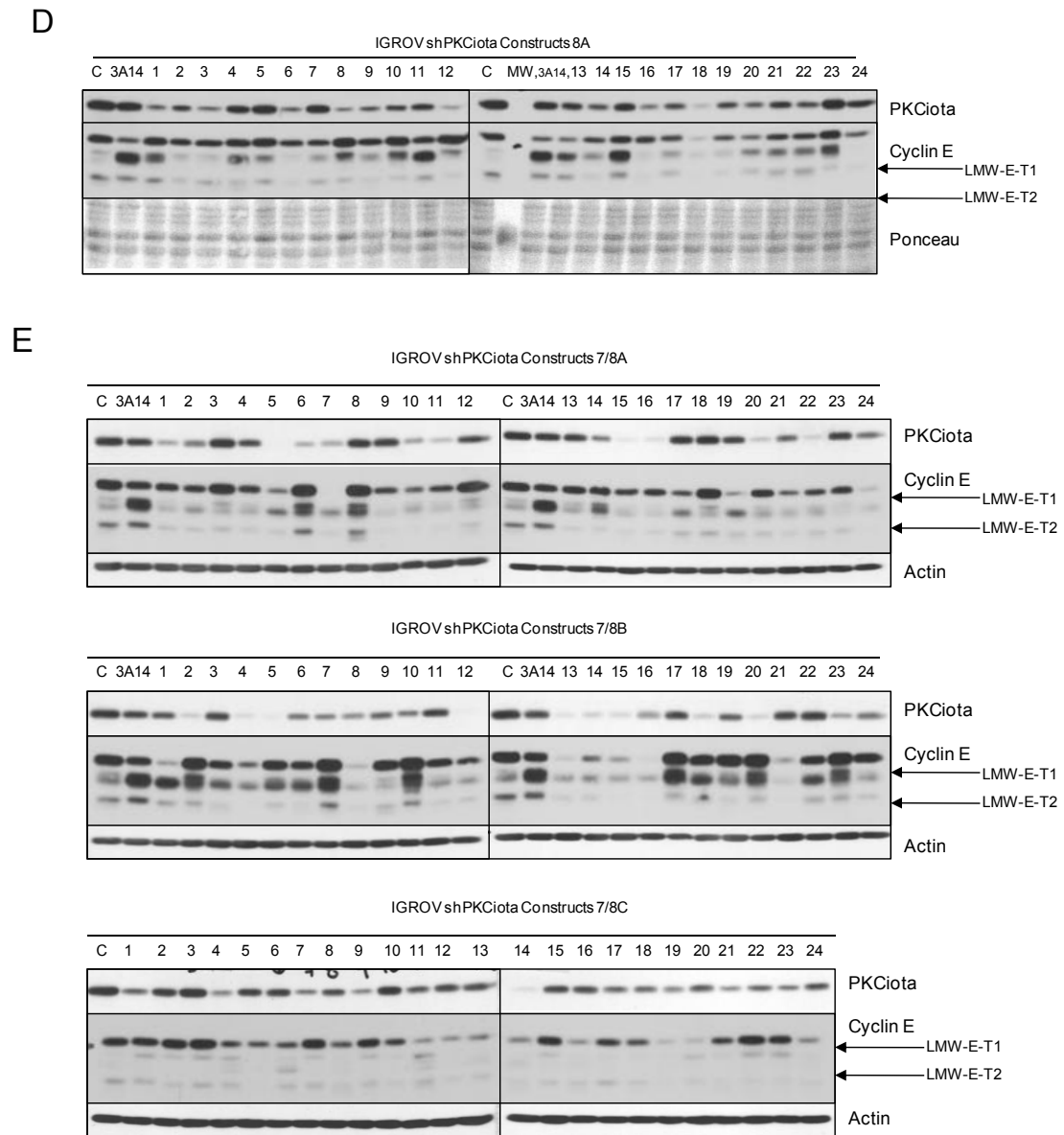


Figure S1: Selection of Stable IGROV shRNA Clones

(A) shControl or shPKCiota (constructs 7, 8, or 7/8) were transfected into IGROV cells, and stable pools were generated using increasing concentrations of Puromycin (0.25, 0.5, and 1 $\mu\text{g/mL}$). Western blot analysis using PKCiota and cyclin E (HE-12) antibodies was performed. Ponceau was used as a loading control. Stable clones of shControl (B), shPKCiota construct 7 (C), 8 (D), and 7 and 8 (E) were also examined by Western blot analysis. Each blot contained a parental control (C) and/or a stable shControl control (clone 3A14). Either Ponceau stain or actin were used as loading controls. Several clones were determined to be 'potential positives' and were examined further.

Supplemental Figure S2 and S3.

To validate the effect of PKC ϵ knockdown on cyclin E in another cell line, we used OVCAR3 cells based on the initial findings that cyclin E and PKC ϵ are highly overexpressed in these cells (Figures 1 and Table 1). OVCAR3 shPKC ϵ clones were generated similarly to the IGROV shPKC ϵ clones. However, OVCAR3 cells were more sensitive to Puromycin than IGROV cells (IGROV- Figure S1a; OVCAR3- Figure S2a). In OVCAR3 cells, 0.1 μ g/mL Puromycin was determined to be the optimal concentration to obtain single shPKC ϵ clones. Similar to the IGROV cells, the stable pools of OVCAR3 cells with shPKC ϵ 7, 8, or 7/8 appeared to have decreased cyclin E levels compared to the shControl clones. OVCAR3 shPKC ϵ clones were picked and analyzed by Western blot analysis using antibodies against PKC ϵ and cyclin E (Figure S2b). Several OVCAR3 shPKC ϵ 'potential positive' clones were identified and of these, several were chosen. 5 shControl clones and 9 shPKC ϵ clones were re-grown and analyzed at comparable confluencies (Figure S2c). Unlike IGROV shPKC ϵ clones, the OVCAR3 shPKC ϵ clones did not exhibit changes in the level of cyclin E (either cyclin E or LMW-E). Thus, an unknown factor between IGROV and OVCAR3 cells is involved in PKC ϵ -mediated cyclin E regulation. Another possible explanation for the difference between regulation of cyclin E by PKC ϵ in these two cell lines could be in the ability of OVCAR3 cells to revert.

Several IGROV shPKC ϵ clones reverted after only one or two passages post transfection. Because cyclin E is essential for cell growth, many of these clones were able to up-regulate cyclin E in a PKC ϵ -independent manner. This selection for cyclin E-expressing cells could be due to the inability of the cells with down-regulated cyclin E to proliferate. To test this theory, OVCAR3 cells would be transiently transfected with siRNA to PKC ϵ and the results would be measured over a period of 24-72 hours post transfection to prevent possible selection for cyclin E-expressing cells. OVCAR3 cells transfected with siRNA (siPKC ϵ) over increasing time (Figure S3a) and increasing amount of siPKC ϵ (Figure S3b) were analyzed by Western blot analysis with antibodies directed against PKC ϵ and cyclin E. In both conditions (increasing time and increasing siRNA), the levels of cyclin E (both cyclin EL and LMW-E) decrease. Examining increasing amounts of siRNA to PKC ϵ over increasing time resulted in mixed results (Figure S3c). The level of cyclin E did not correlate with the level of PKC ϵ knockdown in this experiment. Examination of OVCAR3 cells at differing confluencies revealed that these cells vary too greatly to examine down-stream effects on cyclin E expression quantitatively (Figure S3d). Thus, OVCAR3 cells were not used in experiments from this point forward where cyclin E was measured as the downstream effect.

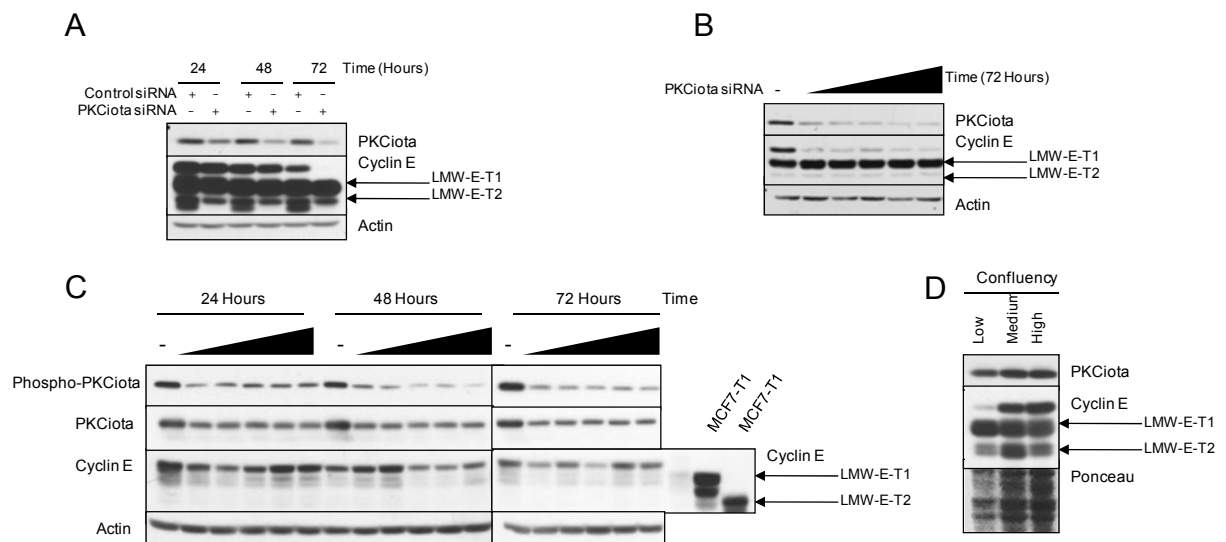


Figure S3: Transient Knock-down of PKCιota in OVCAR3 Cells

(A) Transient siRNA (either control- scrambled or PKCιota) was delivered over 24, 48, and 72 hours. Western analysis was performed using PKCιota, cyclin E, and actin (loading control) antibodies. Upon silencing of PKCιota, both full length cyclin E and LMW-E decreased. (B) Similar results were observed upon increasing amounts of PKCιota siRNA at 72 hours. Western blot analysis was similar to above. (C) When parts (A) and (B) were repeated, cyclin E levels did not correlate with PKCιota knock-down as before. Western blot analysis was similar to above plus phospho-PKCιota antibody. (D) Examination of PKCιota and Cyclin E protein levels over different cell confluencies revealed that PKCιota and cyclin E levels change substantially, perhaps accounting for the differences between (A), (B), and (C).

Supplemental Figure S4.

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Identities = 987/998 (98%), Gaps = 8/998 (0%)
Strand=Plus/Plus

PKCiota Control
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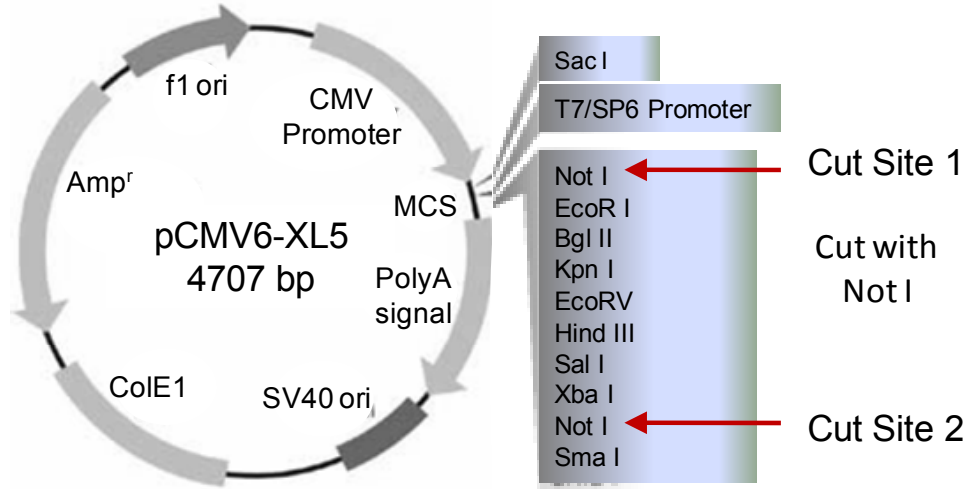
Figure S4: Sequence Results for Generation of PKCiota CA and DN Constructs.

The sequencing data for colonies CA3a and DN3a as well as a control (Wt) are pictured here. This confirms site-directed mutagenesis of Figure 8.

Supplemental Figure S5.

Before selection of IGROV cells transfected with PKC α could be performed, the pCMV-XL5-PKC α Wt, CA, and DN constructs would need to be subcloned into the pcDNA3.1+ vector (Figure S5a). The pCMV6-XL5-PKC α Wt, CA, and DN constructs could be cut with the restriction enzyme, NotI. The PKC α inserts and the cut pcDNA3.1 vector would be gel purified (Figure S5b) and ligated at a 1:3 (vector to insert ratio). The colonies that transformed into bacteria were sent for sequencing. In the first round of sequencing, all inserts were in the reverse orientation (data not shown). However, the second round of sequencing revealed 2 to 4 clones in the positive orientation (Figure S5c- white arrows; Figure S5d- sequencing data). Constructs circled in red (Wt-14C; CA-15B; and DN-16B) were oriented properly (Figure S5d), and were used in further experimentation.

A pCMV6-XL5-PKCiota WT, CA, and DN



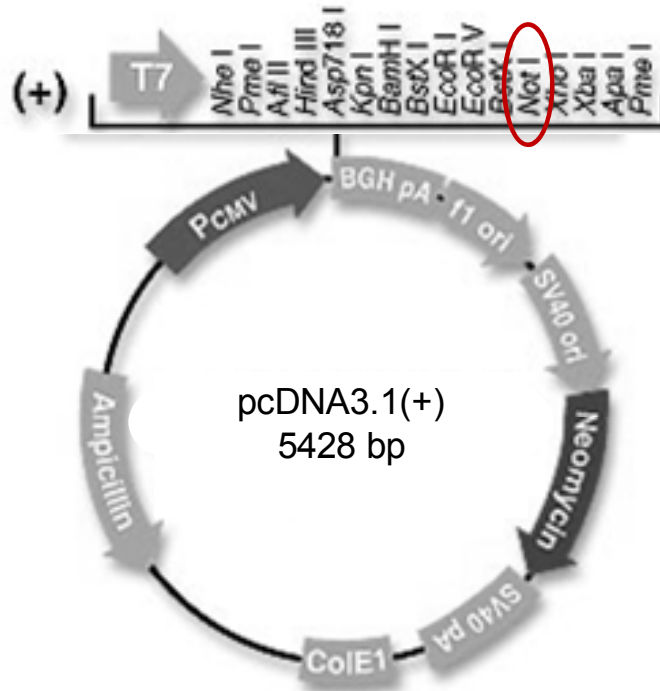
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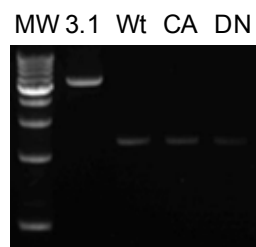
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Sequence for Orientation

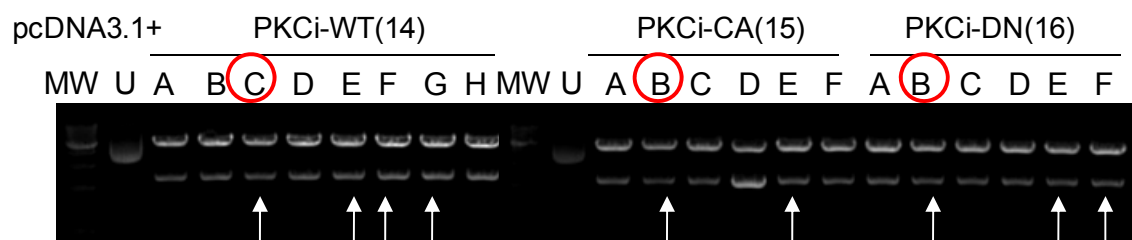
pcDNA3.1+ PKCiota WT, CA, and DN



B



C



D

GENE ID: 5584 PRKCI | protein kinase C, iota [Homo sapiens]
(Over 10 PubMed links)

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Strand=Plus/Plus

pcDNA3.1+ PKCiota CA
(Clone 15B)

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(Over 10 PubMed links)

Score = 1668 bits (903), Expect = 0.0
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Strand=Plus/Plus

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(Clone 16B)

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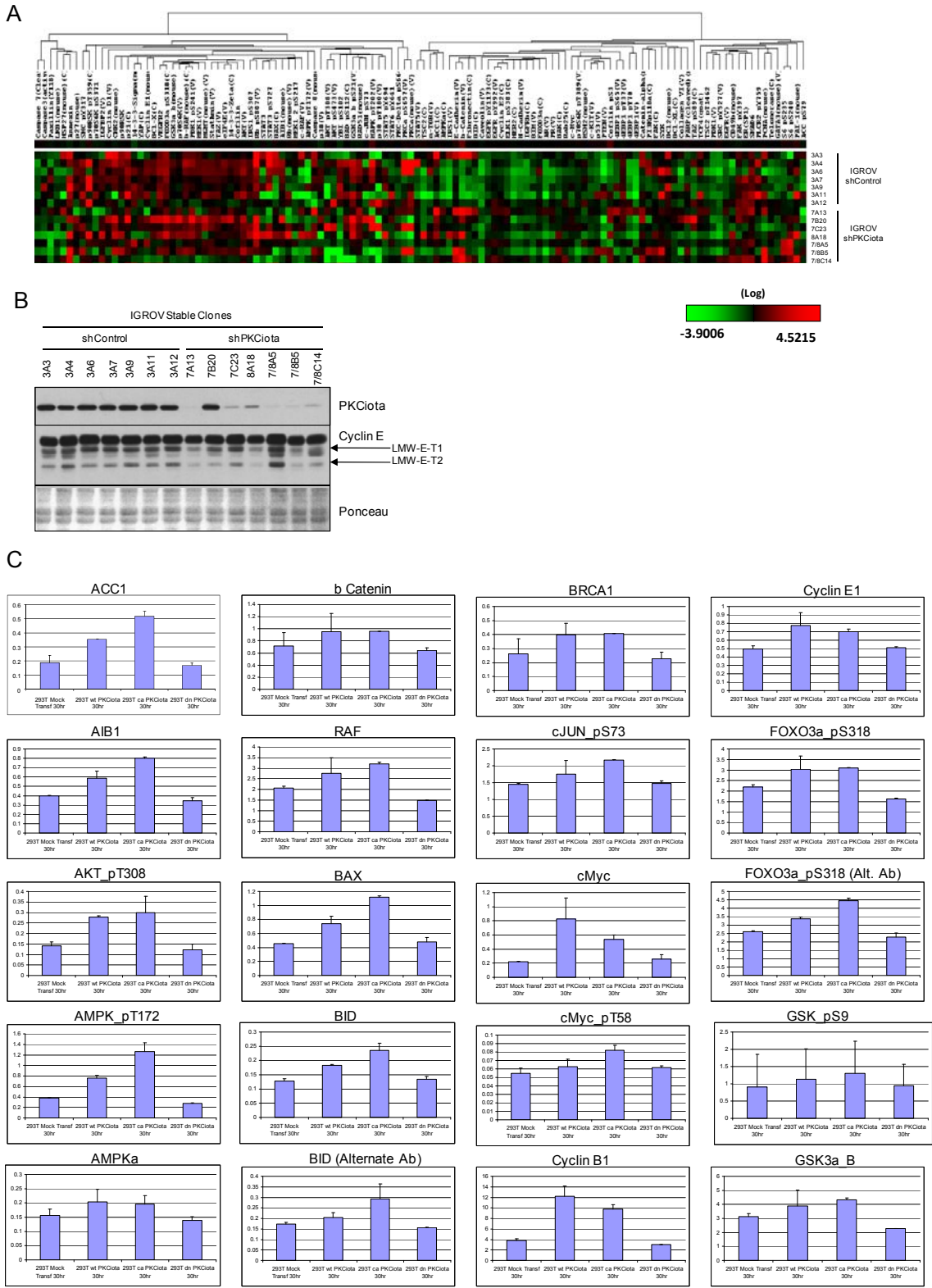
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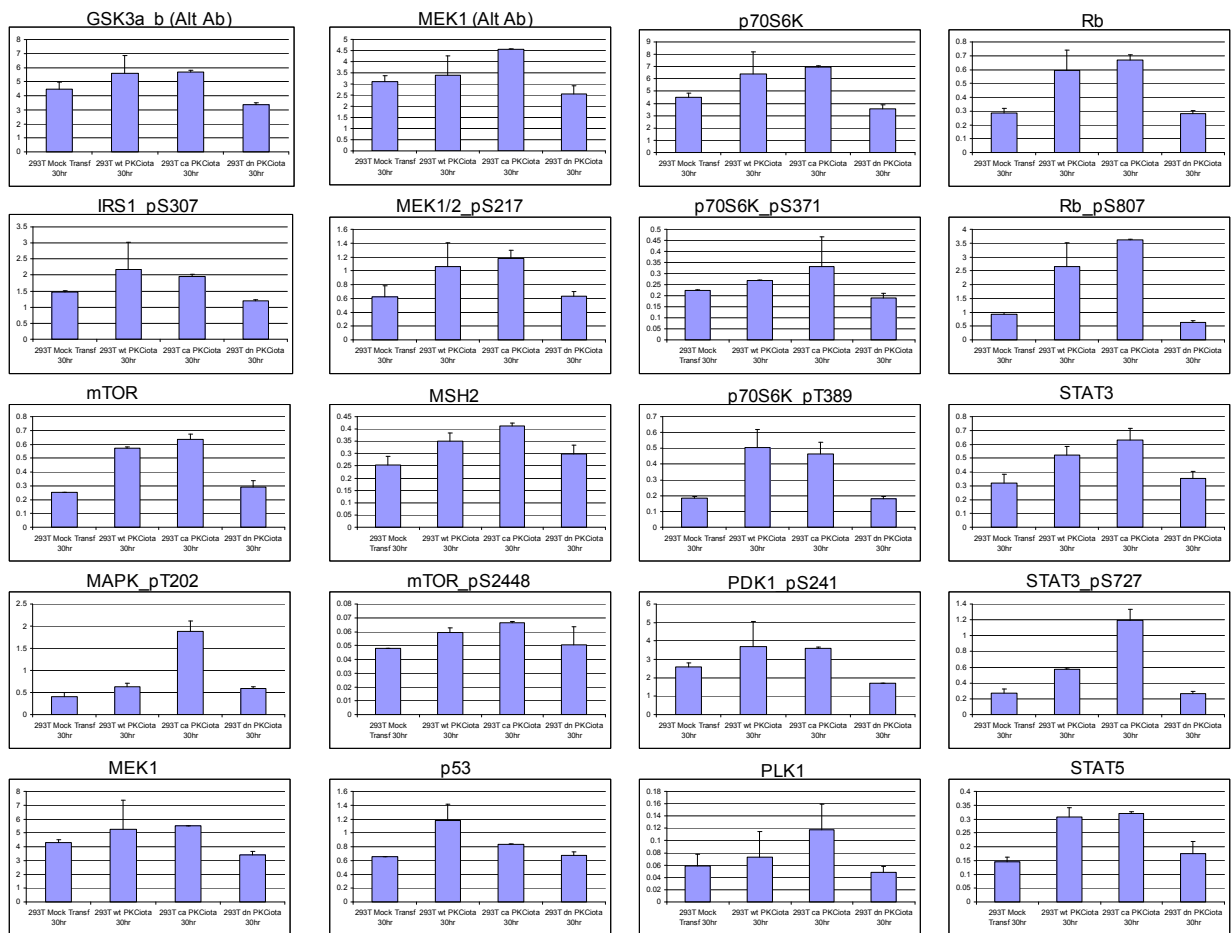
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Figure S5: Subcloning of the PKCiota Gene into the pcDNA3.1+ Vector

(A) Schematic of the steps involved in subcloning of pCMV6-XL5 PKCiota Wt (wildtype), CA (constitutively active), and DN (dominant negative) into pcDNA3.1(+)-PKCiota Wt, CA, and DN. pCMV6-XL5-PKCiota was cut at NotI sites, the insert was gel purified, and then ligated at a 1:3 (pcDNA3.1+ vector: PKCiota insert) ratio and sent out for sequencing. (B) The vector (pcDNA3.1+) and inserts (PKCiota Wt, CA, and DN) were run on a 1% agarose gel and purified for ligation. pcDNA3.1(+)-PKCiota Wt, CA, and DN colonies were sent for sequencing (D) and analyzed by gel electrophoresis (C). In (C), the arrow indicates that the clone was in the correct orientation and contained the necessary mutation. The red circle indicates this clone was chosen for DNA amplification for future experiments. "MW" is the molecular weight ladder, and "U" indicates uncut pcDNA3.1+ vector control. (D) The sequences for clones 15B (positive pcDNA3.1(+)-PKCiota CA) and 16B (positive pcDNA3.1(+)-PKCiota DN).

Supplemental Figure S6.





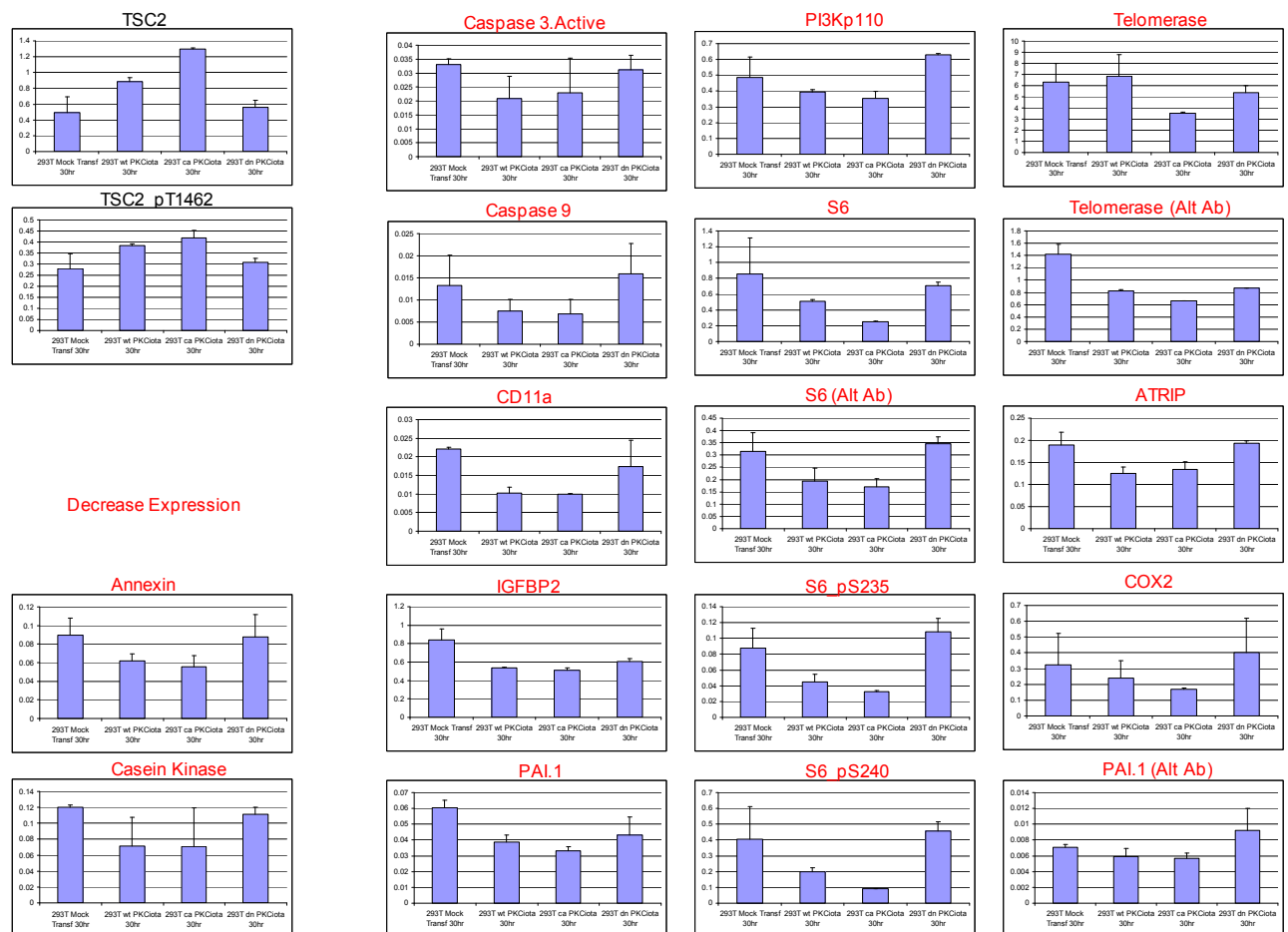


Figure S6: RPPA Analysis of IGROV Cells with PKCιota Knockdown

(A) Reverse Phase Protein Array (RPPA) heatmap for IGROV samples analyzed. IGROV shControl (first 7 samples) and shPKCιota (next 7 samples) were compared. (B) Western blot analysis depicting the IGROV shRNA stable clones used in the RPPA assay (first 14 lanes in (A)). Antibodies against PKCιota and cyclin E were used, while Ponceau stain was used as a loading control. (C) Graphs of proteins from the chart in (Figure 13c) whose expression levels either increased (black letters in title) versus decreased (red letters in title) in response to PKCιota overexpression. 'Alt Ab' or alternate antibody after a title indicates that two antibodies against this particular protein were examined.

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VITA

Angela Nanos Webb was born in San Diego, CA in 1981. Angela attended several private schools, in elementary school, before being homeschooled from grades 4th through 9th. For high school, Angela attended a college preparatory school in San Antonio, TX names SACS, where she graduated in the top 10% of her class. Angela went on to study at Texas A&M University in the spring semester of 2000, where she double majored in biochemistry and genetics. Angela joined a laboratory in the medical school, under the direction of Van Wilson, PhD. Under his guidance, Angela researched the characterization of SUMO1 and UBC9 promoters. Upon graduation (*cum laude*), Angela joined Dr. Wilson's lab as the lab manager, and continued working on her undergraduate project for another 1 and ½ years, before marrying an aspiring architect and beginning graduate school, at the University of Texas- Graduate School of Biomedical Sciences (UT-GSBS). In graduate school, Angela was quite involved with the Cancer Biology Program, and she went on to study ovarian cancer and PKC α regulation of cyclin E under the mentorship of Khandan Keyomarsi, Ph.D., who initially observed and characterized the tumor-specific forms of cyclin E (LMW-E). After graduate school, at MD Anderson Cancer Center, Angela plans on testing the effects of different drug combinations on synergistic cancer cell death in a breast cancer model system under the guidance of Drs. Khandan Keyomarsi, Ph.D. and Kelly Hunt, M.D.