THE EFFECT OF PHOSPHOLIPASE D ON TGF-beta SIGNALING IN CANCER SURVIVAL

by

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ABSTRACT

THE EFFECT OF PHOSPHOLIPASE D ON TGF-beta SIGNALING

IN CANCER SURVIVAL

by

Noga Gadir

Advisor: Professor David A. Foster

MDA-MB-231 human breast cancer cells have a survival signal generated by phospholipase D (PLD) that involves the activation of the mammalian target of rapamycin (mTOR) and mitogenic activated protein kinase (MAPK). In the absence of serum, rapamycin induces apoptosis in MDA-MB-231 human breast cancer cells. However, in the presence of serum, rapamycin induces G1 cell cycle arrest – indicating that a factor(s) in serum suppresses rapamycin-induced apoptosis. We find that transforming growth factor-β (TGF-β) suppresses rapamycin-induced apoptosis in serum-deprived MDA-MB-231 cells in a protein kinase Cδ (PKCδ)-dependent manner. Importantly, if TGF-β signaling or PKCδ was suppressed, rapamycin induced apoptosis rather than G1 arrest in the presence of serum. If cells were allowed to progress into S-phase, rapamycin induced apoptosis in the presence of serum. We also examined the effect of rapamycin on cancer cell lines harboring genetic defects in TGF-β signaling, and as expected, rapamycin induced apoptosis in these cells in the presence of either

serum or TGF-β. Thus, in the absence of TGF-β signaling, rapamycin becomes cytotoxic rather than cytostatic.

MDA-MB-231 breast cancer cells are resistant to the growth arresting effects of TGF-β. Since mTOR is a target of PLD signals and mTOR suppresses TGF-β signaling, we investigated whether the elevated PLD activity in MDA-MB-231 cells is critical for the reported suppression of TGF-β signaling in these cells. Suppression of PLD activity and/or expression resulted in increased activation of Smads and in addition suppressed phosphorylation of Smad2 on sites that are phosphorylated by MAP kinase and negatively regulate TGF-β signaling. Suppression of PLD also led to a predictable response of known TGF-β downstream targets like $p21^{Cip1}$, $p27^{Kip1}$and pRb.

The data presented in this work indicate that the suppressed TGF-β signaling in MDA-MB-231 cells is due to elevated PLD activity and is mediated by mTOR and MAP kinase. These results point out that the survival signals generated by PLD involve the suppression TGF-β signals and prevention of G1 arrest. Importantly, this study provides evidence indicating that tumors with defective TGF-β signaling – common in colon and pancreatic cancers – will be selectively sensitive to rapamycin or other strategies that target mTOR.

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And above all, to the love of my life, Ofer – this is for you.

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INTRODUCTION

1.1 TRANSFORMING GROWTH FACTOR β (TGF-β) AND SMADS

1.1.1 TGF-β FUNCTION: THE DUAL ROLE OF TGF-β IN CANCER

Transforming growth factor-β (TGF-β) is a 25 kDa multifunctional autocrine/paracrine growth regulator belonging to the large TGF-β superfamily (Massagué, 1992). Almost all cell types produce and secrete TGF-β and have receptors that respond to it. TGF-β is involved in a large range of biological processes, including embryonic development, wound-healing and angiogenesis. In its normal state, the TGF-β pathway restricts cell growth, differentiation, and cell death (Guo and Kyprianou, 1999; Tang *et al*, 1999; Akhurst and Derynck, 2001; Wakefield and Roberts, 2002). TGF-β was first identified in 1978 as a factor secreted from cancer cells that could produce a transformed phenotype in a nontransformed cell line in culture (Roberts and Sporn, 1985). There are five isoforms, TGFβ-1 through 5; all share the same receptor, when TGFβ-1 is the most predominant (Massagué, 1998). With the finding that TGF-β was a potent growth inhibitor of epithelial cells, and the identification of inactivating mutations within the TGF-β signaling pathway in cancers it became clear that TGF-β signaling is a tumor suppressor pathway for early stages of cancer. However many human carcinomas overexpress TGF-β and this is associated with poor patient prognosis and increased frequency of metastasis (Kretzschmar *et al.*, 1999). Thus, there is a growing body of evidence for a duality of function for the role of TGF-β in cancer that is “stage specific”.

TGF-β plays a dual role in tumorigenesis. On one hand, TGF-β inhibits the proliferation of normal epithelial, endothelial, and hematopoietic cells, thus being crucial for the homeostasis of these tissues (Massagué 1992; Roberts and Sporn 1993; Alexandrow and Moses 1995). On the other hand, TGF-β can intensify the malignant phenotype at later stages of tumorigenesis (Cui *et al*. 1996; Barrack 1997; Factor *et al*. 1997; Reiss and Barcellos-Hoff 1997). TGF-β is abundantly expressed in various tumors of epithelial origin (Derynck *et al*. 1985; Keski-Oja *et al.* 1987) in which it can suppress immune surveillance (Letterio and Roberts 1998), promote tumor invasion (Cui *et al*. 1996), and advance the development of metastases (Welch *et al*. 1990; Yin *et al*. 1999). These effects become evident in tumor cells that retain TGF-β receptors but have lost the capacity to respond to TGF-β-induced growth arrest. Such a state of altered TGF-β responsiveness is observed in Ras-transformed cells. These cells typically exhibit a limited growth inhibitory response to TGF-β (Schwarz *et al*. 1988; Houck *et al*. 1989; Valverius *et al*. 1989; Longstreet *et al*. 1992; Filmus and Kerbel 1993) but may respond to TGF-β with invasive activity (Oft *et al*. 1996) and metastatic behavior (Oft *et al*. 1998; Yin *et al*. 1999).

The main event that leads to TGF-β- mediated growth arrest is TGF-β-induced expression of the cyclin kinase inhibitors $p15^{INK4b}$,(Hannon and Beach, 1994), $p21^{CIP1}$ (Datto *et al*., 1995), and $p27^{KIP1}$ (Polyak *et al*., 1994), which block cyclin (Cyclin A or Cyclin E) and cyclin-dependent kinases from phosphorylating the retinoblastoma protein (Rb), thus allowing the hypophosphorylated form of Rb to bind and sequester the E2F transcription factor, and second by directly

suppressing c-Myc expression. (Reynisdottir *et al*., 1995; Sandhu *et al*., 1997; Massagué *et al*., 2000; Donovan and Singerland, 2000) (Figure 1.1.1).

Depending on cell type, there are further mechanisms that contribute to TGF-β- mediated growth arrest. For example, TGF-β inhibits the expression of CDK4 and CDK6 (Iavarone and Massagué, 1997; Iavarone and Massagué 1999) and as mentioned above, c-Myc. High levels of c-Myc (common in cancer) provide the cells with resistance to the growth inhibitory activity of TGF-β, and downregulation of c-Myc is required for the induction of $p15^{INK4B}$.

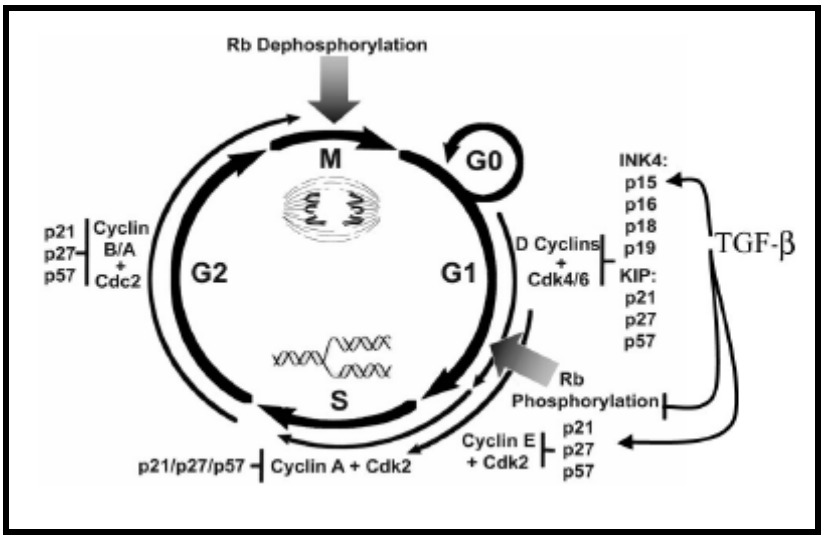

Figure 1.1.1: Mechanisms of cell cycle arrest by TGF-β (Donovan and Singerland, 2000)

Cell cycle progression is governed by cyclin-dependent kinases (cdks), the activities of which are regulated by binding of cyclins, by phosphorylation and by the cdk inhibitors [the inhibitor of cdk4 (INK4) family: p15, p16, p18 and p19; and the kinase inhibitor protein (KIP) family: p21, p27 and p57].

TGF-β has also been shown to induce apoptosis in several cell types (Rotello *et al*., 1991; Oberhammer *et al*., 1992; Chaouchi *et al*., 1995) by unclear

mechanisms and Smad3 or Smad4 shown to induce apoptosis as well (Atfi *et al*., 1997; Dai *et al*., 1999).

In spite of the wide range of cellular processes that the TGF-β signaling pathway is involved in, the pathway is relatively simple and will be elaborated in part 1.1.3. TGF-β signals mainly through two cell surface signaling receptors, TGF-β receptor type I (TβRI) and TβRII, whereby binding of ligand to TβRII promotes receptor heteromerization (Massagué, 1992), allowing the TβRII kinase to activate TβRI (Wieser *et al*, 1995). TβRI then phosphorylates and activates Smad2 and Smad3 by phosphorylating their C-terminal serines (Abdollah *et al*, 1997), a process shown to involve accessory proteins (Tsukazaki *et al*, 1998). This causes the receptor-activated Smads to multimerize (Wu *et al*, 1997) and then translocate to the nucleus, where they activate gene transcription (Xiao *et al*, 2000).

TGF-β as a tumor suppressor. The preservation of homeostasis in rapidly proliferating cells such as epithelial cells requires delicate balance between the increase in cell number by proliferation and the loss of cells by apoptosis (Hanahan and Weinberg, 2000). Although TGF-β was originally reported as an oncogene, it has been also shown to be a potent inhibitor of cell growth and an inducer of apoptosis. The fact that TGF-β primarily inhibits the proliferation of many cell types attributed to this cytokine and its signaling pathway a tumor suppressor role (Alexandrow *et al*., 1997; Markowitz and Roberts, 1996). Consequently, during tumor progression, cancer cells tend to acquire resistance

to the growth inhibitory response of TGF-β, through mutations or inhibition of components along the TGF-β signaling pathway (Kretzschmar and Massagué, 1998). For example, when TGF-β is overexprssed to the keratinocytes of mice and their skin is exposed to chemical carcinogens TGF-β initially inhibits the formation of benign skin tumors. However, in the benign tumors that form, progression to invasive carcinomas is increased (Cui *et al.*, 1996). The tumor-suppressive effects of TGF-β have been clearly demonstrated in transgenic mice models. Hemizygous or homozygous TGF-β-null animals show increased incidence of chemically or spontaneously induced tumors, respectively (Derynck *et al*., 2001; Wang *et al*., 1999; Tang *et al*., 1998; Engle *et a*l., 1999). Similarly, targeting a dominant negative TβRII to mammary or skin epithelia also enhances tumorigenesis whereas TGF-β-overexpressing mice have decreased incidence of tumors (Akhusrt and Derynck, 2001).

TGF-β as a tumor promoter. The major cause of mortality in cancer is the formation of metastases, the spread of tumor cells to distant sites in the body via the lymph system or the bloodstream. A critical regulator of breast tumor growth and metastasis formation is TGF-β. During the course of the disease, a switch in TGF-β -responsiveness of the tumor cells often occurs. TGF-β then induces the acquisition of a more invasive and tumorigenic cell phenotype with an elevated potential to form metastases. The molecular basis for this switch is mostly unclear (Akhusrt and Derynck, 2001).

Many tumor cells secrete non-physiological levels of TGF-β which may affect in an autocrine manner, the differentiation of tumor cells and the surrounding cellular environment in a paracrine manner, leading to tumor progression and metastasis (Roberts and Sporn, 1990; Massagué, 1992). The excess TGF-β affects the cell in two ways. First, it promotes angiogenesis (the overgrowth of blood vessels) that is a hallmark of tumors. Tumors use angiogenesis to grow new blood vessels that sustain their growth and metastasis. Second, excess TGF-β suppresses T cells and other components of the immune system that would normally attack aberrant cells (Elliott and Blobe, 2005). TGF-β has been implicated in each one of the hallmark steps to cancerous phenotype portrayed by Hanahan and Weinberg in 2000 (see table 1.1.1).

Table 1. TGF-β and the Hallmarks of Cancer

Hallmark	Effect of TGF-β	Example
Resistance to growth-inhibitory factors	Loss of TGF-β–induced growth inhibition	About half of human pancreatic cancers are not growth inhibited by TGF-β because of mutation of the *Smad4* gene[2]
Proliferation in absence of exogenous growth factors	TGF-β stimulates proliferation of some cancer cells	In colon carcinoma cells, TGF-β stimulates proliferation through a Ras-dependent mechanism[3]
Invasion and metastasis	TGF-β promotes invasiveness and metastasis	In prostate cancer cells, exogenous TGF-β increases secretion of plasminogen activator, the expression of which promotes extracellular matrix degradation and is correlated with a more invasive phenotype[4]
Limitless replicative potential	Loss of TGF-β–induced repression of *hTERT*	TGF-β induces telomere shortening followed by senescence in lung cancer cells[5]
Evasion of apoptosis	Loss of TGF-β–mediated apoptosis	Blocking TGF-β signaling inhibits tamoxifen-induced apoptosis in human breast cancer cells[6]
Induction of angiogenesis	TGF-β induces angiogenesis	Neutralizing TGF-β antibody decreases the angiogenesis and tumorigenesis of TGF-β–insensitive renal cell carcinoma cells in an animal model[7]
Immune system evasion	TGF-β is a potent immunosuppressent	In animal models increased TGF-β expression allows highly immunogenic cancer cells to escape immune surveillance and form tumors[8]

Abbreviation: TGF-β, transforming growth factor β.

Table 1.1.1: TGF-β and the Hallmarks of Cancer (Elliott and Blobe, 2005)

Presented are various characteristics of TGF-β in a tumor promoting context. Corresponding references are presented in the text.

One aspect of tumor cell biology that is thought to contribute to metastasis is epithelial-to-mesenchymal transformation (EMT) (Thiery and Chopin, 1999). EMT allows cells to move out of the primary tumor into circulation. TGF-β-induced EMT has been reported in Ras-transformed breast, ovarian and skin cancer (Oft *et al.*, 1996; Portella *et al.*, 1998; Kitagawa *et al.*, 1996). Transfection of dominant negative TGF-β receptors into tumors inhibited this transformation.

Tumor angiogenesis is vital for tumor growth and invasion since blood deliver nutrients and oxygen into the tumor and give the tumor access to circulation. In human breast tumors, high levels of TGF-β are associated with increased microvessel density, and poor prognosis (de Jong *et al.*, 1998). TGF-β has also been shown to induce expression of the angiogenesis-inducing factor vascular endothelial cell growth factor (VEGF) (Pertovaara *et al.*, 1994) and local administration of TGF-β neutralizing antibody strongly reduced VEGF secretion and tumor angiogenesis in nude mice (Ueki *et al.*, 1992, Stearns *et al.*, 1999).

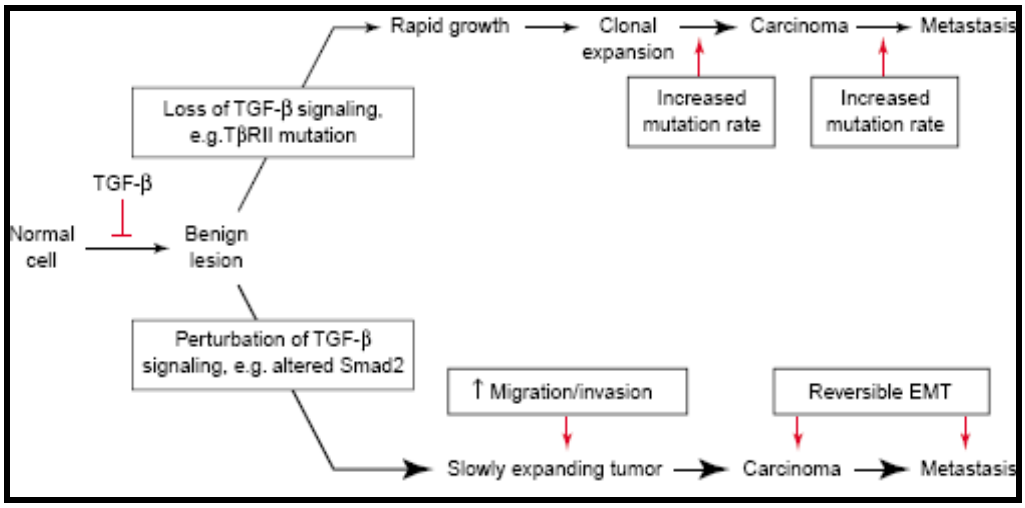

Figure 1.1.2: Dual role of TGF-β signaling in cancer progression

(Akhurst and Derynck, 2001)

Various alterations in the TGF-β signaling pathway can contribute to increased tumor progression, invasion and metastasis. Two general routes can be taken: (1) Early genetic loss of signaling components, such as TβRII, leads to rapid growth. Increased cell division increases the probability of further cancer-contributing mutations and cytogenetic changes that ultimately drive tumor progression. (2) More frequently, the TGFβ signaling pathway remains intact but is perturbed by other mechanisms. Altered Smad signaling leads to a direct increase in tumor cell plasticity, invasion and metastasis, involving reversible epithelial-to-mesenchymal transformation (EMT). Thick lines indicate the more prevalent pathway.

1.1.2 THE TGF-β RECEPTOR

Members of the TGF-β superfamily (TGF-βs, activins, bone morphogenic proteins (BMPs)) signal via heteromeric serine/threonine kinase transmembrane receptor complexes (Derynck and Feng, 1997; Massagué, 1998; ten Dijke and Hill, 2004). Based on their structural and functional properties, the TGF-β receptor family is divided into two subfamilies: type I receptors and type II receptors (Fig. 1.1.3). Binding of the ligand to its primary (type II, TβRII) receptor, a constitutively active kinase, allows the recruitment, transphosphorylation and

activation of the signaling (type I, TβRI) receptor. The latter is then able to exert its phosphorylation-dependent serine–threonine kinase activity to phosphorylate cytoplasmic protein mediators of the Smad family (Derynck *et al*., 1998; Miyazono *et al*., 2000; Shi and Massagué, 2003).

Type I and II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively (Matthews and Vale, 1991). The extracellular region is relatively short (approximately 150 amino acids), N-glycosylated (Cheifetz *et al*., 1988; Wells *et al*., 1997) and contains 10 or more cysteines.

The GS domain. A distinctive feature of type I receptors is a highly conserved 30– amino acid region immediately preceding the protein kinase domain (Figure 1.1.3). This region is called the GS domain because of a characteristic SGSGSG sequence it contains (Wrana *et al*., 1994). Ligand-induced phosphorylation of the serines and threonines in the TTSGSGSG sequence of TβRI by the type II receptor is required for activation of signaling (Wieser *et al*., 1995). Following the SGSGSG sequence, all type I receptors have a Leu-Pro motif that serves as a binding site for FKBP12 (Chen *et al*.,1997). FKBP12 may act as a negative regulator of the receptor signaling function. The GS domain is a key regulatory region that control the catalytic activity of the type I receptor kinase or its interaction with substrates (Massagué, 1998).

The kinase domain. The kinase domain in type I and II receptors match the recognized sequence of a serine/threonine protein kinase domain (Mathews and Vale, 1991). Consistent with this, TβRI receptors have been shown to phosphorylate their substrates Smad proteins on serine residues (Kretzschmar *et*

al., 1999; Abdollah *et al*., 1997), whereas TβRII receptor phosphorylate themselves and type I receptors on serine and threonin residues but not tyrosine residues (Wrana *et al*., 1994; Wieser *et al*.,1995; Matthews and Vale, 1991). Type II receptors typically contain a very short C-terminal extension following the kinase domain, whereas type I receptors have none. The regulatory region contains two serines in TβRII whose phosphorylation may enhance or inhibit the signaling activity of the receptor (Massagué *et al*., 2000).

Mechanism of receptor activation. Signals originate from TβRI when it is phosphorylated by its activator, TβRII. As first shown with TGF-β receptors (Wrana *et al*., 1992) ligand binding induces the formation of a heteromeric complex of type I and II receptors (Wrana *et al*., 1994; Lebrun and Vale, 1997; Elliotte and Blobe, 2005) (Figure 1.1.3).The heteromeric receptor complex and, in particular, the phosphorylation and activation of the type I receptor are highly dependent on ligand binding.

The cytoplasmic domain of various type I receptors interacts with FKBP12 in yeast and mammalian cells (Wang *et al*., 1994; Kawabata *et al*., 1995). FKBP12 binding to TβRI inhibits TGF-β signaling by inhibiting TβRI phosphorylation by TβRII within the receptor complex. (Chen *et al*., 1997; Wang *et al*., 1996).

The events that transduce TGF-β signals start with type II receptor–mediated activation of the type I receptor. This receptor then phosphorylates and activates SMAD proteins, which carry the signal to the nucleus. The TGF-β signaling pathway will be discussed next in part 1.1.3.

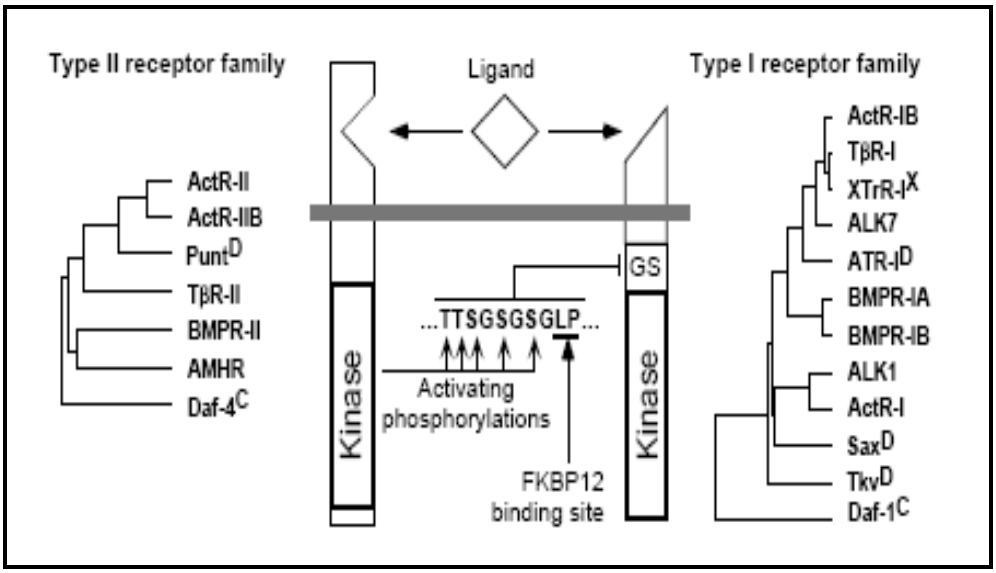

Figure 1.1.3: Type I and II TGF-β receptor families (Massagué, 1998)

In type I receptors, the protein kinase domain is preceded by the GS domain (GS). The characteristic GS sequence motif of TβRI is shown, indicating the phosphorylation sites and the FKBP12-binding site. Listed members are from vertebrates unless otherwise indicated: D, *Drosophila*; C, *Caenorhabditis elegans*; X, *Xenopus*. The dendrograms indicate the relative level of amino acid sequence similarity in the kinase domain.

1.1.3 TGF-β SIGNALING PATHWAY

The TGF-β signaling pathways are important during embryonic development, and in adult tissue are known to regulate homeostasis (Roberts and Sporn, 1993; Kretzschmar and Massagué, 1998). TGF-β family signal through receptor serine/threonine kinases and intracellular Smads proteins (Moustakas *et al*., 2001; Shi and Massagué, 2003). In addition, there are several proteins that participate in the TGF-β pathway and are mediated by receptor tyrosine kinases, G-protein coupled receptors or cytokine receptors (Massagué, 1998; Derynck *et al*., 1998).

The only well-characterized signaling effector pathway that is initiated by TGF-β is provided by the Smads, a small family of structurally related proteins (Massagué, 2000; Derynck *et al*., 1998). The Smad pathway is evolutionary conserved and is regulated by phosphorylation, ubiquitination and Smad-inhibitory events (Shi and Massagué, 20003). Smads can fall into three subgroups: (1) Smads that are direct substrates of the TGF-β receptor kinase (R-Smads), (2) Smads that associate with the R-Smads, and (3) antagonistic Smads that inhibit the signaling function of the previous groups (Massagué, 2000; Derynk *et al*., 2001). Receptor mediated Smads (R-smads) Smad2 and smad3 are activated via carboxy-terminal phosphorylation by TβRI receptor kinase. The phosphorylated R-Smads form complexes with a common mediator, Smad4, (Massagué, 1998) and enter the nucleus where they bind DNA and interact with transcription factors to regulate gene expression (see Figure 1.1.4).

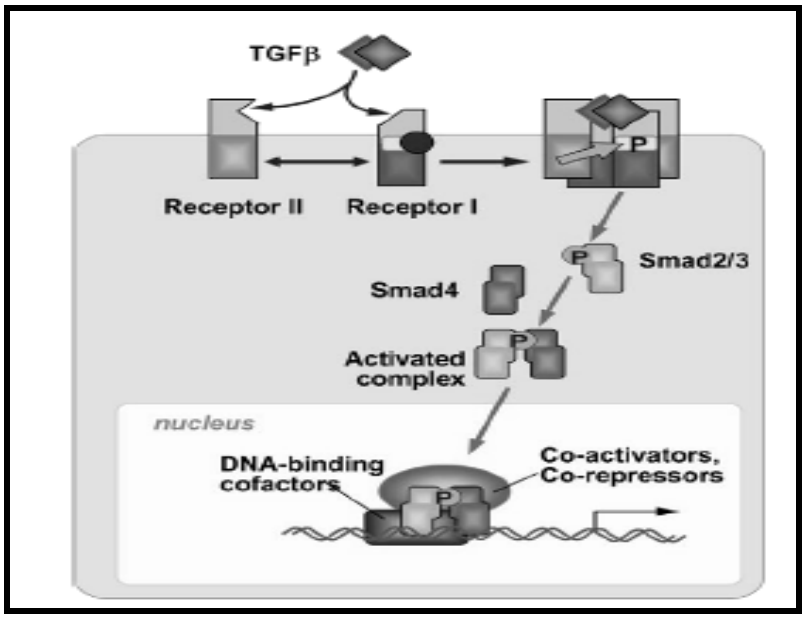

Figure 1.1.4: The TGF-β signaling pathway (Massagué and Gomis, 2006)

TGF-β binds to the Type II receptor and recruits Type I, whereby Type II receptor phosphorylates and activates Type I. The Type I receptor, in turn, phosphorylates receptor-bound Smad (Smad2/3) transcription factors at the carboxy-terminal SXS motif, releasing them from retention in the cytoplasm and allowing them to translocate into the nucleus. Smad4 acts as a common partner of activated Smads to help execute their function. Smad proteins continuously undergo nucleocytoplasmic shuttling. Once in the nucleus, activated Smad proteins form final complexes that regulate target gene transcription, generating approximately three hundred early gene responses.

Smad signaling is essential for most but not all TGF-β gene responses and non-Smad signaling has been reported (Shi and Massagué, 2000). For example, TGF-β had been shown to activate extracellular-signal-regulated kinase (ERK)-1, ERK2, p38 and c-Jun amino-terminal kinase (Jnk), as well as Mitogen-activated protein kinases (MAPKs) in various cell lines and are non-Smad dependent.

Ras/MAPK signaling also induces expression of TGF-β (Geiser *et al.*, 1991; Kretzschmar *et al.*, 1999; Derynck *et al.*, 2001), which can be enhanced

further by TGF-β signaling and can explain the often observed TGF-β increase by Ras-transformed tumor cells. Cells harboring oncogenic Ras mutations often show a loss of TGF-β antiproliferative responses. Oncogenic Ras can achieve inhibition of TGF-β signaling in mammary epithelial cells by negatively regulating Smad2 and Smad3; that is, by inhibiting their TGF-β -induced nuclear accumulation and transcriptional activity (Kretzschmar *et al.*, 1999).

Ras- inhibition of TGF-β signaling is specifically mediated by Erk MAPK which has been shown to phosphorylate Smad2 and Smad3 on a site that prevents their nuclear translocation and accumulation (Kretzschmar *et al.*, 1999) (Figure 1.1.5). These sites are separate from the carboxy terminal sites targeted by TβRI. Mutation of these MAP kinase sites in Smad3 yields a Ras-resistant form that can partially rescue the growth inhibitory response to TGF-β in Ras-transformed cells (Kretzschmar *et al*, 1999; Calonge and Massagué, 1999). This mechanism explains why many Ras-transformed cells loose their TGF-β response capacity (Kretzschmar *et al.*, 1999). MDA-MB-231 human breast cancer cells have a survival signal generated by phospholipase D (PLD) that involves the activation of mTOR and MAP kinase. TGF-β signals are suppressed in MDA-MB-231 cells and our lab has recently reported that the suppressed TGF-β signaling in MDA-MB-231 cells is due to elevated PLD activity and is mediated by mTOR and MAP kinase (Gadir *et al.,* 2007) (see Chapter IV).

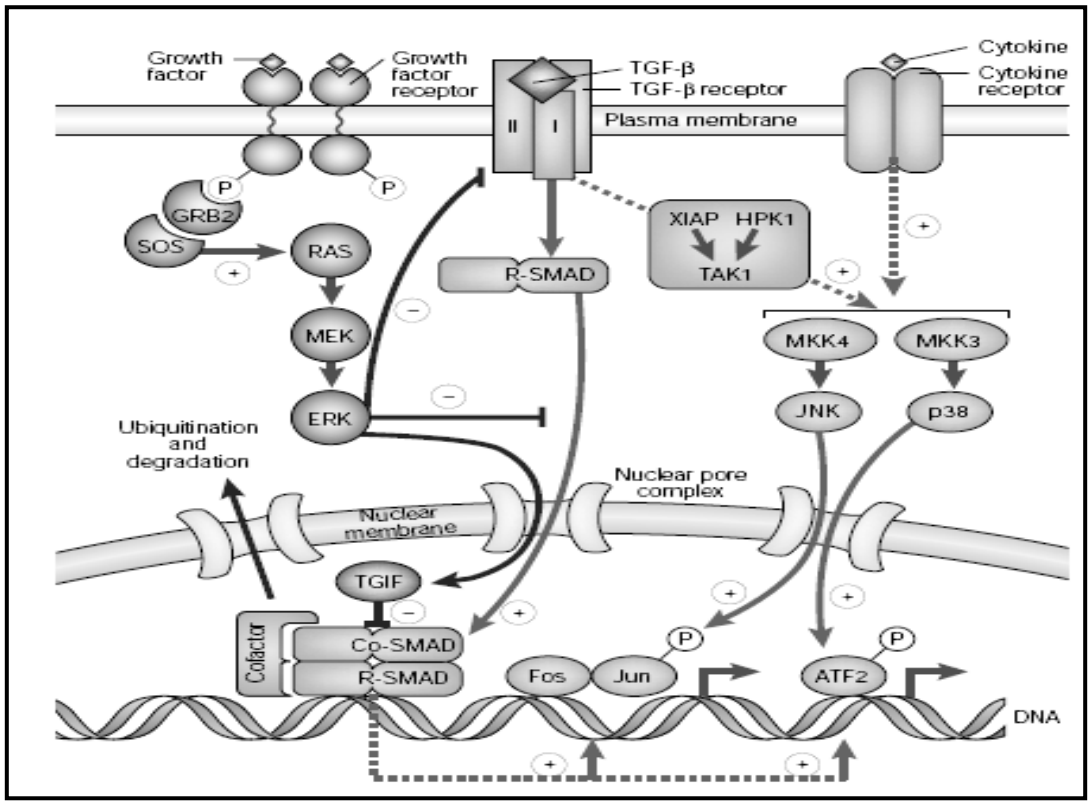

Figure 1.1.5: Crosstalk between Smad and MAPK pathway

(Massagué, 2000)

The three principal MAPK pathways in mammalian cells may affect the SMAD pathway through various mechanisms. The Ras–MEK–ERK pathway can decrease TGF-β receptor levels by controlling expression, attenuate SMAD accumulation in the nucleus by phosphorylating SMADs in the linker region and increase the level of the SMAD corepressor TGIF by stabilizing this protein. The MKK4/JNK and MKK3/p38 pathways, which can be activated by various cytokines, enhance the activity of Jun and ATF2 transcription factors that may cooperate with SMADs through direct physical contacts. In certain cell types and conditions, the MKK4/JNK and MKK3/p38 pathways are reportedly activated by TGF-β itself, and the proteins XIAP, HPK1 and TAK1 might be involved in this link. The direct nature and physiological relevance of these interactions remain to be established.

Cancer	TβRII Gene	Smad2 Gene	Smad4 Gene
Breast	Frequently downregulated, rarely mutated[149]	No mutations detected in approximately 100%[150]	No mutations detected in approximately 100%[150]
Colon	Mutated in 68%-82% of RER-positive tumors and 15% of RER-negative tumors[33,151]	Mutated in 6%[152]	Mutated in 20% invasive tumors and 6% noninvasive tumors[152]
Lung	Frequently downregulated, particularly in SCLC, but rarely mutated[26]	Mutated in 2% NSCLC; normal in almost all SCLC[153]	Mutated in 7% NSCLC; normal in almost all SCLC[154]
Pancreatic	Mutated in 4%[155]	No mutations detected in ~100%[33]	Mutated in 50%[7]
Prostate	TβRII protein not detected in 24%[135]	No mutations detected in ~100%[157]	No mutations detected in approximately 100%[158]

Table 1.1.2: Mutation of components of the TGF-β signaling pathway in cancer (Elliott and Blobe, 2005)

1.1.4 TGF-β AND SMAD REGULATION IN BREAST CANCER

The growth and differentiation of normal breast cells are two closely related processes that are regulated by growth factors and TGF-β plays an important role in the regulation of breast cell differentiation. The growth of normal breast cells is tightly regulated, and in response to environmental stimuli, breast cells can differentiate to perform special functions such as producing milk. Cancer cells, in addition to uncontrolled growth, no longer respond to environmental signals and fail to perform breast-specific functions.

In breast cancer, TGF-β has been suggested to play a dual role (Reiss and Bracellos-Hoff, 1997). It acts as a tumor suppressor in early stages of the disease when it inhibits the outgrowth of carcinomas using its antiproliferative functions. This has been established in transgenic mouse models, in which overexpression of TGFβ-1 was targeted to the mammary gland, and tumor

formation was induced by simultaneous overexpression of TGF-β and administration of a carcinogen (Welch *et al*., 1990). In later stages of the disease, TGF-β is believed to promote tumor progression, in part by enhancing tumor cell motility and invasiveness (Oft *et al*., 1996; Welch *et al*., 1990) and the ability to metastasize (Welch *et al*., 1990; Yin *et al*., 1999; Oft *et al*., 1998).

Tumor promoting functions of TGF-β correlate with increased secretion of TGF-β by the cancer cells during tumor progression (Reiss and Bracellos-Hoff, 1997). Similar to other types of carcinomas, many malignant breast carcinoma cells have lost most or all sensitivity to TGF-β -induced growth inhibition, while tumor cells derived from early stages of the disease are usually inhibited (Kertzschmar, 2000; Siegel and Massagué, 2003). This loss of antiproliferative responsiveness thus predisposes to or causes cancer progression.

Inactivating mutations in TβRII and Smad4 are involved in significant proportions of gastrointestinal and pancreatic cancers, respectively, but are rare in breast cancers. In some breast cancer cell lines, limited expression of TβRII has been correlated with the lack of TGF-β responsiveness (Kalkhoven *et al*., 1995; Sun *et al*., 1994). Stable expression of TβRI in such cell lines can restore TGF-β -induced growth inhibition, indicating that all other signaling components are functional (Kalkhoven *et al*., 1995; Sun *et al*., 1994; Siegel and Massagué, 2003).

A different mechanism for evasion of TGF-β cytostatic gene responses appears to operate in metastatic breast cancers. In mammary and other epithelial cell types, the induction of $p15^{INK4b}$ and the repression of c-Myc in response to

TGF-β are essential to maintain the TGF-β cytostatic function. In these cells, TGF-β receptors and Smad proteins remain intact and capable of inducing many other gene responses. The remaining responses include induction of interleukin-11 (IL11) and connective-tissue growth factor (CTGF), two genes that have been independently implicated in bone metastatsis (Kang *et al*., 2005; Kang *et al*., 2003).

Tumor cells that have selectively lost the TGF-β cytostatic response may use their remaining responsiveness in favor of metastasis. TGF-β enhances the ability of MDA-MB-231 human breast cancer cells to form osteolytic bone metastasis in nude mice (yin *et al*., 1999). These cells, which have been used in this thesis, have typically lost TGF-β cytostatic responses (Chen *et al*., 2001). A group of genes whose overexpression mediates bone metastasis in MDA-MB-231 cells has been identified (Kang *et al*., 2005; Kang *et al*., 2003).

1.1.5 TGF-β IN CANCER THERAPY

Strategies to manipulate TGF-β signaling for cancer therapeutics must consider its bifunctional mode of action and attempt more complicated and specific targeting of drugs to inhibit tumor outgrowth and also evade metastasis. For example, activating TGF-β signaling might suppress tumor growth but might also promote tumor invasion and metastasis, especially if cells are no longer sensitive to the growth inhibitory effects of TGF-β. Enhanced or exogenously-administered TGF-β signaling would require that the tumors are sensitive to the

growth inhibitory effects of TGF-β. Unfortunately, loss of the TGF-β growth inhibitory effects is lost early in caner progression, most likely before diagnosis (Arteaga, 2006). Approaches that inhibit TGF-β might induce regression of advanced cancers, but may stimulate the growth of otherwise quiescent pre-malignant tissue. Importantly, disrupting the TGF-β pathway in normal tissue may be harmful and so blocking it raises many concerns (Massagué, 2003).

There are three main strategies for TGF-β inhibition: (1) compound that interferes with the binding of ligands to the TGF-β receptors, (2) drugs that block intracellular signaling, and (3) antisense oligonucleotides (see table 1.1.3) (Iyer *et al*., 2005; Arteaga, 2006). A second group of strategies is aimed at directly blocking the catalytic activity of TβRI. Theses strategies include the use of small molecules such as SB-431542 that was used in this thesis (Halder *et al*., 2005; Gadir *et al*., 2007). Ligand-neutralizing antibodies are used as well but because of their large molecular weight cannot distribute rapidly and evenly in tumor tissue as small molecules do.

Inhibition of Ras/Raf and/or PI3K pathways in addition to blocking the cell survival and mitogenic effects of these pathways might also attenuate the adverse effects of TGF-β (Derynck *et al*., 2001).

Strategy	Selected examples of tumor models tested (in vivo)	Ongoing clinical trials
TGFβ-1, TGFβ-2 antisense oligonucleotides	Breat, ovarian, glioma, colon, hepatocellular carcinoma	Phase II/III
TGFβ-1 antibody	Breast, prostate, colon, renal cell carcinoma	Phase III
Dominant negative receptor (DNRII)	Breast, prostate, melanoma, colon, ovarian, lung	None
Soluble domain of TGFβRII	Breast, colon, pancreas, hepatoma	None
Small-molecule drugs (inhibitors of TβRI)	Breast, glioma colon	Phase I

Table 1.1.3: Cancer therapy strategies targeting TGF-β signaling (Iyer *et al.*, 2005)

Presented are various strategies of targeting TGF-β signaling in a tumor therapy..

Because the mechanism governing the switch in TGF-β from a tumor suppressor to a tumor promoter remains unknown, questions remain about the clinical use of TGF-β inhibitors. However, studies are now showing that inhibition of TGF-β signaling *in vivo* does not induce tumor formation. Inhibition of TGF-β signaling through expression of mutant TβRII or through the continual administration of TGF-β-specific antibody showed no significant side-effects (Yand *et al*., 2002; Ruzek *et al*., 2003). These findings, coupled with progressing clinical trials all indicate that TGF-β inhibition may, indeed, be a viable option to cancer therapy.

1.2 PHOSPHOLIPASE D (PLD) AND CANCER SURVIVAL

1.2.1 PLD FUNCTION

Phospholipase D was first discovered in carrot plants extracts as a distinct phospholipid-specific enzyme that hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (Figure 1.2.1) (Hanahan and Chaikoff, 1947; Hanahan and Chaikoff, 1948). The activation of PLD is believed to play an important role in the regulation of cell function and fate. Multiple PLD activities were characterized in eukaryotic cells, and, more recently, several PLD genes have been cloned (Liscovitch *et al*., 2000; Exton, 2002).

Besides simple hydrolysis, PLD may catalyse a transphosphatidylation reaction utilizing short-chain primary alcohols as phosphatidyl-group acceptors (Yang *et al*., 1967; Ella *et al*., 1967). The resultant phosphatidylalcohols are produced only by PLDs, and are not normally found in biological membranes. Because of their unique origin, their low basal levels and their relative metabolic stability, the formation of phosphatidylalcohols has served as a convenient and sensitive marker for PLD activation in cultured cells (McDermott *et al*., 2004;; Morris *et al*, 1997, see materials and methods, Chapter II).

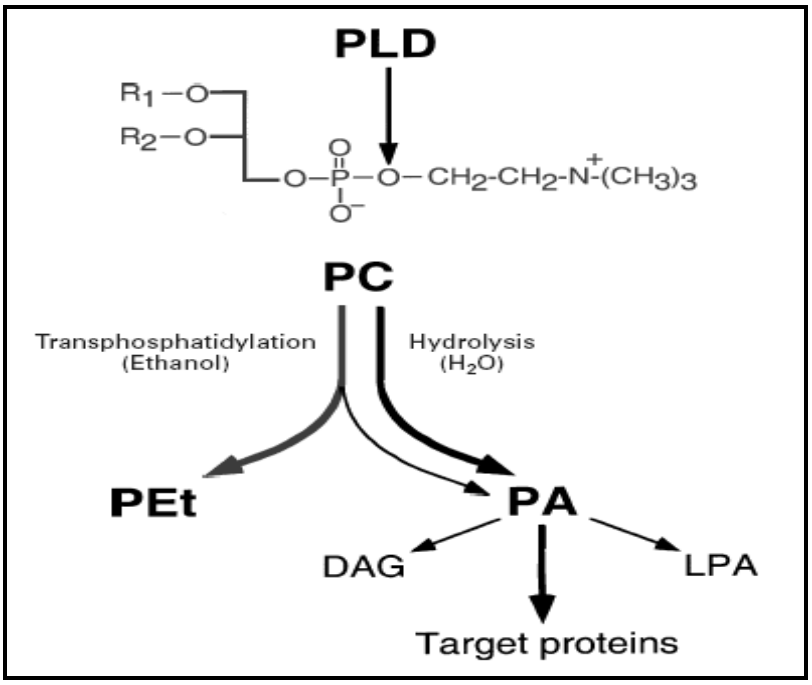

Figure 1.2.1: Phospholipase D – catalyzed reactions (Liscovitch *et al.*, 2000)

PLD hydrolyses the phosphodiester bond in phospholipids such as PC. A phosphatidyl-enzyme intermediate is believed to form transiently which normally is hydrolysed by water, generating PA. Primary short-chain alcohols (e.g. ethanol) can substitute for water in a competing, transphosphatidylation, reaction. In the presence of ethanol the product of PLD-catalysed transphosphatidylation is or phosphatidylethanol (PEt). This reaction (thick arrow pointing to the left) occurs at the expense of the hydrolytic reaction (thin arrow pointing to the right), decreasing PA formation. PA can also be produced by diacylglycerol kinase and by acylation of glycerol 3-phosphate. In contrast, phosphatidylalcohols are uniquely formed by PLD. PA can be further metabolized (thin arrows) to diacylglycerol (DAG) and lyso-PA (LPA). In contrast, phosphatidylalcohols are metabolically stable and would accumulate in cells upon PLD activation. (references provided in the text).

PLD has been implicated in membrane trafficking, cytoskeletal reorganization, receptor endocytosis, exocytosis, and cell migration (Exton *et al* 2002). PLD has also been shown to be involved in cell proliferation (Foster and Xu, 2003). The activity of PLD is elevated in response to platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF),

insulin, and insulin-like growth factor 1 (IGF-1) (Plevin *et al.*, 1991; Motoike *et al.*, 1993; Song *et al.*, 1994; Karnam *et al.*, 1997; Sa *et al.*, 1999; Banno *et al.*, 2003). PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src, v-Ras, v-Fps, and v-Raf (Song *et al.*, 1991; Carnero *et al.*, 1994; Jiang *et al.*, 1994; Jiang *et al.*, 1995; Frankel *et al.*, 1999). Hence, there is a growing body of evidence linking PLD activity with mitogenic signaling and elevated PLD has been shown to contribute to cell transformation and survival (Foster and Xu, 2003). It has been reported that elevated expression of either PLD1 or PLD2, in combination with overexpression of c-Src or EGF receptor, transforms rat fibroblasts (Lu *et al.*, 2000; Joseph *et al.*, 2001). Fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 were able to provide survival signals that prevented apoptosis (Zhong *et al.*., 2003). The capability of PLD to cooperate with a tyrosine kinase such as c-Src or EGFR to transform cells or provide survival signals would implicate PLD as a good candidate oncogene in cancer, especially those cancers that have elevated tyrosine kinase such as c-Src or EGFR (Foster and Xu, 2003).

Elevated PLD expression and activity has been reported in several human cancer tissues including breast cancer (Uchida *et al.*, 1997; Noh *et al.*, 2000), gastric and renal cancers (Uchida *et al.*, 1999; Zhao *et al.*, 2000). Hence, it is of interest whether the elevated PLD in human cancer tissues plays any role in tumorigenesis. Our lab has shown that blocking PLD activity by using either a catalytic inactive mutant of PLD or primary butanol will induce apoptosis in

breast cancer cell line MDA-MB-231 (Zhong *et al*., 2003) and renal cancer cells (Toschi *et al*, unpublished data) . These cells have very high level of PLD activity, and this finding further implicates PLD in survival signaling in cancer cells. The role of PLD in cancer survival will be discussed more in depth in part 1.2.4.

1.2.2 PLD STRUCTURE

Mammals contain two separate PLD genes PLD1 and PLD2 both with splice variants (Hammond *et al*., 1995, Colley *et al*., 1997). Despite homology between the two genes, there are significant differences in the regulation and subcellular distribution of PLD1 and PLD2. The approximately 120 kDa PLD1 is found mainly in intracellular membranes while the approximately 106 kDa PLD2 is localized almost exclusively on the plasma membrane (Liscovitch *et al*., 2000; Freyberg *et al*., 2001). The highly conserved domain in the PLD family is the HKD motif, which is used to define the PLD superfamily. It was termed “HKD” because the domain contains the motif HxKxxxxD/E, which is found twice without exception in all cloned PLDs (Hammond *et al*., 1995), Mammalian PLD1 and PLD2 both contain two HKD motifs, which are critical for enzymatic activity *in vitro* and *in vivo* (Sung *et al.,* 1997).

Other highly conserved regions of the PLD genes are the phox consensus sequence (PX), the plekstrin homology (PH) domain and the PI-4-P 5-kinase binding site (Table 1.2.1). The PH domain is thought to function in the localization of the protein (Sciorra *et al.,* 2002) but is not required for PLD enzymatic activity. The PX domain is thought to mediate protein-protein interactions or to bind

phosphatidylinositol phosphates (PIP) (Xu *et al.,* 2006). Finally, PLD1 has a conserved loop region that is not found in PLD2. The loop region has been proposed to function as a possible negative regulatory element, since deletion of this region from PLD1 increased its basal activity threefold (Sung *et al.,* 1999).

PLD1 and PLD2 both have an absolute requirement for Phosphatidylinositol bisphosphate (PIP2) (Hammond *et al.*,1997), however, reports indicate that PLD1 has low basal activity while PLD2 has high basal activity (Colley et al., 1997). In addition, PLD1 is activated by the ADP-ribosylation factor (ARF)-, Ral- and Rho-family GTPases, as well as by protein kinase Cα (PKCα) while PLD2 is activated by fatty acids (Colley *et al.*, 1997). Table 1.2.1 summarizes the distinguishing characteristics of the two PLD isoforms.

Characteristic	PLD1	PLD2
Molecular Weight	~120 kDa	~106 kDa
Substrate Specificity	PC	PC
PIP2-dependent	yes	yes
RalA-associated	yes	no
Basal Activity	low	high
Subcellular localization	Mainly intracellular membranes	Plasma membrane
Activators	ARF-, Ral-, Rho-GTPases, PKCα	fatty acids, ARF

Table 1.2.1: Biochemical properties of Phospholipase D 1 and 2 (Foster and Xu, 2003)

Presented are various characteristics of PLD 1 and 2, corresponding references are presented in the text.

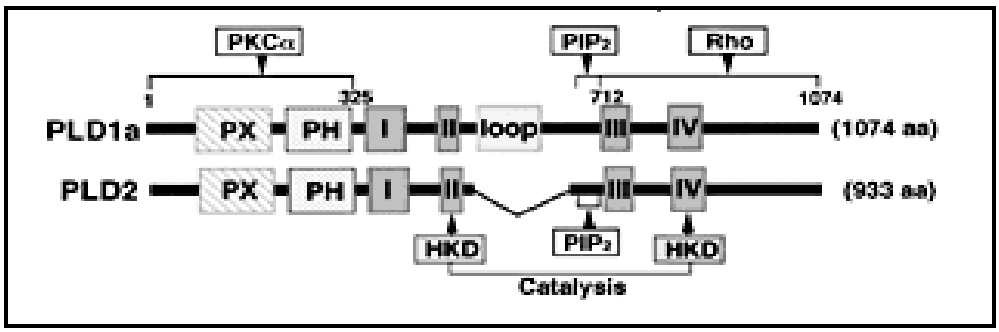

Figure 1.2.2: PLD genes

Basic structure of PLD1 and PLD2. Regions of conserved sequences of PLD1 and PLD2 are shown. PX, phox homology domain; PH, pleckstrin homology domain; motifs I, II, III and IV, regions of sequence conserved among all PLD isozymes. Motifs HKD are found in II and IV. Regions of PLD1a identified as interacting with protein kinase C and Rho, and PIP_2 binding regions of PLD1 and PLD2 are indicated.

1.2.3 PLD SIGNALING PATHWAY

Mammalian PLD activity is highly regulated by a large number of factors. Fatty acids, phosphoinositides, small GTP binding proteins, protein kinase C, Ca^{2+}, phosphorylation, and negative regulators all regulate PLD activity. Many of these factors act in concert to positively or negatively regulate PLD activation (McDermott *et al*., 2004).

Several small GTPases have been reported to activate PLD1 *in vivo* and *in vitro*, including ARF-, Ral-, and Rho-family members (Exton, 2002). These GTPases are involved in cell membrane trafficking, cytoskeleton regulation, and survival signal pathways, and PLD plays a role in these functions as well.

The ARF family of small GTP-binding proteins plays a central role in membrane trafficking and cytoskeletal remodeling (Liu *et al*., 2005). ARF proteins

were reported to be required for PLD activities that are elevated by various mitogenic factors, including PDGF, EGF, insulin, phorbol esters, and H-Ras (Foster and Xu, 2003). Among the members of ARF family, ARF6 co-localizes with PLD1 (Powner *et al*., 2002) in the lipid raft fractions, while ARF4 was recently implicated in the activation of PLD2 (Kim *et al*., 2003).

Rho-family GTPases regulate cell membrane trafficing and actin dynamics. Rho-family members were implicated in mitogenic signaling through PLD (Hess *et al*., 1997), and more commonly Rho-family members were shown to mediate PLD responses through agonists that stimulate secretion (Powner *et al*., 2002).

RalA mediates Ras signaling, and directly interacts with PLD1 (Jiang *et al*., 1995; Luo *et al*., 1997). Although RalA alone can not activate PLD1 either *in vitro* or *in vivo*, it is required for the activation of PLD activity by EGF, PDGF, insulin, Src, Ras, Raf, and phorbol esters (Foster and Xu, 2003). These evidence suggest that RalA is critical in PLD response stimulated by mitogenic or oncogenic signals, possibly by working together with Arf6 (Foster and Xu, 2003).

Other than small GTPases like PIP2 is also required for both PLD1 and PLD2 activation (as mentioned above). PIP2 can be generated by various PI kinases including PI-4-P 5-kinase, while PI3-kinase, on the other hand, converts PIP2 to PIP3. PI-4-P 5-kinase is a downstream target of PLD signaling, so by generating PIP2, it may provide a positive feedback loop (Foster and Xu, 2003; Foster, 2006). Since both ARF6 and RalA stimulate PLD activity through interaction with PLD1, but evidences suggest that PLD2 is the major responsible

isoform for the mitogenic signal stimulated PLD activity (Xu *et al*., 2000), and that PLD2 activation was suggested to be dependent on the activation of PLD1 (Mwanjewe *et al*., 2001), we proposed a model (Figure 1.2.3) in which mitogenic signals stimulate PLD1 activation through the interaction of ARF6 and RalA, which then lead to the activation of PLD2 which mainly reside on the lipid rafts of the plasma membrane (adapted from Foster and Xu, 2003).

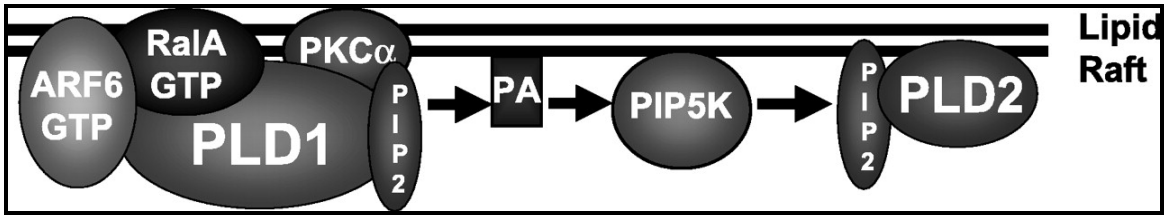

Figure 1.2.3 Mitogenic signaling through PLD1-dependent activation of PLD2 (Foster and Xu, 2003)

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

While lots of evidence indicates PLD involvement in various cell activities, the relevant downstream targets of PLD are still mostly elusive. Raf has a PA binding site and its association with PA was implicated to facilitate the recruitment of Raf to the plasma membrane, where it can participate in the activation of the mitogen-activated protein (MAP) kinase pathway (Foster and Xu, 2003; Rizzo *et al*., 1999).

The mammalian target of rapamycin (mTOR) has recently emerged as an important downstream target of PLD (Figure 1.2.4). mTOR is a protein kinase directly involved in both cell cycle progression and cell growth by regulating translation, transcription, membrane traffic, and protein degradation (Kuruvilla and Schreiber., 1999; Schmelzle and Hall., 2000), and mTOR is also a downstream target of PI-3-kinase/Akt survival pathway (Sekulic *et al*., 2000; Nave *et al*., 1999). It has recently been reported that PA binds to mTOR competitively with rapamycin and is required for activation of mTOR (Fang *et al*., 2001; Chen and Fang 2002). Consistently, elevated PLD activity seems to confer resistance to rapamycin in some human cancer cells (Chen *et al*., 2003). All these evidence suggest the role for mTOR as a particularly important downstream target of PLD signaling pathway.

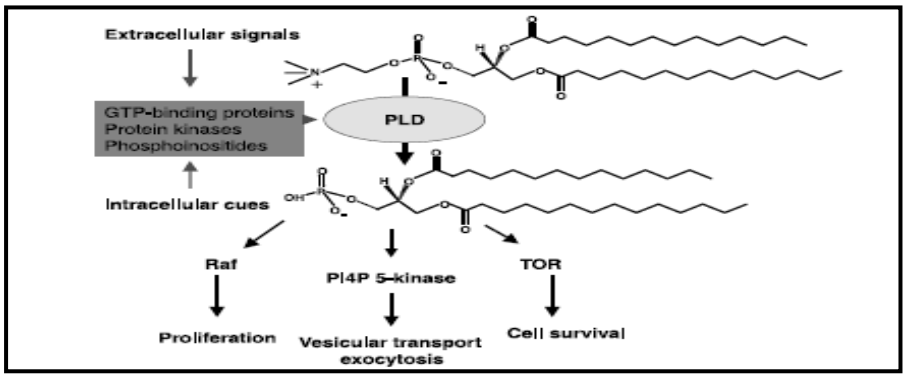

Figure 1.2.4 PLD substrates, products, regulators and targets (McDermott *et al*., 2004)

The structure of the PLD substrate and product phosphatidylcholine and phosphatidic acid are shown, and the figure summarizes the regulation of PLD by cell surface receptors and intracellular signals through actions of intermediate protein and lipid activator

1.2.4 PLD REGULATION IN BREAST CANCER

During the last several years, it has become apparent that PLD is a critical regulator of cell proliferation and survival. PLD has been shown to facilitate cell cycle progression, suppress apoptosis, contribute to the transformation of mice fibroblasts, and enhance cell migration – all critical steps in tumorigenesis. Moreover, Elevated PLD activity has been reported in several human cancers.

A critical barrier for an emerging tumor is the generation of "survival" signals that suppress default apoptotic programs (Hanahan and Weinberg, 2000). High intensity Raf signals induces cell senescence (Samuels and McMahon, 1994; Kerkhoff and Rapp, 1998) or, in the absence of serum, apoptosis (Joseph *et al*., 2002). Either PLD1 or PLD2 was able to suppress the cell cycle block and apoptosis in cells with high intensity Raf signals (Joseph *et al*., 2002). Similarly, fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 prevented this apoptosis (Zhong *et al*., 2003). More recently, we have shown that in the highly malignant MDA-MB-231 breast cancer cell line, PLD provides a survival signal that suppresses apoptosis induced by the withdrawal of serum (Chen *et al*., 2005). Importantly, this PLD survival signal was dependent upon mTOR, which has been widely implicated in survival signal signals mediated by PI3K (Sawyers, 2003; Foster, 2004). The survival signal generated by PLD in MDA-MB-231 cells could be distinguished from PI3K dependent survival signals in another breast cancer cell line MDA-MB-435s (Chen *et al.,* 2005). These data indicate that PLD

generates a distinguishable survival signal in breast cancer cells that overcomes default apoptotic programs.

Tumor invasion is a required step in metastasis and it has been proposed that the ability to invade is actually a dysregulated cell migration (Kassis *et al*. 2001). Cytoskeleton networks, which play a central role in cell migration, have been reported to be regulated by PLD (Aguirre Ghiso *et al*. 1997; Kam and Exton 2002). Interestingly, MDA-MB-231 human breast cancer cells, which have very high levels of PLD activity, migrate and invade matrigel in culture, whereas MCF-7 breast cancer cells, with relatively low PLD activity do not (Zheng *et al*., 2006). Protease secretion is also a property of invasive cancer cells and PLD activity has also been correlated with elevated protease secretion (Aguirre Ghiso *et al*., 1999). A dominant negative mutant of RalA, which blocks PLD activity in v-Src- and v-Ras-transformed cells (Jiang *et al*., 1995), also blocked protease secretion and tumor formation in nude mice (Aguirre-Ghiso *et al*., 1999).

More recently our lab has found that the ability of MDA-MB-231 cells to migrate and invade Matrigel is dependent upon PLD activity (Zheng *et al*., 2006). The link between PLD and cytoskeletal organization, protease secretion, and cell migration and invasiveness suggest that PLD plays an important role in metastasis of cancer cells. Our lab has proposed that the link between survival signals and cell migration is part of a “survival program” that suppresses apoptosis during stress and facilitates migration to less stressful sites (Zheng *et al*., 2006).

A major focus of our lab has been survival signals generated in breast cancer cells by PLD. There are several targets of PA generated by PLD, but perhaps the most significant with regard to cancer is mTOR, which has been widely implicated in survival signals in many cancers (Sawyers, 2003; Foster, 2004). Jie Chen and colleagues reported that mTOR has a PA requirement (Fang *et al*., 2001), which implicated PLD in mTOR-mediated survival signals. Our lab has gone on to demonstrate that elevated PLD activity in the human breast cancer cell line MDA-MB-231 cells leads to an mTOR-dependent suppression of both p53 (Hui *et al*., 2004) and protein phosphatase 2A (PP2A) activity (Hui *et al*., 2005). PLD activity also led to an mTOR-dependent increase in the expression of Myc (Rodrik *et al*., 2005). In this thesis, we have provided evidence indicating that the elevated PLD activity in MDA-MB-231 cells contributes to the suppression of TGF-β signaling via two apparently independent mechanisms – one mediated by mTOR and the other mediated by MAP kinase (Chapter IV, Gadir *et al*., 2007). These studies have strongly implicated PLD in many of the critical hallmarks needed for progression to a malignant tumor (Hanahan and Weinberg, 2002). Suppression of the TGF-β signals that suppress cell cycle progression may be another hallmark needed for tumorigenesis. Moreover, elevated PLD activity in human breast cancer cells is able to alter the expression and activity of key regulators of cell cycle progression and survival in ways that are consistent with a novel PLD/mTOR survival pathway that could be distinguished from the better-characterized PI3K/Akt

survival pathway (Cantley, 2002; Luo *et al*., 2003), which also targets mTOR indirectly.

A model for two alternative strategies for activating mTOR is shown schematically bellow (adapted from Foster, 2006) where mTOR can be activated indirectly by PI3K or directly by PA generated by PLD (Figure 1.2.5). Interestingly, the two mechanisms may be linked by differential dependence on PIP2 and PIP3, indicating that the two pathways may be integrated. The mTOR signaling pathway and its application to PLD will be further discussed in part 1.3.

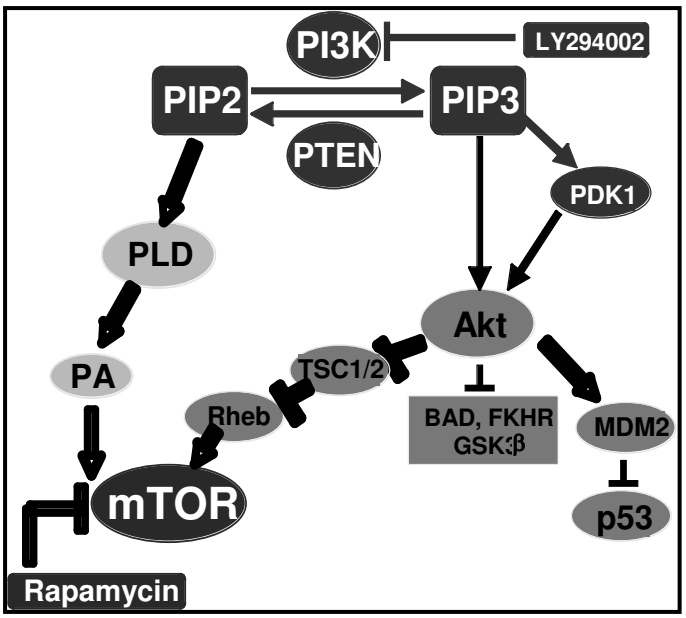

Figure 1.2.5: Alternative PLD and PI3K survival signals (Foster, 2006)

PI3K generates PIP3 from PIP2. The presence of PIP3 leads to the recruitment of PDK1 and Akt. PIP3 levels are also regulated by PTEN, a phosphatase that dephosphorylates PIP3 to PIP2. PDK1 phosphorylates and inactivates several substrate proteins that negatively regulate cell proliferation or stimulate apoptosis. These include GSK3β, BAD, MDM2 and indirectly, mTOR. PLD generates PA, which also leads to the activation of mTOR. mTOR then phosphorylates several substrate proteins that regulate protein synthesis. The two pathways are also connected by their dependence on PIP2.

Consistent with the model above, PLD suppressed apoptosis in MDA-MB-231 cells that were deprived of serum in a rapamycin-dependent manner (Chen *et al*., 2005; Gadir *et al*., 2007). In this thesis, rapamycin induced apoptosis in MDA-MB-231 cells in the absence of serum; however, in the presence of serum, rapamycin did not induce apoptosis – indicating that a factor(s) in serum was preventing rapamycin-induced apoptosis, discussed in Chapter III and IV.

1.2.5 PLD IN CANCER THERAPY

Hydrolysis of PC by PLD produces the release of PA, a mitogenic second messenger which shown to be involved in cell proliferation and transformation (Foster, 2006; Foster, 2007). Therefore, targeted inhibition of PLD may slow down the process of tumour formation and proliferation and may thus be an interesting target for the development of effective anti-cancer drugs. However, because PLD is considered to play an important role in diverse cellular processes such as nuclear vesicle dynamics during mitosis (Liscovitch *et al*., 1999), vesicular traffic in the Golgi (Exton *et al* 2002), formation of budding secretory vesicles in the trans- Golgi (Chen *et al*., 1997), exocytosis (Brown *et al*., 1998), cytoskeleton and cell motility (Colley *et al*., 1997), these basic cellular functions may also be affected by PLD inhibition and so appropriate targeting of tumour-related cells would be necessary.

Despite the fact that negative regulation of PLD is still poorly understood, a series of compounds of diverse origin have shown anti-PLD activity during the past few years like APCs (alkylphosphocholines) and certain synthetic 1,3-diacylglycerols (1,3-DAGs) (Unger *et al*., 1990; Terwogt *et al*., 1999; Lucas *et al*., 2001; Rodríguez-González, 2003). Although clear anti-tumoral activity has not as yet been observed with these compounds, a clear correlation is seen in some instances. Such is the case when inhibiting PLD activation showed hopeful anti-tumour effects in breast cancer patients with skin metastases (Unger *et al*., 1990; Terwogt *et al*., 1999; Lucas *et al*., 2001).

Elevated PLD activity has been implicated in a large number of human cancers including breast, colon, gastric, and kidney (Foster, 2006). PLD activity has been reported to suppress apoptosis in human cancer cells subjected to the stress of serum withdrawal (Foster, 2006) and PLD activity is elevated in several cancer cells in response to the stress of serum withdrawal (Zheng *et al*., 2006). mTOR has been implicated as a key regulator of stress responses by shutting down under conditions of poor nutrition or hypoxia. For a cancer cell to survive and proliferate, it is becoming apparent that it must overcome this stress response (Foster, 2007). The PLD activity elevated in response to serum withdrawal in cancer cells may be a mechanism to activate mTOR that was selected for to overcome the shutdown of mTOR. In this regard, it is significant that the survival signals generated by PLD in the breast cancer cell line MDA-MB-231 cells are dependent upon mTOR (Chen *et al*., 2005). .

Together, the data linking PA production – especially via PLD mediated hydrolysis of phosphatidylcholine – with mTOR activation represents a potentially important mechanism for activating this critical regulator of responses to environmental stress and survival signals in cancer. The significant percentage of cancers with elevated PLD activity strongly suggest that this pathway for activating mTOR be considered – especially since targeting mTOR with rapamycin or rapamycin derivatives in cancer would be strongly influenced by the level of PLD activity in the cancer cells.

Thus, the findings suggest that targeting the signals mediated by PLD will be a promising therapeutic strategy in an apparent large number of cancers where elevated PLD activity is promoting cell cycle progression and suppressing apoptosis. The need for the stressful condition of serum withdrawal indicates that successful strategies for inducing apoptosis may require combination strategies that suppress growth factor signals in addition to suppressing PLD signals; however, cancer cells – especially solid tumors – are subjected to a variety of stressful conditions during tumorigenesis prior to vascularization. Our lab has been working to establish approaches for therapeutic targeting human cancer cells that are dependent on PLD activity for survival.

Rapamycin based therapies: Our previous work has established that rapamycin can induce apoptosis in MDA-MB-231 cells deprived of serum (Chen *et al*., 2005). However, we also demonstrated that elevated PLD activity confers rapamycin resistance in human cancer cells (Chen *et al*., 2003) leading to higher concentrations of rapamycin being required to suppress cell growth and induce

apoptosis. We have demonstrated that suppressing PLD activity with a dominant negative PLD2 mutant reduces the concentration of rapamycin to suppress cell proliferation and to inhibit S6 kinase phosphorylation. These preliminary studies indicate that suppressing PLD activity can increase the efficacy of rapamycin.

Honokiol: we have found that a compound isolated from *magnolia grandiflora* known as honokiol suppresses PLD activity in both MDA-MB-231 and T24 cells. This compound also suppresses both tumor growth and angiogenesis (Bai *et al*., *2003*). In the absence of serum, honokiol induces apoptosis in MDA-MB-231 and T24 cells (our unpublished results). Since suppression of PLD also induces apoptosis in these cells, the data indicate that the effect honokiol is due at least in part to its ability to suppress PLD survival signals. Since honokiol is well tolerated by mice (Bai *et al*., 2003), honokiol may be an ideal candidate drug to target PLD signals in human cancers were PLD is implicated. Reducing PLD activity in order to increase the efficacy of rapamycin and honokiol should be ideal in this regard. Moreover, the ability to target PLD survival signaling both upstream and downstream with two different drugs (honokiol and rapamycin) may be important therapeutically since single drug approaches usually result in the development of resistant cells (Shah and Sawyers, 2002).

1.3 MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

1.3.1 mTOR FUNCTION

mTOR, the mammalian target of rapamycin (also known as FRAP) is an evolutionary conserved large (~290 kDa) serine/threonine protein kinase. It was originally discovered in the yeast *saccharomyces cerevisiae*, during a screen for resistance to the immunosuppressant drug rapamycin (Kunz *et al*., 1993; Helliwell *et al*., 1994). Rapamycin acts by forming an inhibitory complex with its intracellular receptor, the FKBP12 which binds a region in the C terminus of TOR proteins termed FRB (FKBP12-rapamycin binding), and thereby inhibiting TOR activity (Chen *et al*. 1995; Choi *et al*. 1996).

mTOR integrates signals from growth factors, nutrients, and energy to regulate essential cellular processes such as cell growth (increase in cell mass and size) and cell cycle progression (see Figure 1.3.1) (Fingar and Blenis, 2004). The mTOR pathway controls the translation of mRNAs that encode proteins that are required for G1 cell-cycle progression and S-phase initiation, and thus inhibition of mTOR signaling results in a slowing or arrest of cells in G1. mTOR might therefore also be viewed as a gatekeeper, which only allows G1 progression under low nutrients conditions (reviewed by Bjornsti and Houghton, 2004). In the last decade, a role for mTOR in tumorigenesis is emerging as mTOR comprises a critical target of “survival signals” in cancer cells (Sawyers, 2003; Tee and Blenis, 2005).

Most of the work done in mammalian cells focused on the rapamycin-sensitive mTORC1 complex (raptor) and it has been suggested that raptor function as an adaptor for recruiting mTOR substrates. Two well-characterized substrates of the mTORC1 complex that control translation and cell growth are the eukaryotic initiation factor 4E-binding protein (4E-BP1) family of proteins and the S6 protein kinases (S6K1 and S6K2). mTOR regulates protein synthesis through the phosphorylation and inactivation of the repressor of mRNA translation 4E-BP1, and through the phosphorylation and activation of S6K1 (reviewed by Hay and Sonenberg, 2004). Thus, S6K1 or 4E-BP1 phosphorylation is often used as a marker for mTOR activity.

The mTORC2 complex (rictor) has been shown to control cytoskeleton organization (Jacinto *et al*., 2004). This rapamycin-insensitive complex is required for phosphorylation of the hydrophobic motif at Ser473 of Akt, a site analogous to the hydrophobic motif site in S6K that is known to be regulated by the mTORC1 complex (Sarbassov *et al*., 2004). On growth factor stimulation, PI3K activation results in recruitment of Akt to the plasma membrane, in which phosphorylation at Akt Ser473 is mediated by the mTORC2 complex and phosphorylation at Thr308 is mediated by PDK1. The mTORC2-activated Akt then phosphorylates and inactivates tuberin, resulting in increased mTORC1 activity (Sarbassovd *et al*., 2004; Shaw and Cantley, 2006). The mTOR signaling pathway will be further discussed in part 1.3.3.

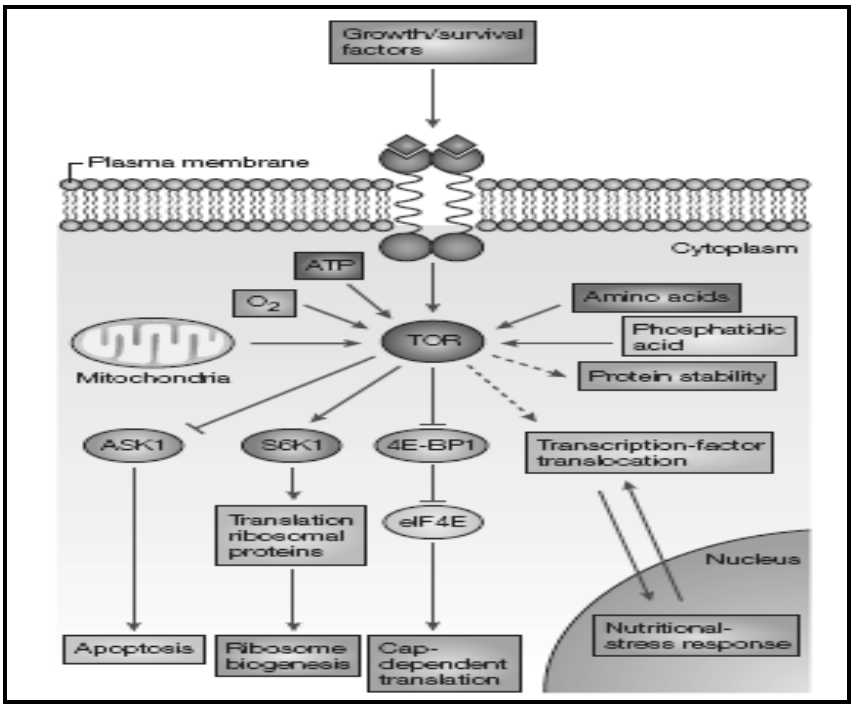

Figure 1.3.1: Target of rapamycin is a central regulator of cell growth and proliferation in response to environmental and nutritional conditions. (Bjornsti and Houghton, 2004)

Target of rapamycin (TOR) signalling is regulated by growth factors, amino acids, ATP and O2 levels; second messengers (for example, phosphatidic acid); and, possibly, mitochondrial stress. Signalling through TOR seems to regulate several downstream pathways that impinge on cell-cycle progression, translation initiation, transcriptional stress responses, protein stability and survival. Dashed lines indicate pathways that are best described in yeast.

The role of mTOR in autophagy is conserved from yeast to mammals where it acts to regulate the induction of the autophagic process (Levine and Klionsky, 2004). Autophagy is implicated in the pathology of cancer and although a reduction in autophagy appears to be common in tumor cells, some level of autophagy may be required for the development of cancer (Easton and Houghton, 2006). One mechanism tumor cells may use to cope with nutrient deprivation is to temporally recover nutrients autonomously by activating

autophagy (Guertin and Sabatini, 2007). Confirmation for this is provided in breast cancer in which one copy of the essential autophagy gene BECN1, which codes for the protein BECLIN 1, is frequently deleted. If mTOR is inactive autophagy proceeds, and conversely, when mTOR is activated the autophagic process is inhibited. Treatment with rapamycin elevated BECLIN 1 expression in MDA-MB-231 breast cancer cells (our unpublished data).

1.3.2 mTOR STRUCTURE

mTOR is a high molecular-weight protein contains 2549 amino acids and comprises several conserved structural domains. The N terminus possesses 20 tandem HEAT (for Huntignton, EF3, A subunit of PP2A, TOR1) repeats. Tandem HEAT repeats are present in many proteins and are implicated in protein-protein interactions (Andrade and Bork, 1995; Hay and Sonenberg, 2004). The C-terminal half of mTOR contains the kinase domain, which has sequence similarity with the catalytic domain of PI3K and therefore makes it a member of the phosphoinositide 3-kinase–related kinase (PIKK) family, whose members (ATM, ATR, DNA-PK, hSMG1, mTOR, and TRAPP in mammalian cells) transmit signals related to cell growth, proliferation, and stress responses (Abraham and Gibbons, 2007). In addition, the C-terminal end contains a FAT domain (for FRAP, ATM, TRAP), designated FATC which is necessary for mTOR activity, and the deletion of even a single amino acid from this domain abrogates the activity (Peterson *et al*. 2000; Takahashi *et al*. 2000). mTOR also contains a presumed negative

regulatory domain (NRD) between the catalytic and FATC domains (Figure 1.3.2) (Sekulic *et al*. 2000). Figure 1.3.2 below portrays the mTOR structure.

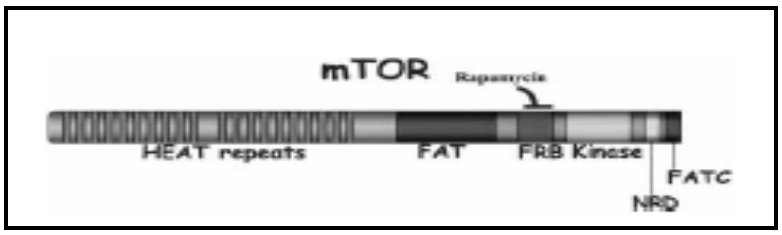

Figure 1.3.2: The primary structure of mTOR

(Hay and Soneneberg, 2004)

The protein consists of a catalytic kinase domain (CD), an FKBP12-rapamycin-binding (FRB) domain, a putative auto-inhibitory domain near the carboxyl terminus and up to 20 tandemly repeated HEAT motifs at the amino terminus, as well as FAT and FATC (FATC terminus) domains.

In yeast and mammals, there are two distinct TOR complexes, each composed of TOR, a common regulatory subunit called LST8, and at least a third subunit that specifies the downstream substrates (Shaw and Cantley, 2006). Among the PIKK family members, the FRB domain is found only in the TOR proteins, and gives an exclusive specificity of rapamycin and its derivatives for mTOR in mammalian cells. The drug directly attacks only one subpopulation of mTOR proteins residing in a complex, termed mTORC1 (Sarbassov *et al*., 2004). In mammals, the substrate-defining subunits are raptor (the mTORC1 complex) and rictor (mTORC2) (Sarbassov *et al*., 2004; reviewed by Guertin and Sabatini, 2007). Whereas mTORC1 complexes are strongly inhibited by rapamycin, mTORC2 is not affected by the drug (Sarbassov *et al*., 2004) (discussed also in part 1.3.1).

1.3.3 mTOR SIGNALING PATHWAY

Upstream signaling. The PI3K-AKT pathway is involved in the delivery of growth factor–derived stimulatory signals to the mTORC1 complex. Ligand binding to various growth-factor receptors results in activation of PI3K, which catalyses the conversion of PIP2 to PIP3. The protein and lipid phosphatase PTEN (phosphatase and tensin homolog deleted from chromosome 10), negatively regulates this pathway. Akt, a serine/threonine protein kinase (also known as PKB) is a downstream effector of PI3K. The neighboring target for Akt in this pathway is the tuberous sclerosis 2 (TSC2) protein, which functions in a heterodimeric complex with TSC1. The TSC1/2 complex expresses GTPase-activating protein activity toward the Ras-related GTPase Rheb, and this activity is inhibited by Akt -dependent phosphorylation of TSC2 (Abraham and Gibbons, 2007). When active, TSC1/2 converts the GTP-bound form of Rheb to its inactive, GDP-bound state. When TSC1/2 activity is suppressed, GTP-bound Rheb stimulates mTORC1 signaling through a poorly understood mechanism that may involve a direct interaction between Rheb and mTORC1 (Long *et al*., 2005). The location of mTOR as a downstream target in the PI3K- Akt pathway provides a clear link to oncogenesis. Deregulated signaling through the PI3K pathway is a feature of most, if not all, types of cancer cells (Shaw and Cantley, 2006). Second messengers such as phosphatidic acid, ATP levels and polyphosphates might also regulate mTOR signaling in addition to nutrients and mitogens, and will be discusses presently.

Downstream signaling. The mTOR–Raptor complex signals directly to important translational regulators, the translational repressor protein eukaryotic initiation factor 4E-BP1 and S6K1. Binding of 4E-BP1 to eIF-4E is controlled by mTOR dependent phosphorylation of specific serine and threonine residues (Hay and Soneneberg, 2004). Once 4E-BP1 is completely phosphorylated, it dissociates from eIF-4E allowing the formation of an initiation factor complex eIF-4F (Hay and Soneneberg, 2004) which then activate translation of a subset of capped mRNA and encoding proteins involved in G1- to S-phase progression, such as c-Myc (West *et al*., 1998) and cyclin D1 (Noh *et al*., 2000; Gera *et al*., 2004). The second important mTOR target, S6K1, has been implicated in translational regulation of mRNAs such as those that encode ribosomal proteins, proteins involved in translation (Hay and Soneneberg, 2004). Considering the importance of the proteins that are subject to mTOR mediated translational control in cell proliferation and growth, it is expected that cancer cells undergo alterations that impact on mTOR activity.

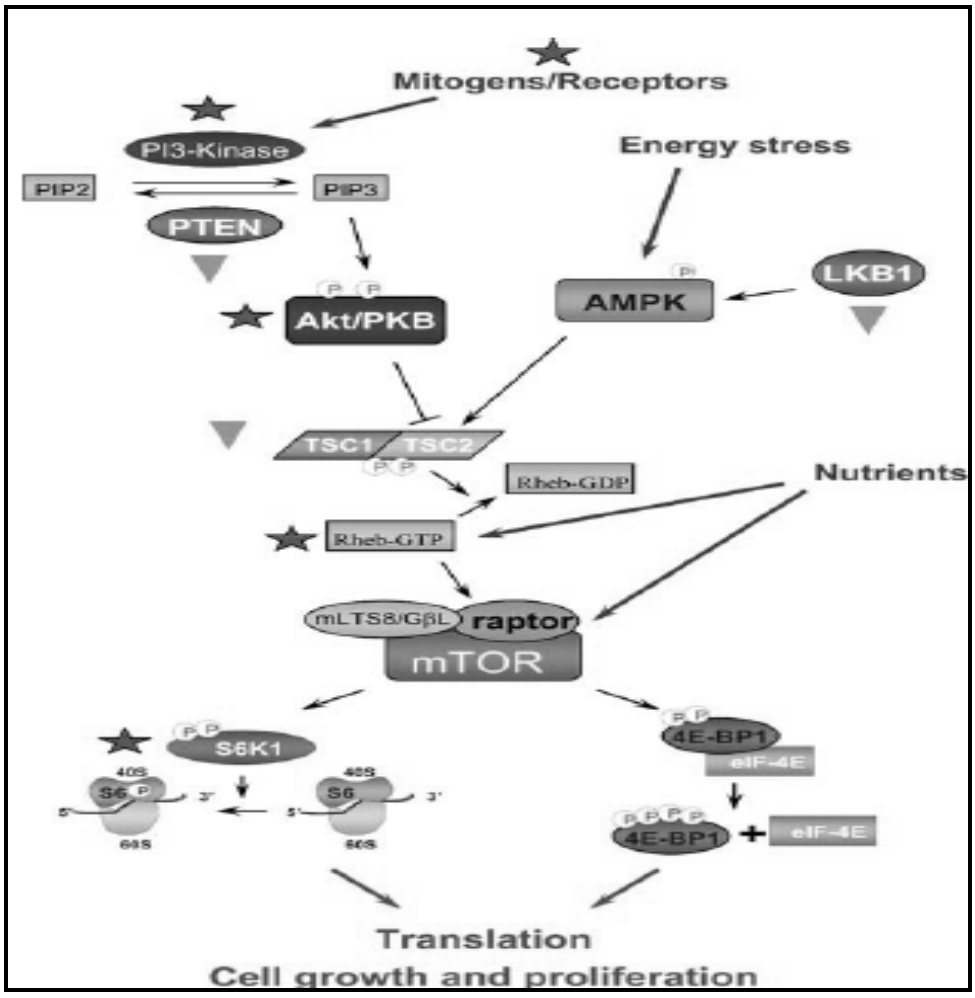

Figure 1.3.3: Model of the mTOR pathway; its regulation and cancer specific alterations. (Hynes and Boulay, 2006)

mTOR lies at the border of two major signaling pathways, one initiated by PI3K and the other by AMPK. Mitogen signaling to receptor tyrosine kinases activates PI3K and Akt, which phosphorylates TSC2 leading to activation of Rheb GTPase and mTOR activation. AMPK is a regulator of cellular energy metabolism. In the presence of high AMP, AMPK phosphorylates TSC2, strengthening the ability of the TSC complex to block Rheb GTPase activity and lowering mTOR activity. The asterisk (*) indicates mutations leading to pathway activation in cancer. These include constitutive activation of receptor tyrosine kinases, overexpression or mutational activation of PI3K and Akt as well as overexpression of Rheb and S6K1. The inverted triange (▽) indicates proteins that are lost in cancer cells. These include PTEN, the negative regulator of PI3K, TSC complex proteins hamartin and tuberin and the LKB1 kinase.

Signaling through phosphatidic Acid (PA) – link for PLD in mTOR activation. While the regulation of mTOR through TSC1/2 and Rheb has been widely described and reviewed, another mechanism contributing to the activation of mTOR by phosphatidic acid has been proposed. Jie Chen and colleagues reported a requirement for PA for the activation of mTOR (Fang *et al*., 2001). PA is most commonly generated by the hydrolysis of phosphatidylcholine by PLD (Exton, 2002; see part 1.2). PLD, like PI3K, has been implicated in survival signals in human cancer cells (Foster, 2006). Chen's lab have showed that mTOR bound to PA in a manner that was competitive with rapamycin complexed with the FKBP12 leading them to propose a model for the action of rapamycin whereby rapamycin/FKBP12 binds to mTOR and preventing interaction with PA (see Figure 1.3.4, adapted from Foster, 2007) (Chen and Fang, 2002). Consistent with the proposal that the rapamycin/FKBP12 complex acts competitively with PA, elevated PLD activity in human breast cancer cells increased the concentration of rapamycin required to suppress mTOR (Chen *et al*., 2003).

While the model for the action of rapamycin as a competition with PA for mTOR is consistent with available data, a role for PA and PLD in the regulation of mTOR remains controversial. Both PLD1 and PLD2 have been implicated in regulating mTOR. Exogenously expressed PLD2 was shown to increase S6 kinase phosphorylation in MCF7 cells (Chen *et al*., 2005), and elevated expression of PLD1 was reported to increase S6 kinase phosphorylation in rat fibroblasts (Hui *et al*., 2004). lysophosphatidic acid (LPA) -induced mTOR was

shown to be dependent on PLD1 and Rho (Kam and Exton, 2004). The activation of mTOR by Cdc42 was also dependent on PLD1 (Fang *et al*., 2003). Interestingly, it was very recently reported that PLD2 forms a functional complex with mTOR and its binding partner Raptor through a TOS (TOR signaling) motif in PLD2 and that interaction with PLD2 was essential for mitogen stimulation of mTOR (Ha *et al*., 2006).

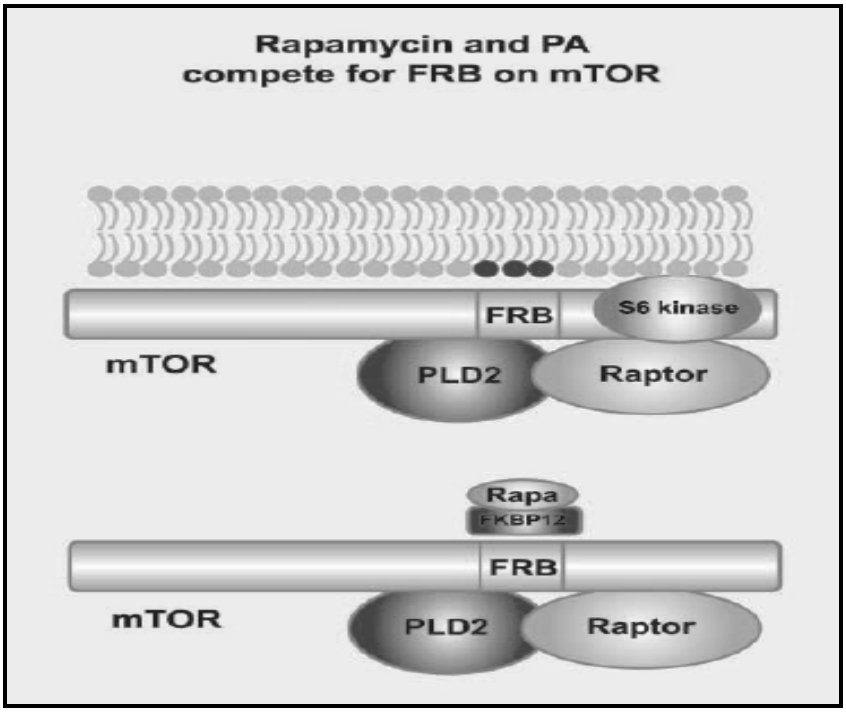

Figure 1.3.4: Regulation of mTOR by phosphatidic acid (PA) (Foster, 2007)

mTOR in complex with raptor and PLD2 associates with PLD2-generated PA through its FRB domain. In the presence of rapamycin (Rapa)/FKBP12 complex, the FRB domain of mTOR is unable to bind PA, and mTOR is unable to access and phosphorylate appropriate substrates, such as S6 kinase. Excess levels of PA on the membrane would provide more sites for binding mTOR and compete with rapamycin/FKBP12 complexes.

1.3.4 mTOR REGULATION IN BREAST CANCER

The mTOR pathway is abnormally activated in many tumors. Multiple alterations, both upstream and downstream of mTOR, leading to pathway activation have been described indicating that this pathway has an essential role in maintaining a transformed phenotype (see figure 1.3.3).

In view of breast cancer pathology, elements of the PI3K/Akt pathway have been demonstrated to be activated by membrane receptors, including the HER (or ErbB) family of growth factor receptors, the insulin-like growth factor (IGF) receptor, and the estrogen receptor (ER) (Saunders *et al*., 2001; Haynes and Boulay, 2006). Stimulation of the PI3K/Akt pathway can also occur through oncogenic Ras. Over-expression of IGF-I receptor and IGF-I commonly occurs in breast cancers and been associated with poor prognosis (Lee *et al*., 1998). Elevated Akt1 and Akt2 kinase activity have been observed in various human tumors, including breast cancer (Haynes and Boulay, 2006). Rheb, the direct upstream activator of mTOR , has also been found to be overexpressed at the RNA level in many human tumors (Hynes and Boulay, 2006) and it has been shown that overexpression of Rheb increases the activity of the mTOR effector protein S6K1 (Castro *et al*., 2003). Additionally, the S6K1 gene is amplified in approximately 9% of primary breast cancers (Wu *et al*., 2000), and elevated levels of S6K1 RNA are found in almost 40% of the tumors (Hynes and Boulay, 2006). An upstream regulator of the PI3K/Akt pathway is the tumor suppressor gene PTEN which inhibits the activity of PI3K. As a result, loss of PTEN

suppressor gene function has been associated with Akt activation and been recorded in breast cancer. Thus, most of the positive regulators of mTOR activity have been found mutated and/or overexpressed in human breast cancer. The most common alteration found is activating mutations in the PI3K pathway (Saunders *et al*., 2001; Haynes and Boulay, 2006).

Transformation of epithelial cells by Ras and c-Myc is often associated with loss of responsiveness to the negative growth regulation by TGF-β (Kretzschmar *et al*., 1999). In a nontumorigenic rat epithelial line, the ability of IGF-1 to inhibit TGF-β-dependent transcription also seems to be mediated through mTOR: IGF-1 selectively inhibited TGF-β-induced activation of Smad3, but not Smad2. This effect was mediated through Akt and was reversed by rapamycin (Song *et al*., 2003; Song *et al*., 2006). These results suggest that signaling through mTOR is required for TGF-β suppression. In human breast cancer cells, rapamycin also restored TGF-β responsiveness. Moses and colleagues demonstrated that cell cycle arrest induced by TGF-β could be markedly enhanced by rapamycin in MDA-MB-231 breast cancer cells (Law *et al*., 2002). We have found that TGF-β is required for suppression of rapamycin-induced apoptosis by serum in MDA-MB-231 cells and that TGF-β is also capable of suppressing rapamycin-induced apoptosis in MDA-MB-231 cells deprived of serum (Gadir *et al*., 2007; see part III). Thus, suppression of TGF-β signaling in MDA-MB-231 cells makes rapamycin a cytotoxic, rather than cytostatic drug. The important implication here is that cells with defective TGF-β signaling may be selectively sensitive to rapamycin. We have tested this

hypothesis by examining the effect of rapamycin in cancer cell lines with defective TGF-β signaling and rapamycin killed these cells in the presence of either TGF-β or serum (Gadir *et al*., 2007; see part III).

1.3.5 mTOR IN CANCER THERAPY

As discussed above two of the major mTOR substrates, 4E-BP1 and S6K1 are directly involved in translational control of mRNA coding for important cell cycle regulators and cell growth regulators, for example, cyclin D1 and ribosomal proteins, respectively. Considering that cancer cells are dependent upon many of these proteins for their malignant phenotype, it is expected that mTOR is considered to be an important target for cancer therapy. A number of mTOR inhibitors, as well as inhibitors of proteins on the mTOR pathway, are in clinical development.

Treatment with rapamycin or its derivatives CCI-779, RAD001, and AP23573 inhibits proliferation of a large number of cell lines and in some instances leads to apoptosis (Carraway and Hidalgo, 2004). Rapamycin and its analogs are the only specific inhibitors of mTOR reported. Rapamycin forms a complex with FKBP12 and mTOR, resulting in potent inhibition of mTOR signaling (see part 1.3.2 and 1.3.3). Most breast cancer cell lines that show to be sensitive to CCI-779 were found to be estrogen dependent, over-expressed ErbB2, and/or had PTEN deletions (Carraway and Hidalgo, 2004). The resistant breast cancer cell lines lacked these features. Additionally, sensitive breast cancer cell lines generally had higher levels of activated Akt, leading to

downstream activation of mTOR and perhaps consequent sensitivity to mTOR inhibitors.

ErbB2 is frequently overexpressed in breast cancers and is correlated with poor prognosis ((Hynes and Boulay, 2006). ErbB2 overexpression provides a strong stimulus for PI3K/Akt pathway activation and has been associated with increased phosphorylation of mTOR effectors in primary breast cancers indicating that ErbB2-driven tumors might be more dependent on mTOR signaling (Hynes and Boulay, 2006). Indeed, in phase II trial, 2 of 3 ErbB2-overexpressing patients showed an objective response to the rapamycin analogue, indicating that ErbB2 overexpression might be a marker for choosing patients to treat with mTOR inhibitors (Zhou *et al*, 2004; Klos *et al*., 2006).

One of the potential problems with the use of rapamycin as an anti-cancer drug is that in most cases, rapamycin is cytostatic rather than cytotoxic and induces G1 arrest, rather than apoptosis in most cancer cells (Neshat *et al*., 2001; Podsypanina *et al*., 2001; Law *et al*., 2002). Therefore, while rapamycin targets a protein critical for survival signals in many human cancers, under most conditions reported to date, rapamycin does not reverse the survival of these cells and leads to the suppression of cell cycle progression in G1 instead of cell death. The synergistic action of rapamycin and TGF-β on cell cycle arrest (Law *et al*., 2002; Gadir *et al*, 2007) may provide some insight into why rapamycin is cytostatic in most cancer cells and provide an opportunity to target cancers where there is a high incidence of defective TGF-β signaling. Since defects in TGF-β signaling is common in pancreatic, and colon cancer it is conceivable that

rapamycin would be selectively effective for treatment of a high percentage of theses cancers. It is also possible that rapamycin in combination with strategies for suppressing TGF-β signaling could be effective in treating cancers with intact TGF-β signaling.

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CHAPTER II

MATERIALS AND METHODS

2.1 CELLS, CELL CULTURE CONDITIONS AND TRANSFECTION

The MDA-MB-231, SW480, MCF-7, MDA-MB-468 , Panc1 and BT549 cells used in this study were obtained from the American Tissue Type Culture Collection (ATCC) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone).

For transfection of siRNAs, Cells were plated on 12-well plates at 30% confluence in medium containing 10% serum without antibiotics. After one day, cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer directions. After 24 hr the media was changed to fresh media containing 10% serum. Three days later cells were lysed and analyzed by Western blot. Transfection efficiency was determined by transfection of pEGFP-C1 (Clontech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 90%.

2.2 MATERIALS

Rapamycin, the PKCδ inhibitor rottlerin, and the MAPK inhibitor U0126 were obtained from Calbiochem. LY-294002 was from Biomol International. Aphidicolin and the TGF-β inhibitor SB-431542 were from Sigma. Recombinant human TGF-β1 and the TGF-β1 blocking antibody (MAB2401) were obtained from R&D systems.

All antibodies used in this work were obtained from Cell Signaling Technology except the phospho-specific antibodies for pRb, the ribosomal subunit S6 kinase (p70S6K), and the Smad3 and p27 antibodies, which were obtained from Santa Cruz Biotechnology. PLD2 antibody and non-targeted negative control siRNA duplexes were obtained from Ambion. PKCδ and non-targeted negative control siRNA duplexes were obtained from Qiagen.

2.3 FLOW CYTOMETRIC ANALYSIS

Cell suspensions were recovered and resuspended in the following fixing solution: 20ml 1X PBS, 2% BSA, 0.1% NaN_3. 9ml of 100% ethanol was added drop wise. Fixed cells were centrifuged, washed, and then resuspended in 500µl sorting buffer: 1X PBS, 0.5% BSA, 1mM EDTA, 40µg/ml Propidium Iodide, 100µg/ml RNAse A, and filtered through 40-mm diameter mesh to remove clumps of nuclei. Percentages of cells within each of the cell cycle compartments (G0-G1, S, or G2-M) were determined by flow cytometry (FACSCalibur; Becton Dickinson).

2.4 CELL VIABILITY AND APOPTOSIS ASSAYS

Cell viability was determined by trypan blue exclusion as described previously (Chen *et al.,* 2005). Apoptosis was evaluated by examination of cleavage of the caspase 3 substrate PARP as described previously (Chen *et al.,*

2005). Apoptosis was also determined by examination of sub-genomic DNA using flow cytometry as described above.

2.5 WESTERN BLOT ANALYSIS

Samples were adjusted into gel-loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol) and then heated for 5 min at 100℃ prior to separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After samples were transferred to nitrocellulose membranes (Osmonics), membrane filters were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.05% Tween 20 and then incubated with the appropriate antibody diluted in 5% nonfat dry milk in PBS with 0.05% Tween 20. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used, and the bands were visualized by using an enhanced chemiluminescence detection system (Pierce).

2.6 PHOSPHOLIPASE D ASSAYS

PLD activity was measured by the ability of cells to convert the metabolically labeled [^{3}H]-PC into PBt in the presence of exogenously provided 1-BtOH. Cells were grown in 60 mm culture dishes to confluency and made quiescent by shifting into DMEM containing 0.5% bovine calf serum for overnight. These quiescent cells were labeled for 4-6 hr with [^{3}H]-myristate (40 Ci/mmol) at

final concentration of 1 µCi/ml, and followed by 15 min of incubation with 1% (v/v) 1-BtOH. Afterwards, cells were placed on ice, washed twice with cold PBS and collected in 0.5 ml of methanol / 6 M HCl (50:1, v/v). Lipids were extracted by adding 0.5 ml of chloroform. Phase separation was achieved by the addition of 155µl of 1M NaCl and the organic phase was recovered after centrifugation. This was followed by reextraction through the addition of 350µl H_2O, 115µl of 1M NaCl and 115µl methanol. An aliquot of the obtained organic phase was counted in a liquid scintillation counter and the volume of each sample that had the same intensity of radioactivity was calculated according to the readings. Lipids containing an equal amount of radioactivity were then dried under a stream of nitrogen and redissolved in 50µl of chloroform / methanol (9:1, v/v). Samples were then spotted on a precoated silica (60A) plates and separated by thin layer chromatography (TLC) with a solvent system of ethylacetate/trimethylpentane/acetic acid/H_2O (9:5:2:10, v/v, upper layer). The transphosphatidylation product PBt was visualized by autoradiography of the TLC plates and the films were scanned in a densitometer for quantification.

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CHAPTER III

DEFECTIVE TGF-β SIGNALING SENSITIZES HUMAN CANCER CELLS TO RAPAMYCIN

(THIS WORK HAS BEEN PUBLISHED ON *ONCOGENE,* 2007)

3.1 INTRODUCTION

Early stages of tumorigenesis frequently involve the suppression of default apoptotic signals that represent the first line of defense against cancer (Hanahan and Weinberg, 2000). Signals that suppress apoptotic programs have been referred to as survival signals (Foster, 2004; Guertin and Sabatini, 2005). Phosphatidylinositol-3-kinase (Luo *et al.*, 2003) and phospholipase D (PLD) (Foster, 2006), both of which generate survival signals in a variety of human cancers, target mTOR, the mammalian target of rapamycin. mTOR is a protein kinase that is sensitive to environmental stresses that restrict cell growth and proliferation (Guertin and Sabatini, 2005; Sawyers, 2003). Targeting mTOR in anti-cancer therapeutic strategies represents a promising approach to attack cancer cells by down-regulating protective apoptotic programs (Foster, 2004; Guertin and Sabatini, 2005; Sawyers, 2003). Although there have been some successes, clinical trials with rapamycin and rapamycin derivatives such as CCI-779 and RAD-001 have been largely disappointing (Guertin and Sabatini, 2005; Sawyers, 2003). One of the problems with the use of rapamycin as an anti-cancer drug is that in most cases, rapamycin is cytostatic rather than cytotoxic and induces G1 cell cycle arrest, rather than apoptosis (Law *et al.*, 2002; Neshat *et al.*, 2001; Podsypanina *et al.*, 2001).

MDA-MB-231 human breast cancer cells have a survival signal generated by PLD that is dependent on mTOR (Chen *et al.*, 2005; Foster, 2007; Zhong *et al.*, 2003). Rapamycin treatment of MDA-MB-231 cells resulted in apoptosis in

the absence of serum, but not in the presence of serum (Chen *et al.*, 2005). These data indicated that a factor(s) in serum was suppressing rapamycin-induced apoptosis. Since previous reports indicated that rapamycin induces a G1 cell cycle arrest, we investigated the effect of serum growth factors on cell cycle progression and the ability to suppress rapamycin-induced apoptosis. Transforming growth factor-β (TGF-β) is a factor present in serum that has been implicated in the suppression of cell cycle progression in MDA-MB-231 cells (Chen *et al.,* 2005; Siegel and Massague, 2003). We therefore investigated whether TGF-β in serum is responsible for the cell cycle arrest induced by rapamycin in MDA-MB-231 cells in the presence of serum and whether TGF-β could suppress the apoptosis induced by rapamycin in the absence of serum.

3.2 RESULTS

3.2.1 TGF-β SUPPRESSES RAPAMYCIN-INDUCED APOPTOSIS IN MDA-MB-231 CELLS

We first examined the effect of rapamycin on cell cycle progression using flow cytometric analysis of DNA content in the presence and absence of serum. As shown in Figure 3.2.1a, rapamycin treatment of MDA-MB-231 cells in the presence of serum resulted in an increase in the percentage of cells with a G1 DNA content and a reduction of cells with greater than G1 DNA content. In the absence of serum, rapamycin treatment resulted in virtually all of the cells containing subgenomic levels of DNA - consistent with the previously reported rapamycin-induced apoptosis observed in these cells when deprived of serum (Chen *et al.,* 2005). Thus, while rapamycin induces apoptosis in the absence of serum, in the presence of serum, rapamycin blocks cell cycle progression in G1.

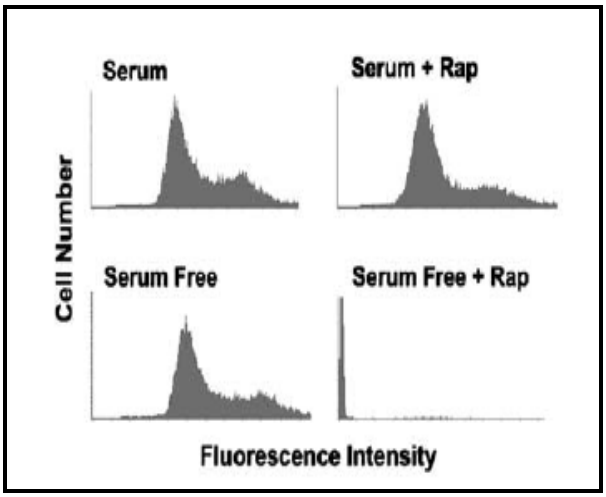

Figure 3.2.1: TGF-β suppresses rapamycin-induced apoptosis in MDA-MB-231 cells. (a) MDA-MB-231 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were provided with either fresh media containing 10% serum, fresh media containing 10% serum and 20 μM rapamycin (Rap), fresh media containing 0% serum, or fresh media containing 0% serum and 20 μM rapamycin as indicated. The cells were then subjected to flow cytometric analysis 18 hr later as described in Materials and Methods. The DNA content per cell shown graphically above is from a representative experiment that was repeated three times. The percentage of cells in G1 was: Serum, 56%; Serum + Rap, 67%; Serum Free, 60%; and Serum Free + Rap, <1%. The flow cytometry in (a) are representative of at least three independent experiments

The data In Figure 3.2.1a indicate that a component(s) in serum causes a block in cell cycle progression when cells are treated with rapamycin. Serum is a complex mix of growth factors, however if the effect is due to suppression of cell cycle progression, then a serum factor capable of suppressing cell cycle progression could be implicated. We therefore examined the effect of TGF-β, which can suppress cell cycle progression (Law *et al.,* 2002; Siegel and Massague, 2003), on the ability of rapamycin to induce apoptosis in serum-starved MDA-MB-231 cells. As demonstrated previously (Chen *et al.,* 2005) and indicated in Figure 3.2.1a, there is a large loss in cell viability in MDA-MB-231 cells subjected to serum withdrawal in the presence of rapamycin (Figure 3.2.1b).

The loss in cell viability was accompanied by an increase in the cleavage of the caspase 3 substrate poly-ADP-ribose polymerase (PARP) (Figure 3.2.1b), indicating that the cell death was due to apoptosis. If TGF-β was added along with rapamycin, the effect of rapamycin on cell viability and PARP cleavage was reversed – almost as effectively as observed for serum (Figure 3.2.1b). The addition of IGF1, a growth factor that facilitates cell cycle progression (Baserga *et al.,* 2003), did not reverse the effect of rapamycin (Figure 3.2.1b). We also examined the effect of TGF-β on DNA content per cell in rapamycin treated MDA-MB-231 cells. As shown in Figure 3.2.1c, TGF-β eliminated most of the sub-genomic DNA caused by rapamycin treatment and led to a profile that indicated a predominant G1 DNA content for surviving cells. These data demonstrate that TGF-β, like serum, suppresses rapamycin-induced apoptosis.

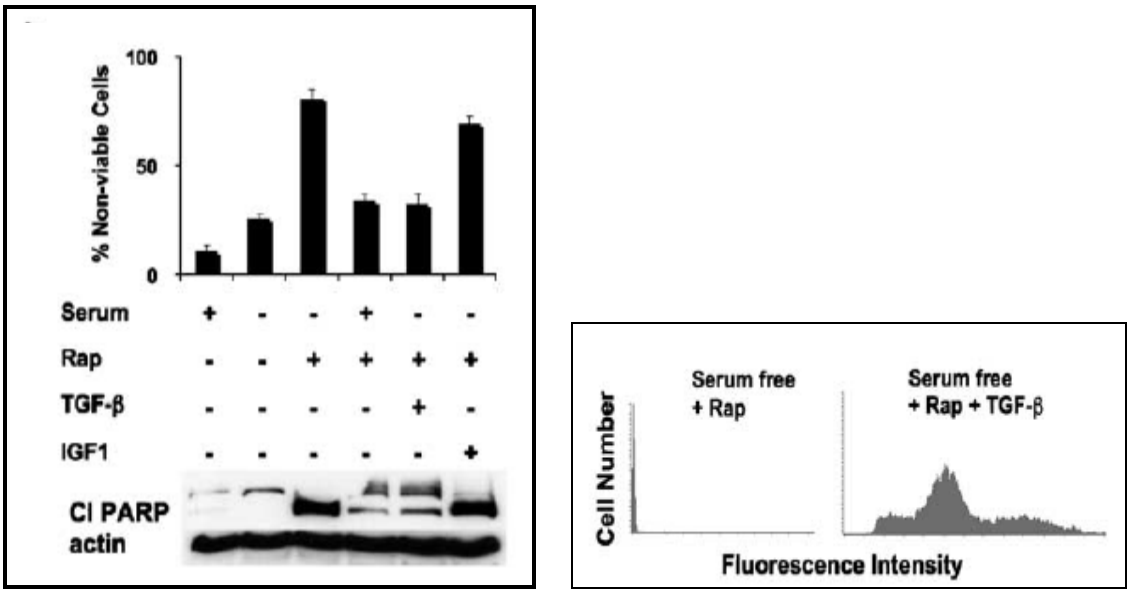

Figure 3.2.1: (Continue). (b) MDA-MB-231 cells were plated as above and then shifted to media containing 10% serum (Ser), 20 μM rapamycin, 10 ng/ml TGF-β, and 10 ng/ml IGF-1 as indicated. 18 hr later cell viability (upper graph) and PARP cleavage (lower blot, Cl PARP) was determined as described in Materials and Methods. The Western blot for PARP was examined for loading by reprobing with an antibody raised against actin. (c) MDA-MB-231 cells in serum free media in the presence of rapamycin (20 μM) with or without TGF-β (10 ng/ml) were subjected to flow cytometric analysis as in (a). Error bars for the graph in (b) represent the standard deviation from three independent experiments. The Western blots in (b) and the flow cytometry in (c) are representative of at least three independent experiments

Because of the enrichment of cells with G1 DNA content upon rapamycin treatment, we analyzed the effect of TGF-β and rapamycin on regulators of the G1/S cell cycle progression. To this end, we examined the effect of TGF-β and rapamycin on the level of the cyclin-dependent kinase inhibitor p21 and the phosphorylation of the tumor suppressor Rb, which is phosphorylated by cyclin-dependent kinases. MDA-MB-231 cells were put in serum free media for 18 hr in the presence of rapamycin and or TGF-β and the levels of p21 and Rb phosphorylation were determined by Western blot analysis. As shown in Figure

3.2.1d, rapamycin and TGF-β synergistically stimulated increases in the level of p21 and decreases in the phosphorylation state of Rb. Since phosphorylation of Rb is critical for progression through G1 (Ho and Dowdy, 2002), these data are consistent with the hypothesis that in the presence of rapamycin, TGF-β signaling is de-repressed and induces G1 arrest – in part by increasing p21 expression and suppressing Rb phosphorylation.

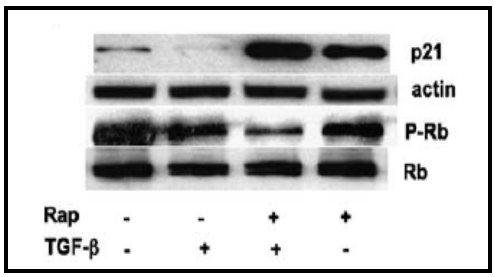

Figure 3.2.1: (Continue). (d) MDA-MB-231 cells were plated as above and put in serum free media 24 hr later. TGF-β (10 ng/ml) and rapamycin (Rap) (20 μM) was added as indicated and the levels of p21 and phosphorylated Rb (P-Rb) were determined 18 hr later by Western blot analysis. Blots were re-probed with an antibody to actin and Rb to control for loading. The Western blots in (d) are representative of at least three independent experiments.

3.2.2 TGF-β SIGNALING IS REQUIRED FOR THE SUPPRESSION OF RAPAMYCIN-INDUCED APOPTOSIS BY SERUM

While the data in Figure 3.2.1 demonstrate that TGF-β is able to suppress the apoptosis induced by rapamycin in MDA-MB-231 cells, it does not indicate whether TGF-β in serum is responsible for suppressing rapamycin-induced apoptosis. We therefore pre-treated the serum with a neutralizing anti-TGF-β antibody previously demonstrated to block the effects of TGF-β (Ho and Dowdy,

2002). As shown in Figure 3.2.2a, rapamycin induced both cell death and PARP cleavage in the presence of the neutralizing anti-TGF-β antibody. To further establish that the anti-TGF-β antibody used was blocking TGF-β signaling, we examined the effect of this antibody on Smad2 phosphorylation, which occurs in response to TGF-β (Siegel and Massague, 2003). As shown in Figure 3.2.2b, the anti-TGF-β antibody also suppressed Smad2 phosphorylation induced by serum. These data indicate that TGF-β in serum was at least partially responsible for the ability of serum to suppress the apoptosis induced by rapamycin in MDA-MB-231 cells.

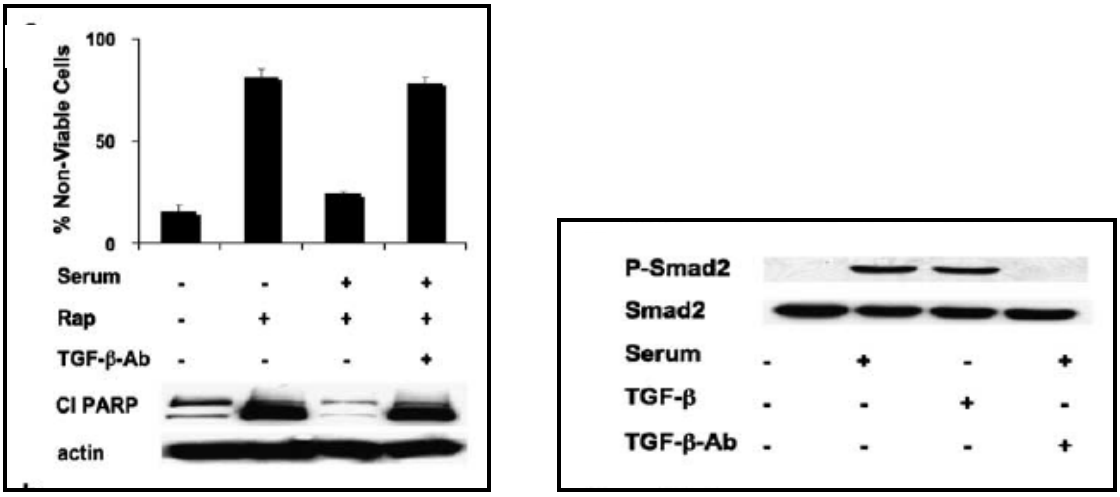

Figure 3.2.2: TGF-β signaling is required for the suppression of rapamycin-induced apoptosis by serum. (a) MDA-MB-231 cells were plated as in Figure 1B and then shifted to media containing 10% serum, 20 μM rapamycin (Rap), and anti-TGF-β1 antibody (2 μg/ml) as indicated. 18 hr later cell viability and PARP cleavage were determined as in Figure 1B. (b) MDA-MB-231 cells were plated as above and put in media containing either 0% or 10% serum, or 0% serum and TGF-β (10 ng/ml). The anti-TGF-β1 antibody (2 μg/ml) was added as indicated. 24 hr later, the levels of phosphorylated Smad2 (P-Smad2) and total Smad2 were determined by Western blot. Error bars for the graph in (a) represent the standard deviation from at least two independent experiments. Western blots are representative of at least two independent experiments.

We also examined the effect of an inhibitor of the TGF-β receptor SB-431542 (Inman *et al.,* 2002) on the ability of rapamycin to induce apoptosis in MDA-MB-231 cells. As shown in Figure 3.2.2c, SB-431542 had the same effect as the neutralizing TGF-β antibody and made rapamycin an apoptotic drug on the MDA-MB-231 cells. These data indicate that the TGF-β present in serum is sufficient to suppress the apoptotic effect of rapamycin.

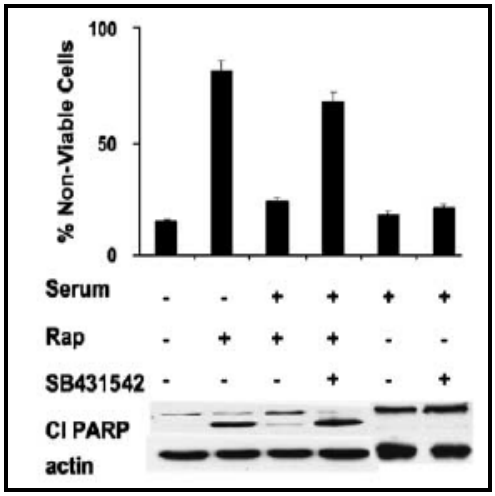

Figure 3.2.2: (Continue) (c) MDA-MB-231 cells were plated as above and then shifted to media containing 10% serum, 20 μM rapamycin (Rap), and SB-431542 (10 μM) as indicated. 18 hr later cell viability and PARP cleavage were determined as (a). Error bars for the graph in (c) represent the standard deviation from at least two independent experiments. Western blots are representative of at least two independent experiments.

Protein kinase Cδ (PKCδ) has been Implicated in signals generated by TGF-β (Perillan *et al.,* 2002; Runyan *et al.,* 2003). Therefore to further establish that de-repression of TGF-β signaling was critical for the G1 arrest induced by rapamycin, we examined whether suppression of PKCδ, like suppression of TGF-β directly, prevented G1 arrest by rapamycin and led to apoptosis. We first examined the effect of rottlerin, a compound that has been shown to suppress

PKCδ activity *in vivo* (Lu *et al.,* 1997) on rapamycin treated MDA-MB-231 cells. As shown in Figure 3.2.2d, in the presence of rottlerin, rapamycin induced apoptosis and PARP cleavage in MDA-MB-231 cells in the presence of either serum or TGF-β. To confirm this result we examined the effect of suppressing PKCδ expression with PKCδ siRNA. MDA-MB-231 cells were transfected with either a non-specific control or PKCδ specific siRNA and the effect of TGF-β and rapamycin on cell viability and PARP cleavage was evaluated. As shown in Figure 3.2.2e, the PKCδ siRNA reversed the protective effect of both serum and TGF-β on rapamycin-induced apoptosis. We also examined the effect of PKCδ siRNA on PKCδ levels and on the induction of p21 by TGF-β. As shown in Figure 3..2.2f, PKCδ siRNA reduced PKCδ expression and suppressed the expression of p21. We also examined the effect of suppressing PKCδ activity on Smad2 phosphorylation, and as shown in Figure 3.2.2g, rottlerin treatment reduced the Smad2 phosphorylation induced by both serum and TGF-β. These data indicate that the effects of TGF-β on cell cycle progression and rapamycin-induced apoptosis are dependent upon PKCδ. Collectively, the data in Figure 3.2.2 suggest that the TGF-β present in serum is required for the G1 arrest induced by rapamycin in the presence of serum, and that suppression of TGF-β signaling results in apoptosis instead of G1 arrest in the presence rapamycin.

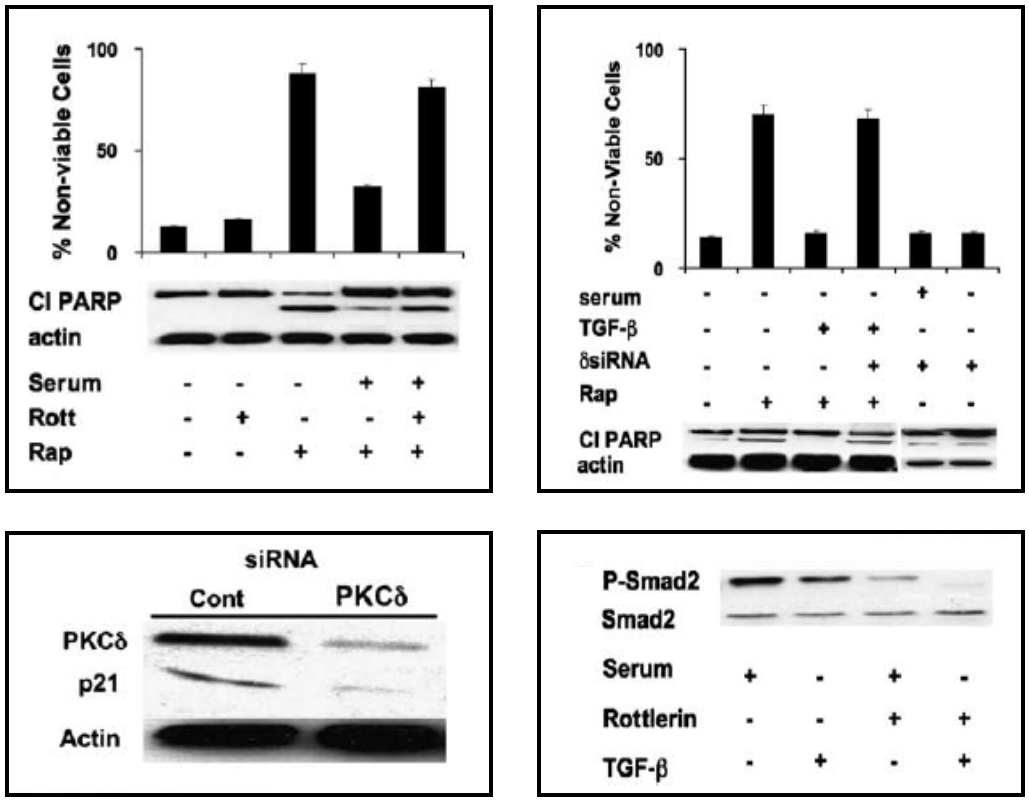

Figure 3.2.2: (Continue) (d) MDA-MB-231 cells were plated as in Figure 3.2.1 and then shifted to media containing 10% serum, TGF-β (10 ng/ml), 20 μM rapamycin (Rap), and 3 μM rottlerin (Rott) as indicated. 18 hr later cell viability and PARP cleavage were determined as in Figure 1. (e) MDA-MB-231 cells were plated as in Figure 1 and put in media containing 10% serum for 24 hr. Cells were then transfected with either control or PKCδ siRNA as described in Materials and Methods. 48 hr later, the cells were put in serum free media containing TGF-β (10 ng/ml) and rapamycin (20 μM) as indicated. Cell viability and PARP cleavage were determined 18 later as Figure 3.2.1. (f) MDA-MB-231 cells were plated as in Figure 3.2.1 and put in media containing 10% serum for 24 hr. Cells were then transfected with either control or PKCδ siRNA as in (d). 48 hr later, the cells were put in serum free media containing TGF-β (10 ng/ml). The levels of PKCδ, p21, and actin were then determined by Western blot analysis 24 hr later as in Figure 1. (g) MDA-MB-231 cells were plated as above and put in media containing either 0% serum 10% serum, or TGF-β (10 ng/ml) as indicated. Rottlerin (3 μM) was added as indicated. 24 hr later, the levels of phosphorylated Smad2 (P-Smad2) and total Smad2 were determined by Western blot. Error bars for the graphs in (d), and (e) represent the standard deviation from at least two independent experiments. Western blots are representative of at least two independent experiments.

3.2.3. BLOCKING CELLS IN S-PHASE RESTORES THE ABILITY OF RAPAMYCIN TO INDUCE APOPTOSIS IN THE PRESENCE OF SERUM

The data provided in Figure 3.2.1 suggest that serum and TGF-β suppress rapamycin-induced apoptosis by blocking passage through the G1/S cell cycle checkpoint. We therefore pretreated MDA-MB-231 cells with aphidicolin, which blocks cell cycle progression after the G1/S cell cycle checkpoint in early S-phase (Borel *et al.,* 2002). As shown in Figure 3.2.3a, aphidicolin treatment lead to an accumulation of cells with a G1 DNA content – indicating that the aphidicolin was inducing cell cycle arrest at the G1/S transition as reported. As shown in Figure 3.2.3b, pre-treating cells with aphidicolin restored the ability of rapamycin to induce apoptosis in the presence of serum. These data indicate that cells that pass the G1/S cell cycle checkpoint and enter S-phase become sensitive to the apoptotic effects of rapamycin.

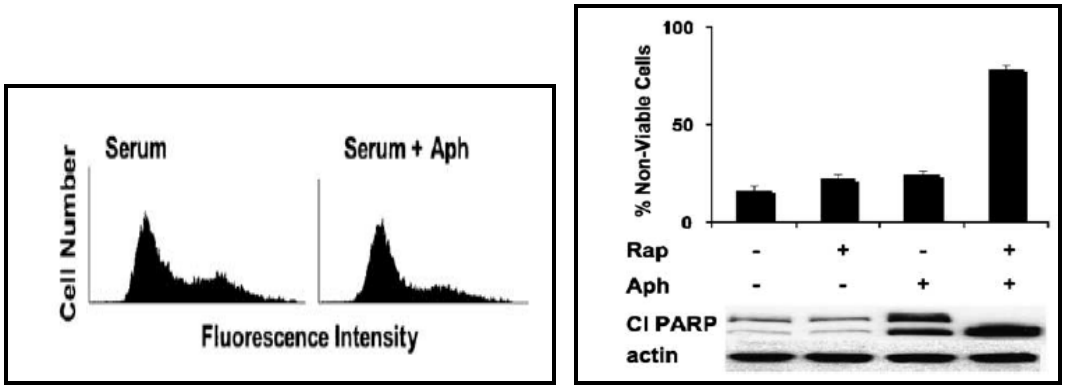

Figure 3.2.3: Aphidicolin restores the ability of rapamycin to induce apoptosis in MDA-MB-231 cells in the presence of serum. (a) MDA-MB-231 cells were plated as in Figure 3.2.1a and put in fresh media containing 10% serum 24 hr later. The cells were treated with 5 μg/ml aphidicolin (Aph) for 18 hr at which point the cells were subjected to FACS analysis as In Figure 3.2.1a to investigate the effect of aphidicolin on cell cycle progression. (b) Rapamycin (Rap) (20 μM) was then added and cell viability and PARP cleavage was determined 18 hr later as in Figure 3.2.1b. The experiment shown is representative of two independent experiments.

3.2.4. HUMAN CANCER CELLS WITH DEFECTIVE TGF-β SIGNALING ARE KILLED BY RAPAMYCIN IN THE PRESENCE OF EITHER SERUM OR TGF-β

Defective TGF-β signaling has been reported in SW480 colon and MDA-MB-468 breast cancer cells by virtue of mutations in the Smad4 gene (Jackson *et al.,* 2005; Runyan *et al.,* 2003). BT-549 breast cancer cells do not express PKCδ (Jackson *et al.,* 2005), which is required for TGF-β signals (Perillan *et al.,* 2002; Runyan *et al.,* 2003). We therefore examined the effect of rapamycin on these cells in the presence and absence of serum. As shown in Figure 3.2.4a, rapamycin induced apoptosis in the SW480, MDA-MB-468, and BT549 cells in the presence of serum as indicated by increased cell death and PARP cleavage. Significantly, TGF-β was unable to suppress the apoptosis observed in the absence of serum (Figure 3.2.4a). The ability of rapamycin to kill the SW480 and MDA-MB-468 cells was somewhat less than that observed for the BT549 cells. This may reflect the ability of TGF-β to partially suppress growth in Smad4 deficient cells (Fink *et al.,* 2004; Fink *et al.,* 2001). These data indicate that cells defective in TGF-β signaling are uniquely sensitive to the effects of rapamycin in that rapamycin induces apoptosis rather than cell cycle arrest in the presence of serum or TGF-β.

We also wanted to determine whether the phenomenon of combining strategies that target mTOR with strategies to suppress TGF-β signaling could be observed in cells other than the MDA-MB-231 cells. To this end we examined the effect of rapamycin MCF-7 human breast cancer cells that have intact TGF-β signals (Kalkhoven *et al.,* 1995) in combination with the anti-TGF-β antibody and

the TGF-β receptor inhibitor SB-431542. The MCF-7 cells were placed in culture in both the presence and the absence of serum. As reported previously, there was significant cell death in the absence of serum and rapamycin also induced cell death in the presence of serum (Figure 3.2.4b). However, treatment with either anti-TGF-β antibody or SB-413524 significantly increased cell death and PARP cleavage in the presence of serum and rapamycin (Figure 3.2.4b). These data further support the concept that the absence of TGF-β signaling increases sensitivity to rapamycin.

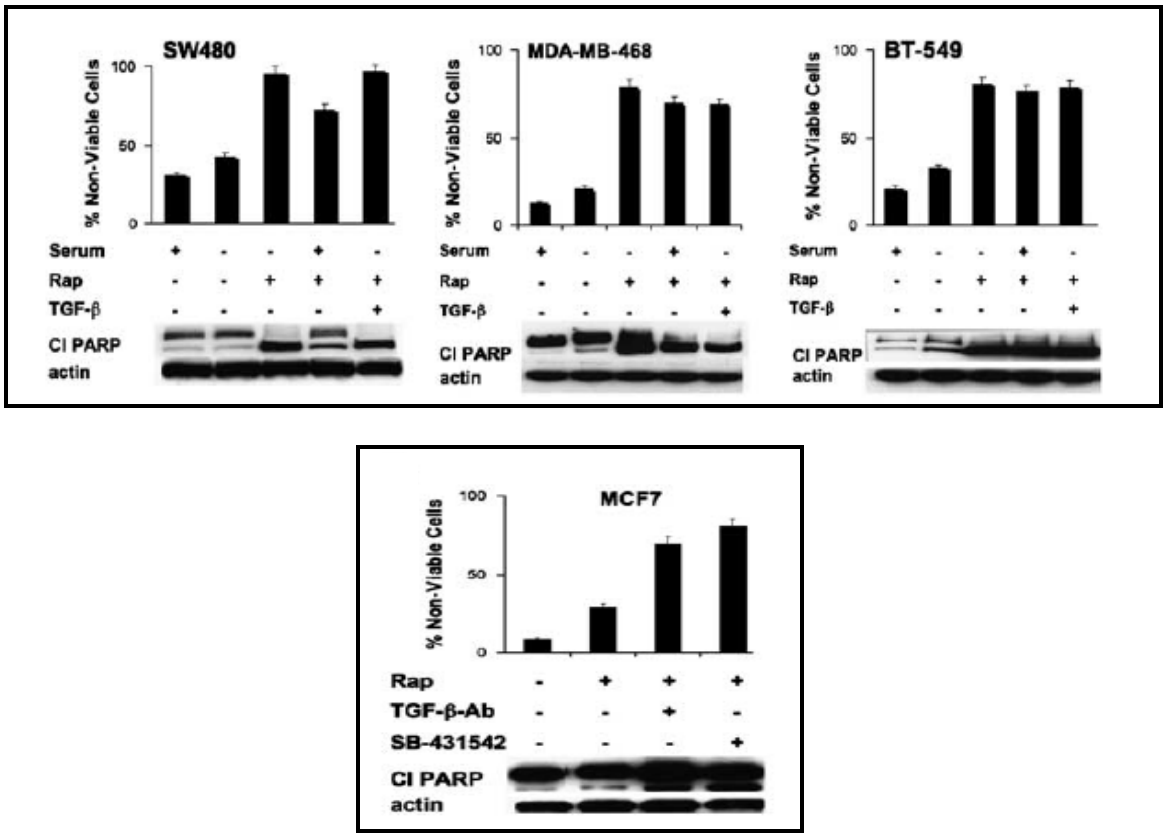

Figure 3.2.4: Human cancer cells with defective TGF-β signaling are killed by rapamycin in the presence of serum. (a) SW480, MDA-MB-468, and BT549 cells were treated with rapamycin (20 μM) and TGF-β (10 ng/ml) in the presence and absence of serum as indicated and cell viability and PARP cleavage was determined as in Figure 3.2.1. (b) MCF-7 breast cancer cells were treated with rapamycin (20 μM) in the presence of serum and TGF-β antibody (2ug/ml) or SB-431542 (10uM) as indicated. Cell viability and PARP cleavage were determined as in (a). The experiments shown are representative of at least two independent experiments.

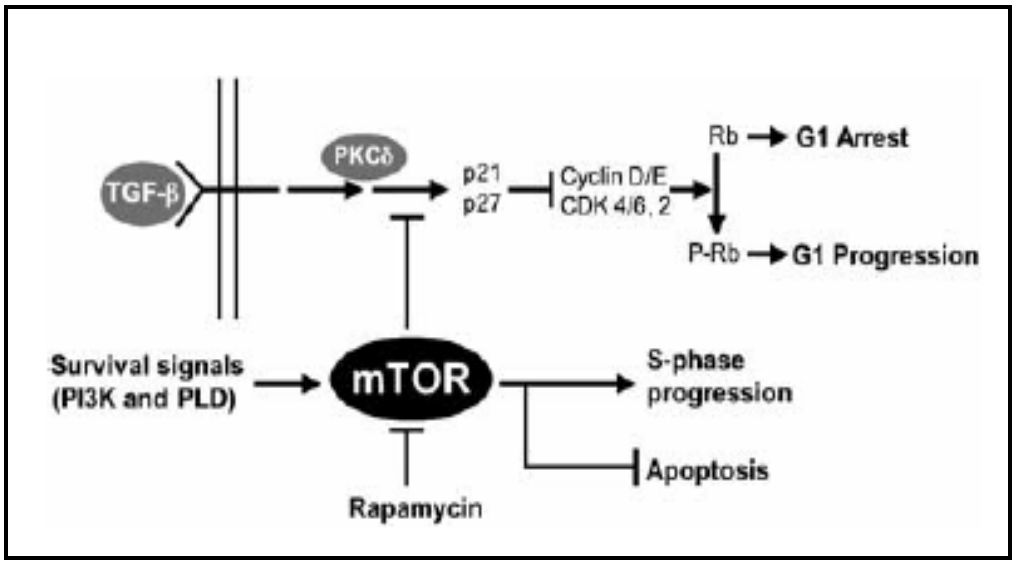

Figure 3.2.5: Model for suppression of rapamycin-induced apoptosis by TGF-β. It is proposed that survival signals generated by phosphatidylinositol-3-kinase (PI3K), PLD, or both activate mTOR. Elevated mTOR activity leads to the suppression of TGF-β signals. In the presence of TGF-β, suppression of mTOR results in the activation TGF-β signals leading to the elevation of p21, p27. p21 and p27, by inhibiting cyclin D and E along with there partner cyclin-dependent kinases (CDK) 4/6 and 2 respectively, suppress the phosphorylation of Rb to P-Rb, leading to G1 arrest rather than progression into S-phase. mTOR promotes progression through S-phase and suppresses apoptosis. Thus, in the absence of mTOR signaling (in the presence of rapamycin), then cells undergo apoptosis as a default pathway activated when cells are unable to complete progression through S-phase.

3.3 DISCUSSION

Rapamycin has great potential as an anti-cancer drug in that it targets mTOR, a critical mediator of survival signals in cancer cells that suppresses protective apoptotic programs (Foster, 2004; Guertin and Sabatini, 2005; Sawyers, 2003). A potential complication in the use of rapamycin in anti-cancer therapies is that in many cases, the effect of rapamycin is cytostatic and does not

kill cancer cells (Law *et al.,* 2002; Neshat *et al.,* 2001; Podsypanina *et al.,* 2001). We reported previously that under conditions of serum withdrawal, rapamycin induces apoptosis in MDA-MB-231 breast cancer cells (Chen *et al*., 2005). Consistent with the previous studies, we find that in the presence of serum growth factors, rapamycin induces G1 arrest, rather than apoptosis. We demonstrated here that the ability of rapamycin to induce G1 arrest in the presence of serum is dependent upon TGF-β, and, that TGF-β is sufficient to suppress rapamycin-induced apoptosis in MDA-MB-231 cells deprived of serum. Importantly, suppression of TGF-β or PKCδ signaling restored the ability of rapamycin to induce apoptosis rather than G1 arrest in the presence of serum. Moreover, human cancer cell lines with defects in TGF-β or PKCδ signaling were killed by rapamycin in the presence of serum. The finding that PKCδ is required for TGF-β to suppress cell cycle progression in G1 is intriguing in that, like TGF-β, PKCδ has been implicated in both tumor suppression and promotion (Jackson and Foster, 2004). These data indicate that rapamycin or other agents that target mTOR could be made a more effective anti-cancer option in combination with the suppression of TGF-β signals that cause G1 arrest. The data also point out that tumors with defective TGF-β signaling may be selectively killed by rapamycin.

It is not clear how rapamycin induces G1 arrest in the presence of TGF-β. However, since TGF-β is present in serum, it is likely that suppression of mTOR by rapamycin de-represses TGF-β signals in MDA-MB-231 cells. Massague's group reported previously that MDA-MB-231 cells are not responsive to the anti-

proliferative effects of TGF-β (Chen *et al.,* 2001). The data reported here indicate that the suppressed TGF-β signaling in these cells is due to mTOR. Consistent with this interpretation, it was recently reported that mTOR suppresses TGF-β signaling by inhibiting Smad3 phosphorylation (Song *et al*., 2006). Importantly, Moses and colleagues have shown that TGF-β and rapamycin work synergistically to induce G1 arrest in MDA-MB-231 cells (Law *et al.,* 2002). Their data and ours indicate that whereas TGF-β by itself was unable to induce cell cycle arrest in MDA-MB-231 cells, rapamycin and TGF-β together did induce G1 cell cycle arrest (Law *et al.,* 2002). Similarly, rapamycin enhanced TGF-β-induced growth arrest in prostate cancer cells (van der Poel *et al.,* 2003; van der Poel, 2004). These studies indicate that rapamycin enhances intracellular signals generated by TGF-β leading to cell cycle arrest. Current evidence is consistent with the hypothesis that survival signals mediated by mTOR involve both suppression of signals generated by TGF-β that result in G1 cell cycle arrest, and the suppression of apoptotic programs that prevent cells in S-phase from progressing to mitosis. A model for the differential effects of rapamycin – in the presence and absence of TGF-β signaling – is shown schematically in Figure 3.2.5. It is proposed that if TGF-β signaling is suppressed, rapamycin stimulates progression through G1 into S-phase where the lack of mTOR activity signals the cells to undergo apoptosis. A rationale for apoptosis in the absence of mTOR activity is that mTOR has been widely implicated as a sensor of nutritional sufficiency (Schmelzle and Hall, 2000).

Thus, in the absence of mTOR, cells sense that there is not sufficient nutrition to complete the cell cycle.

There are many tumors with genetic defects in TGF-β signaling – particularly in the Smad4 gene (Miyaki and Kuroki, 2003), which localizes to a region of chromosome 18q where there is considerable loss-of-heterozygosity especially in colon (Popat and Houlston, 2005) and pancreatic (Jaffe *et al.,* 2002) cancers. Thus, there may be a large number of tumors with defects in the TGF-β signaling pathway that could be effectively targeted with rapamycin or rapamycin derivatives. Another important consideration in the use of rapamycin is that it acts competitively with PLD-generated phosphatidic acid (Foster, 2004; 2006; 2007) necessitating higher levels of rapamycin in cells with elevated PLD activity (Chen *et al.,* 2003; Chen *et al.,* 2005). Since many cancer cells, including all of the cell lines used in this study have elevated levels of PLD activity (our unpublished results), the dose of rapamycin used will also be important for effective use in clinical settings. Thus, the status of TGF-β signaling and PLD activity could be critical factors for determining whether rapamycin will be an effective and appropriate strategy for anti-cancer therapeutic strategies. The suppression of PLD activity could improve the efficacy of rapamycin as has been demonstrated in culture (Chen *et al.,* 2003). The use of rapamycin in therapeutic strategies for targeting cancers has been widely discussed because it is well tolerated, it is highly specific for mTOR, and because it targets survival signals in cancer cells (Foster, 2004; Sawyers, 2003). The data reported here provide a rationale for using rapamycin in combination with strategies that suppress TGF-β

and PKCδ signaling, which makes rapamycin cytotoxic rather than cytostatic. The study also suggests that rapamycin treatment could be especially effective in treating the many cancers where TGF-β and PKCδ signaling are already defective.

In conclusion, this study demonstrates that rapamycin induces apoptosis when cells cannot be arrested in G1 by TGF-β signaling. The ability of TGF-β to suppress apoptosis is due to preventing cells from progressing through G1 into S-phase at which point, the lack of mTOR signaling causes the cells to decide to undergo apoptosis rather than replicate their DNA under these conditions. The implication from this study is that rapamycin could be used to specifically target human cancers with defects in TGF-β signaling, or alternatively rapamycin could be used effectively in combination with strategies for suppressing TGF-β signaling.

CHAPTER IV:

SUPPRESSION OF TGF-β SIGNALING BY PHOSPHOLIPASE D

(THIS WORK HAD BEEN PUBLISHED ON *CELL CYCLE 2007, 6:22:2840-45*)

4.1 INTRODUCTION

A common genetic defect in cancers is the suppression of signals generated by transforming growth factor-β (TGF-β) (Siegel and Massagué, 2003; Donovan and Slingerland, 2000). A key target of TGF-β signaling is $p27^{Kip1}$, which negatively regulates cell cycle progression. It has been proposed that suppressing the effects of $p27^{Kip1}$ is important in tumor progression (Alkarain *et al.*, 2004). $p27^{Kip1}$ is an inhibitor of the cyclin-dependent kinase 2 (CDK2), which is regulated by cyclin E (Donovan and Slingerland, 2000). TGF-β signaling is initiated by phosphorylation of Smad2 and Smad3 by the TGF-β receptor I, and this phosphorylation leads to an interaction between a Smad2/Smad3 complex (Smad2/3) and Smad4 (Siegel and Massagué, 2003). The Smad2/3-Smad4 complex can then go to the nucleus and act as a transcription factor to stimulate the expression of genes such as those that encode $p27^{Kip1}$, which can suppress cell cycle progression (Abdollah *et al.,* 1997; Liu *et al.,* 1997; Souchelnytskyi *et al.,* 1997). Mutations in the Smad4 gene are commonly observed in human cancers – especially in colon and pancreatic cancers (Markowitz *et al.,* 2002; Jaffee *et al.,* 2002). The human breast cancer cell line MDA-MB-231, does not have a mutant Smad4 gene, but TGF-β does not suppress cell cycle progression in these cells (Chen *et al.,* 2001). Thus, TGF-β signaling in MDA-MB-231 cells is being suppressed by another mechanism. MDA-MB-231 cells have high levels of phospholipase D (PLD) activity, which provides a survival signal that promotes cell cycle progression and suppresses apoptosis in the absence of serum growth

factors (Zhong *et al.,* 2003; Chen *et al.,* 2005). A key downstream target of the PLD metabolite phosphatidic acid (PA) is mTOR – the mammalian target of rapamycin (Fang *et al.,* 2001; Foster, 2004; Foster, 2007). mTOR has been widely implicated as a target of survival signals in cancer cells (Foster, 2004; Hornberger *et al.,* 2006). Intriguingly, mTOR has been reported to suppress TGF-β signaling, (Sawyers, 2003; Song *et al.,* 2006) and rapamycin has been shown to induce G1 cell cycle arrest (Gadir *et al.,* 2007; Neshat, 2001). In addition, rapamycin enhances TGF-β-induced growth arrest in prostate cancer cells (Podsypanina *et al.,* 2001; van der Poel, 2003). Thus, it is possible that the mTOR-dependent survival signals generated by PLD are mediated in part by suppressing TGF-β signaling that blocks cell cycle progression. We have investigated whether the high level of PLD activity in MDA-MB-231 cells is responsible for the reduced responsiveness of these cells to TGF-β-induced growth arrest. We report here that the suppression of PLD activity increases phosphorylation on Smad2/3 at positive regulatory sites and reduces phosphorylation on Smad2 at negative regulatory sites. Suppressing PLD also reversed the suppression of TGF-β signaling in MDA-MB-231 cells leading to increased expression of the CDK inhibitors $p27^{Kip1}$ and $p21^{Cip1}$, and reduced phosphorylation of the CDK substrate pRb. These data suggest that the elevated PLD activity observed in a wide variety of human cancer cells (van der Poel, 2004; Foster *et al.,* 2006), provides a means for suppressing TGF-β signaling and facilitating G1 cell cycle progression.

4.2 RESULTS

4.2.1 SUPPRESSION OF PLD SIGNALING INCREASES SMAD PHOSPHORYLATION AT C-TERMINAL POSITIVE REGULATORY SITES

It was previously reported that mTOR suppresses TGF-β signaling (Sawyers, 2003). Massagué and colleagues (Chen *et al.,* 2001), demonstrated that TGF-β signaling is suppressed in the human breast cancer cell line MDA-MB-231. Since PLD activity is elevated in MDA-MB-231 cells and stimulates an mTOR-dependent survival signal in these cells (Zhong *et al.,* 2003; Chen *et al.,* 2005), we examined the effect of PLD on TGF-β signaling in MDA-MB-231 cells. Following stimulation by TGF-β, Smad2 and Smad3 become phosphorylated at carboxyl terminal serine residues (Ser465 and 467 on Smad2; Ser423 and 425 on Smad3) by TGF-β receptor I (Abdollah *et al.,* 1997). We first examined the effect of inhibiting mTOR with rapamycin on Smad 2/3 phosphorylation in the presence of TGF-β. As shown in Fig. 4.2.1A, Smad 2/3 phosphorylation at these activating serine residues was elevated in response to rapamycin treatment. Phosphorylation of the mTOR substrate S6-kinase was blocked in response to rapamycin treatment indicating that rapamycin was suppressing mTOR.

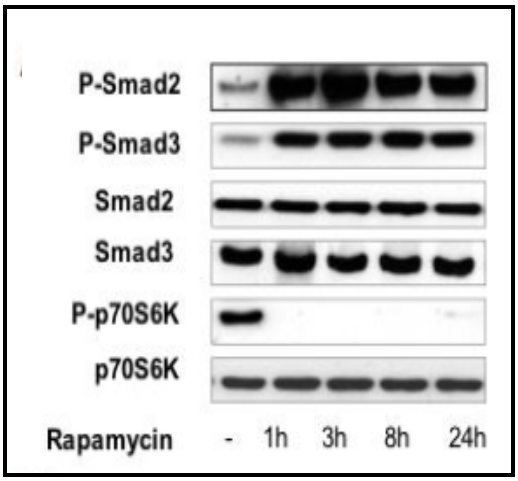

Figure 4.2.1: Supperession of PLD signaling increases Smad phosphorylation at C-terminal positive regulatory sites. (A) MDA-MB-231 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were shifted to fresh media containing 0% FBS and incubated with TGF-β (10ng/ml) overnight. Rapamycin (200 nM) was then added for the indicated times. Cells were harvested, and the level of P-Smad2, P-Smad3 (Ser465 and 467 on Smad2; Ser423 and 425 on Smad3) and P-p70S6K was determined by Western Blot analysis. The blots were reprobed for total Smad2, Smad3, and p70S6K as loading controls. The blot was reprobed for total Smad2/3 as a loading control. These data are representative of at least two independent experiments.

mTOR is activated by phosphatidic acid (PA), the metabolic product of PLD in a manner that is competitive with rapamycin (Fang *et al.,* 2001; Foster and Xu, 2003). To determine the effect of suppressing PLD activity on Smad2/3 activation, two approaches were used. The first approach employed the primary alcohol 1-butanol (1-BtOH), which inhibits the PLD-mediated production of PA and results in production of phosphatidyl-BtOH instead of PA (Chen *et al.,* 2003). Treatment of MDA-MB-231 cells with 1-BtOH, like rapamycin, also elevated Smad2/3 phosphorylation on the activating serine sites in the presence of TGF-β (Fig. 4.2.1B). Tertiary butanol (t-BtOH), which is not utilized by PLD, had no effect on Smad2/3 phosphorylation (Fig. 4.2.1B). To confirm this result, we transfected the cells with PLD2 siRNA. PLD1 siRNA was not tolerated by the

MDA-MB-231 cells and resulted in cell death (data not shown). As shown in Fig. 4.2.1C, the PLD2 siRNA elevated the phosphorylation of Smad2/3 at the activating serine residues.

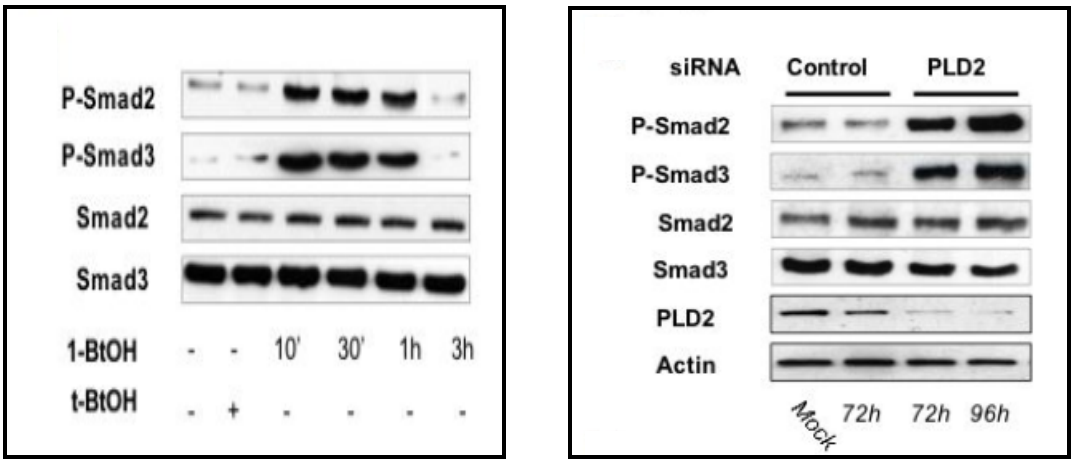

Figure 4.2.1: (Continue). (B) MDA-MB-231 cells were plated as above and incubated overnight in media containing 0% serum and TGF-β (10ng/ml). At this point cells were treated with either 1-BtOH or t-BtOH (0.8%) for the indicated times. The level of P-smad2/3 was then determined as in (A). (C) MDA-MB-231 cells were plated as above and put in media containing 10% serum for 24 hr. Cells were then transfected with either no siRNA (Mock), a control scrambled siRNA, or PLD2 siRNA as described in Materials and Methods. The levels of P-Smad2/3 and PLD2 were determined by Western blot analysis at 72 and 96 hr later as indicated. Total Smad2, Smad3, and actin were determined as loading control. All data are representative of at least two independent experiments.

We also examined the effect of suppressing phosphatidylinositol-3-kinase (PI3K), which also generates signals that target mTOR (Shen *et al.,* 2001). The PI3K inhibitor LY294002 did not lead to increased Smad2/3 phosphorylation (Fig. 4.2.1D), which is consistent with our previous study indicating that MDA-MB-231 cells activate mTOR primarily through PLD (Chen *et al.,* 2005). Interestingly, LY294002 did suppress S6 kinase phosphorylation and also increased the effect

of 1-BtOH on Smad2/3 phosphorylation, indicating that the PI3K pathway may also contribute to mTOR activation in these cells.

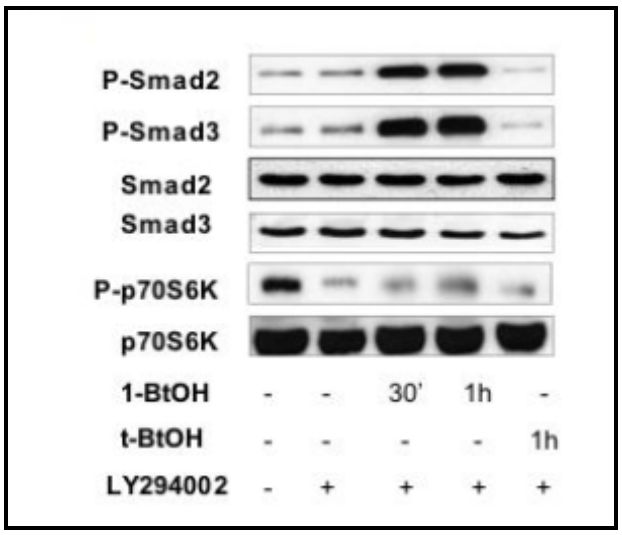

Figure 4.2.1: (Continue). (D) MDA-MB-231 cells were plated as above and incubated overnight in media containing 0% serum and TGF-β (10ng/ml). At this point cells were treated with LY294002 (10 μM) where indicated, and either 1-BtOH or t-BtOH (0.8%) for the indicated times. The levels of P-smad2/3, total Smad2/3, P-S6 kinase, and S6 kinase were then determined as in (A) and (B). These data are representative of at least two independent experiments.

We next examined whether the observed effect of suppressing PLD or mTOR on Smad2/3 phosphorylation was dependent upon TGF-β signaling. To accomplish this, we examined whether the increased phosphorylation on Smad2/3 induced by 1-BtOH and rapamycin was dependent on the presence of TGF-β and TGF-β receptor kinase activity. As shown in Fig. 4.2.1E, the increased phosphorylation of Smad2/3 induced by 1-BtOH and rapamycin was reversed in the absence of TGF-β or by treatment with SB-431542, a compound that suppresses TGF-β receptor I kinase activity (Luo *et al.,*2003). These data

indicate that suppression of PLD survival signals in MDA-MB-231 cells de-represses TGF-β signaling leading to the phosphorylation of Smad2 and Smad3 on the activating carboxyl serine residues Ser465/467, and Ser423/Ser425 respectively.

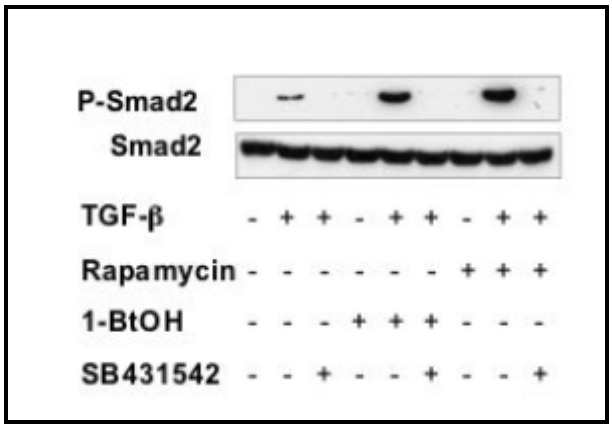

Figure 4.2.1: (Continue). (E) MDA-MB-231 cells were plated as above and incubated overnight in media containing 0% serum in the absence and presence of TGF-β (10ng/ml) as indicated. The TGF-β receptor inhibitor SB-431542 (10 μM) was included with TGF-β where indicated. 1-BtOH (0.8%) and rapamycin (200 nM) were added as indicated and the levels of P-Smad2/3 and total Smad2/3 were determined as above. These data are representative of at least two independent experiments.

4.2.2 SUPPRESSING PLD SIGNALING REDUCES PHOSPHORYLATION AT NEGATIVE REGULATORY SITES IN THE LINKER REGION OF SMAD2

The activity of Smad2/3 in the TGF-β pathway is suppressed by several protein kinases including MAP kinase, CDK2/4, JNK, and others (Inman *et al.,* 2002). These kinases phosphorylate Smad2 at a region that links the C-terminal transcriptional activation domain and the N-terminal DNA-binding domain (Massagué *et al.,* 2000). The MAP kinase-mediated decrease in Smad2 activity

has been attributed to phosphorylation of Smad2 in the linker region (Wringhton *et al.,* 2006; Kretzschmar *et al.,* 1999). Linker region phosphorylation of Smad2/3 during *Xenopus* embryogenesis results in cytosolic retention of Smad2/3 and inhibition of TGF-β signaling (Kretzschmar *et al.,* 1999). Since PLD has been shown to facilitate the activation of MAP kinase in rat fibroblasts (Chen *et al.,* 2003), we investigated whether the PLD suppression of TGF-β signaling was also mediated by a MAP kinase inhibitory phosphorylation on the Smad2/3 linker region. We first examined whether MAP kinase activation was dependent upon PLD activity in MDA-MB-231 cells. As shown in Fig. 4.2.2A, 1-BtOH, but not t-BtOH, suppressed phosphorylation of MAP kinase in the MDA-MB-231 cells, indicating that PLD was required for the activation of MAP kinase in these cells. The phosphorylation of MAP kinase in the MDA-MB-231 cells was also blocked by U0126, which inhibits MEK, the kinase that phosphorylates MAP kinase (Fig. 4.2.2B). The phosphorylation of Smad2 in the linker region at Ser 245/250/255 was also suppressed by U0126 (Fig. 4.2.2B). Phosphorylation of both MAP kinase and Smad2 at the inhibitory sites was not affected by serum conditions indicating that there was constitutive activation of this pathway in the MDA-MB-231 cells. This is consistent with previous reports that Ras, which stimulates MAP kinase via Raf and MEK, is activated in MDA-MB-231 cells (Grimm *et al.,* 2002).

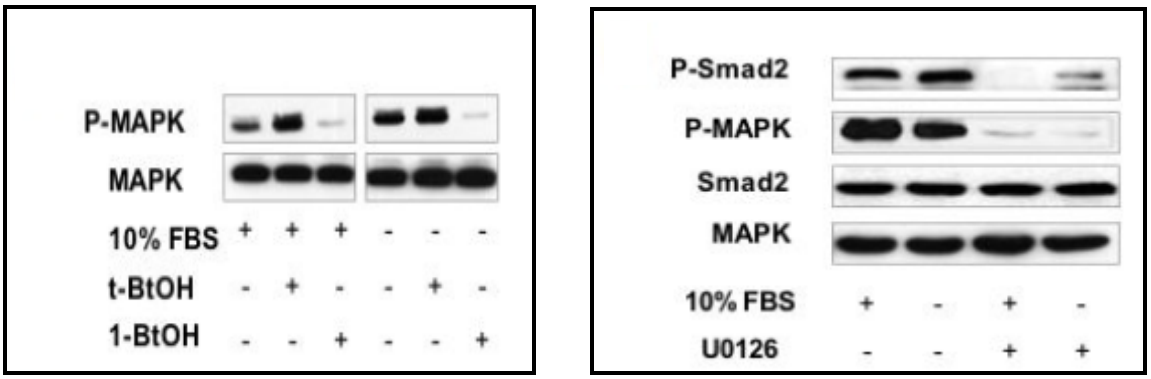

Figure 4.2.2: Suppressing PLD signaling reduces phosphorylation at negative regulatory sites in the linker region of Smad2. (A) MDA-MB-231 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were shifted to fresh media containing 0% FBS overnight. The cells were treated with 1-BtOH and t-BtOH where indicated for 1 hr and the levels of phosphorylated MAP kinase (P-MAPK) (Thr202/Tyr204) and total MAP kinase (MAPK) was determined by Western blot analysis. (B) MDA-MB-231 cells were plated as above and then incubated overnight in media containing 10% FBS, 0% FBS and U0126 (10μM) as indicated. The levels of phosphorylated Smad2 (Ser 245/250/255) and phosphorylated MAP-kinase were determined by Western blot analysis. Total MAP kinase and Smad2 was used as loading control. All experiments were performed at least two times.

Rapamycin, in contrast with the MEK inhibitor, had no effect upon Smad2 phosphorylation at the inhibitory linker region sites, confirming that mTOR was not involved (Fig. 4.2.2C).

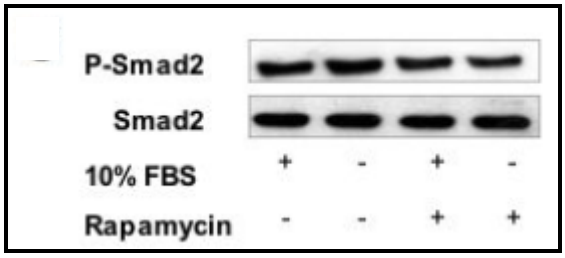

Figure 4.2.2: (continue) (C) MDA-MB-231 cells were plated as above and then incubated overnight in media containing 10% FBS, 0% FBS and Rapamycin (200nM) as indicated. The levels of P-Smad2 were determined by Western blot analysis and total Smad2 was used as loading control. Experiments were performed at least two times.

We next examined whether suppression of PLD affected phosphorylation of Smad2 at the linker region site at Ser 245/250/255. As shown in 4.2.2D, 1-BtOH, but not t-BtOH, suppressed phosphorylation at these inhibitory phosphorylation sites on Smad2. As shown, the level of Smad2 phosphorylation was not significantly influenced by the presence of serum or TGF-β - further establishing that MAP kinase is constitutively activated in these cells. The effect of 1-BtOH on Smad2 phosphorylation was observed in the presence and absence of serum and in the presence of TGF-β (Fig. 4.2.2D). We next examined the effect of PLD2 siRNA on Smad2 phosphorylation at the inhibitory sites, and as shown in Fig. 4.2.2E, PLD2 siRNA similarly suppressed phosphorylation at these sites. We also examined the effect of 1-BtOH and t-BtOH on phosphorylation of Smad2 at Ser245/250/255 in the human pancreatic cancer cell line PANC-1, which, like the MDA-MB-231 cells, have intact TGF-β signaling (Baldwin *et al.,* 1993). As shown in Fig. 4.2.2F, 1-BtOH also suppressed Smad phosphorylation in this human cancer cell line indicating that the effect of suppressing PLD activity on TGF-β signaling is not restricted to a single cell line. The data in Fig. 4.2.2 reveal that in addition to suppressing the activating phosphorylation of Smad2, PLD also facilitates the MAP kinase-dependent phosphorylation of Smad2 at inhibitory sites in the linker region. Moreover, this effect was not restricted to MDA-MB-231 cells and was also observed in PANC-1 human pancreatic cancer cells.

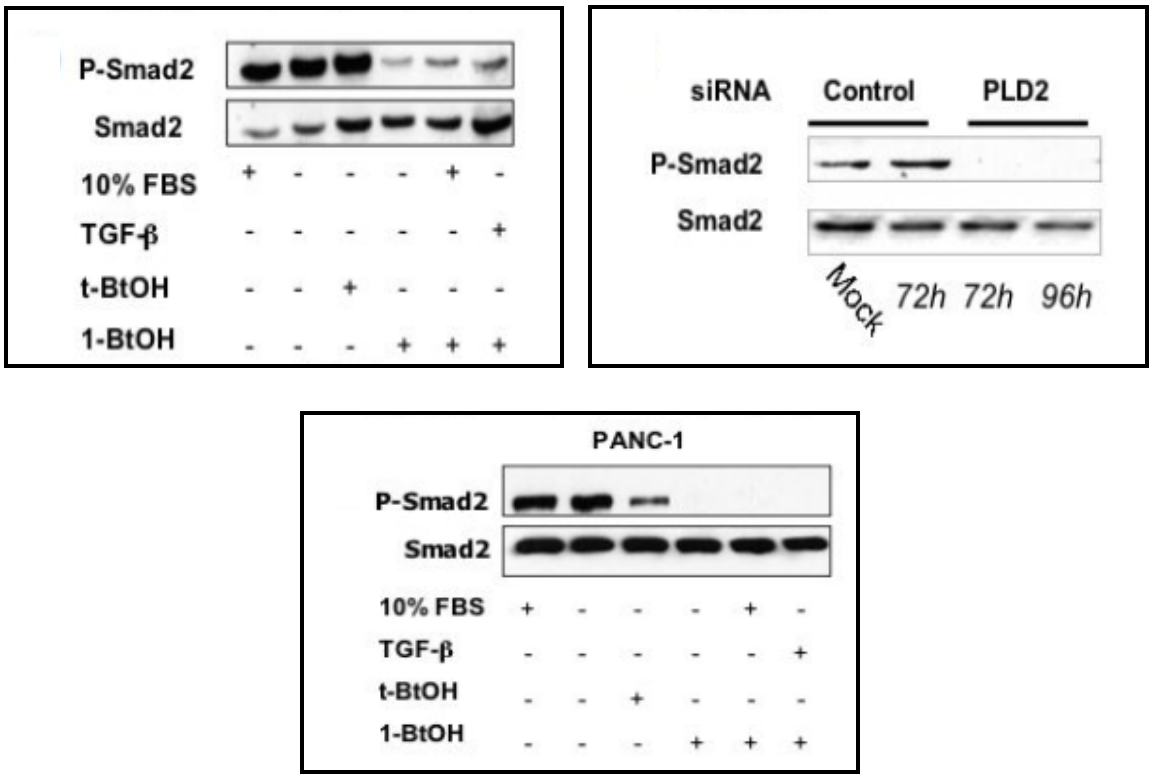

Figure 4.2.2: (continue). (D) MDA-MB-231 cells were plated as above and then paced in media containing either 0% FBS, 10% FBS, or TGF-β (10 ng/ml) overnight. The cells were then treated with 1-BtOH and t-BtOH as indicated for 1 hr, at which time the level of Smad2 phosphorylation was determined by Western blot analysis. (E) MDA-MB-231 cells were plated as above and put in media containing 10% serum for 24 hr. Cells were then transfected as described in Fig. 1C. The level of phosphorylated and total Smad2 was determined by Western blot analysis at the indicated times. All experiments were performed at least two times. (F) PANC-1 cells were plated as above and then placed in media containing either 0% FBS, 10% FBS, or TGF-β (10 ng/ml) overnight. The cells were then treated with 1-BtOH and t-BtOH as indicated for 1 hr, at which time the level os Smad2 phosphorylation was determined as in (D). All experiments were performed at least two times.

4.2.3 SUPPRESSION OF PLD SIGNALING INCREASES EXPRESSION OF THE CYCLIN-DEPENDENT KINASE INHIBITORS $p21^{Cip1}$ AND $p27^{Kip1}$

Rapamycin has been reported to cause cell cycle arrest in G1 (Song *et al.*, *2006;* Ogata *et al.,* 2001), and it has been suggested that the effect of rapamycin

on cell cycle progression is due to the de-repression of TGF-β signaling (Sawyers, 2003; Song *et al.,* 2006). TGF-β has been reported to induce cell cycle arrest through the cooperative action of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (Baldwin *et al.,* 1993). To examine the role of PLD in regulating the expression of TGF-β-inducible gene expression, we examined the effect of 1-BtOH on the expression of $p21^{Cip1}$ and $p27^{Kip1}$. As shown in Fig. 4.2.3A, treatment with 1-BtOH caused an increase in the expression of both $p21^{Cip1}$ and $p27^{Kip1}$ that was not observed with t-BtOH. We also examined the effect of PLD2 siRNA on the expression of $p21^{Cip1}$ and $p27^{Kip1}$. And as shown in Fig. 4.2.3B, PLD2 siRNA also increased expression of both $p21^{Cip1}$ and $p27^{Kip1}$. These data further support a role for PLD in the suppression of TGF-β signaling and suppression of expression of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ and suggest that PLD facilitates cell cycle progression by suppressing the expression of TGF-β-induced expression of CDK inhibitors.

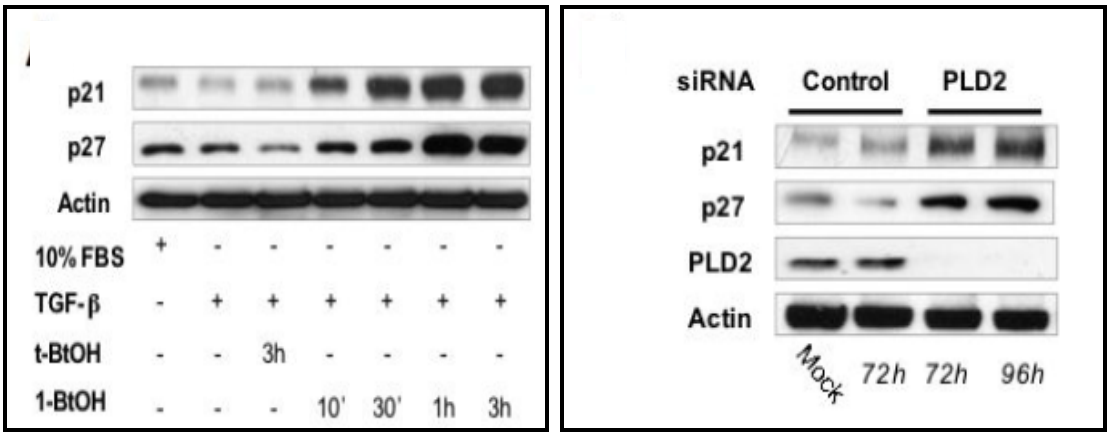

Figure 4.2.3: Suppression of PLD signaling increases expression of the cyclin-dependent kinase inhibitors p21^{Cip1} and p27^{Kip1}. (A) MDA-MB-231 cells were plated as in Fig. 1 and incubated overnight in media containing 10% FBS, 0% FBS, and TGF-β (10 ng/ml) as insicated. 1-BtOH and t-BtOH were added and the levels of p21^{Cip1} (p21) and p27^{Kip1} (p27) were determined by Western blot at the times indicated. Actin was used as loading control. (B) MDA-MB-231 cells were plated as above and put in media containing 10% serum for 24 hr. Cells were then transfected with PLD2 and control siRNAs as described in Fig. 1C. The levels of p21^{Cip1} and p27^{Kip1} were determined at the indicated times by Western blot analysis. Actin was used as a loading control. All experiments were performed at least two times.

4.2.4 SUPPRESSION OF PLD SIGNALING DECREASES PHOSPHORYLATION OF pRb

The CDKs suppressed by p21^{Cip1} and p27^{Kip1} phosphorylate pRB, which negatively regulates progression through G1 of the cell cycle (Law *et al.,* 2002). We therefore investigated the effect of suppressing PLD activity on pRb phosphorylation. As shown in Fig. 4.2.4A, hyper-phosphorylation of pRb phosphorylation was suppressed in MDA-MB-231 cells treated with 1-BtOH, but not with t-BtOH. Similarly, PLD2 siRNA also reduced the level of hyper-phosphorylated pRb (Fig. 4.2.4B). These data indicate that in addition to enhancing the expression of the CDK p21^{Cip1} and p27^{Kip1}, PLD also suppresses

the phosphorylation of the CDK substrate pRb – a critical negative regulator of progression through G1.

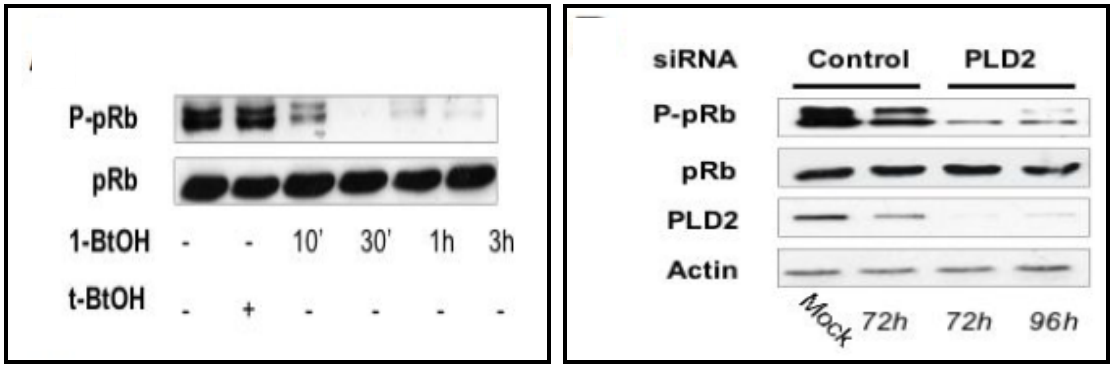

Figure 4.2.4: Suppression of PLD signaling decreases phosphorylation of pRb. (A) MDA-MB-231 cells were plated as above and incubated overnight in media containing 0% serum and TGF-β (10ng/ml). At this point cells were treated with either 1-BtOH or t-BtOH (0.8%) as indicated. The level of phosphorylated pRb (P-pRb) was then determined by Western blot analysis using an antibody that recognized pRb phosphorylated at Ser807/811. The blot was reprobed for total pRb as shown. (B) MDA-MB-231 cells were plated as above and put in media containing 10% serum for 24 hr. Cells were then transfected with either no siRNA (Mock), a control scrambled siRNA, or PLD2 siRNA as in Fig. 1. The levels of P-pRb and total pRb were determined by Western blot analysis at 72 and 96 hr later as indicated. Experiments shown are representative of at least two independent experiments.

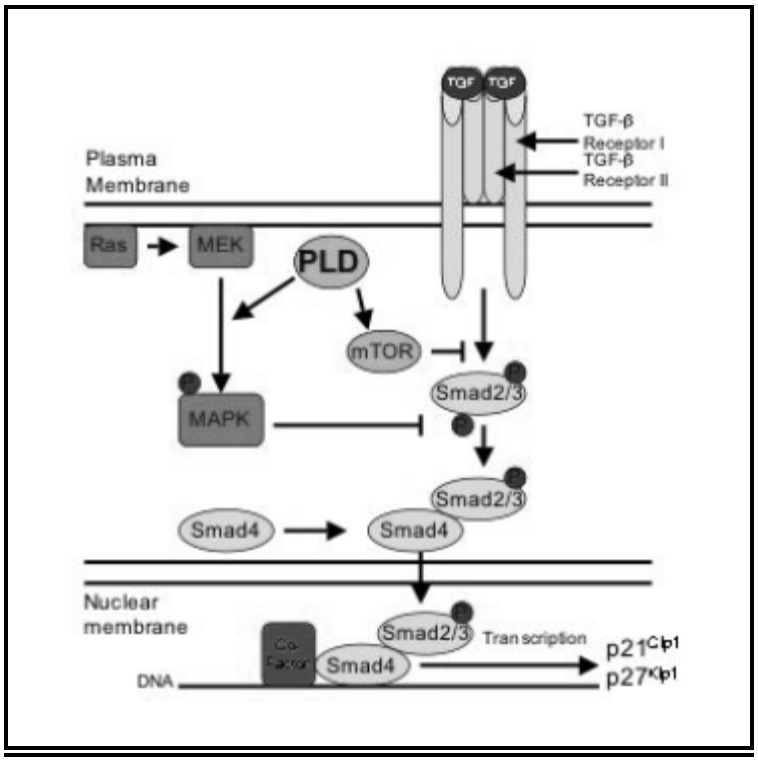

Figure 4.2.5: Model for suppression of TGF-β signaling by PLD. It is proposed that elevated PLD activity leads to the activation of mTOR, which then suppresses the phosphorylation of Smad2/3 by TGF-β receptor I at sites that facilitate interaction with Smad4. By preventing the formation of a complex between Smad2/3 and Smad4, transcription of negative regulators of cell cycle progression like $p21^{Cip1}$ and $p27^{Kip1}$ is suppressed. PLD also facilitates the activation of MAP kinase and is required for the inhibitory phosphorylations on Smad2 by MAP kinase.

4.2 DISCUSSION

The suppression of TGF-β signals is a frequent and perhaps necessary step in tumorigenesis. There are several mutations in human cancers that result in defective TGF-β signaling – most commonly the loss of Smad4 in colon and pancreatic cancers (Markowitz *et al.,* 2002; Jaffee *et al.,* 2002). The MDA-MB-231 breast cancer cell line has no mutations in TGF-β signaling, however these cells are resistant to the growth arresting effects of TGF-β (Chen *et al.,* 2001). Although mTOR is not mutated in human cancers, it is a common target of signals activated in human cancer cells (Florenes *et al.,* 1996). The best-characterized signaling pathway that targets mTOR is the PI3K pathway where several mutations have been reported that lead to increased mTOR activity (Shen *et al.,* 2001). However, the PI3K pathway is not elevated in MDA-MB-231 cells (Chen *et al.,* 2005). Another mechanism for activating mTOR is through elevated PLD activity and the generation of the PLD metabolite PA, which activates mTOR in a manner that is competitive with rapamycin (Fang *et al.,* 2001; Foster, 2007; Foster, 2006). We have reported previously that PLD activity is highly elevated in MDA-MB-231 cells (Zhong *et al.,* 2003; Chen *et al.,* 2005; Foster, 2006) and provides a survival signal that suppresses apoptosis when these cells are deprived of serum (Chen *et al.,* 2005). In this report, we have provided evidence that the suppression of TGF-β signaling in MDA-MB-231 cells is dependent upon PLD. PLD was required for suppression of Smad2/3 phosphorylation that occurs when the TGF-β receptor is activated by TGF-β.

These positive regulatory sites are also sensitive to rapamycin, indicating that the effect of PLD is mediated by mTOR. In addition, phosphorylation of Smad2 at inhibitory sites that is mediated by MAP kinase was also dependent on PLD, as was the activation of MAP kinase. These data indicate that the elevated PLD activity in MDA-MB-231 cells contributes to the suppression of TGF-β signaling via two apparently independent mechanisms – one mediated by mTOR and the other mediated by MAP kinase. A model for the role of PLD in suppressing TGF-β signaling is shown in Fig. 4.2.5. The data presented here provide mechanistic insight for the suppression of TGF-β signaling in MDA-MB-231 cells.

PLD activity is elevated in a wide variety of human cancers, including breast, colon, gastric, and kidney cancer (Foster and Xu, 2003). Elevated PLD activity has also been observed in human cancer cell lines derived from lung, bladder, colon, pancreatic, and prostate tumors (Foster*,* 2004). We have demonstrated previously that elevated PLD activity can stimulate Myc stabilization (Rodrik *et al.,* 2005), suppress protein phosphatase 2A activity (Hui *et al.,* 2005), suppress p53 expression (Hui *et al.,* 2004), and promote cell migration (Zheng *et al*., 2006). These studies have strongly implicated PLD in many of the critical hallmarks needed for progression to a malignant tumor. Suppression of the TGF-β signals that suppress cell cycle progression may be another hallmark needed for tumorigenesis. Thus, the finding reported here that PLD suppresses TGF-β signaling in MDA-MB-231 cells provides further evidence that PLD activity has the ability to promote many aspects of tumorigenesis and suggest that targeting the signals mediated by PLD will be a promising

therapeutic strategy in an apparent large number of cancers where elevated PLD activity is promoting cell cycle progression and suppressing apoptosis.

CHAPTER V:

CONCLUSION

During the last several years, it has become apparent that PLD is a critical regulator of cell proliferation and survival. PLD has been shown to facilitate cell cycle progression, suppress apoptosis, contribute to the transformation of rodent fibroblasts, and enhance cell migration (Foster, 2004; Zheng *et al*., 2006) – all critical steps in tumorigenesis. Moreover, elevated PLD activity has been reported in several human cancers (for review see Foster, 2006).

We have reported previously that PLD activity is highly elevated in MDA-MB-231 cells (Zhong *et al.,* 2003; Chen *et al.,* 2005; Foster, 2006) and provides a survival signal that suppresses apoptosis when these cells are deprived of serum (Chen *et al.,* 2005). MDA-MB-231 cells do not carry mutations in the TGF-β signaling pathway, but TGF-β does not suppress cell cycle progression in these cells (Chen *et al.,* 2001). This study indicate that the elevated PLD activity in MDA-MB-231 cells contributes to the suppression of TGF-β signaling via two apparently independent mechanisms – one mediated by mTOR and the other mediated by MAP kinase. Suppression of PLD activity or PLD expression resulted in increased phosphorylation of Smad2 and Smad3 on Ser 465/467 – sites on Smads that get phosphorylated by the TGF-β receptor and positively regulate TGF-β signaling. Suppression of PLD also suppressed phosphorylation of Smad2 on Ser 245/250/255 – sites that are phosphorylated by MAP kinase and negatively regulate TGF-β signaling. Suppression of PLD also led to increased expression of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, the expression of which is stimulated in response to TGF-β. Consistent with the elevated expression of CDK inhibitors, suppression of PLD also suppressed

phosphorylation of the CDK substrate pRb - a critical inhibitor of cell cycle progression.

These results point out that the survival signals generated by PLD involve the suppression TGF-β signals and prevention of G1 arrest, a step that must be overcome in cancer progression. To further establish a role for PLD in suppression of TGF-β-mediated G1 arrest we examined the effect of suppressing PLD on cyclin D1 expression since regulation of D cyclins is controlled by growth factors like TGF-β (Donovan and Slingerland, 2000). As expected, both 1-BtOH and PLD2 SiRNA suppressed cyclin D expression in MDA-MB-231 cells (our unpublished data). The involvement of PLD in cell cycle regulation is intriguing in that mTOR, a downstream target of PLD, has been implicated in control of cell size and S phase progression (Agbunag *et al*., 2004; Fingar and Blenis, 2004). Cyclin E, a G1-S transition regulator, has been previously reported to be repressed by TGF-β (Donovan and Slingerland, 2000). More important, constitutively active p70S6k - a known target of mTOR, was sufficient to activate cyclin E (Chou *et al.,* 2003). Cyclin E expression is controlled by phosphorylation of pRb (Ho and Dowdy, 2002); therefore, the decrease we observed in pRb phosphorylation in response to PLD suppression should lead to reduced expression of cyclin E. We then examined the effect of suppressing PLD on the expression of cyclin E in MDA-MB-231 cells and suppressing PLD expression and activity indeed suppressed cyclin E expression (our unpublished data). These preliminary findings propose a possible role for PLD in a new emerging

cell cycle checkpoint, (similar to the one observed in yeast) that is controlled by mTOR - thus most likely sensitive to nutrition, and should be further explored.

As with the previous studies, we showed here that in the presence of serum growth factors, rapamycin induces G1 arrest, rather than apoptosis (Law *et al.,* 2002; Neshat *et al.,* 2001; Podsypanina *et al.,* 2001). We demonstrated here that the ability of rapamycin to induce G1 arrest in the presence of serum is dependent upon TGF-β, and, that TGF-β is sufficient to suppress rapamycin-induced apoptosis in MDA-MB-231 cells deprived of serum. Importantly, suppression of TGF-β or PKCδ signaling restored the ability of rapamycin to induce apoptosis rather than G1 arrest in the presence of serum. Furthermore, human cancer cell lines with defects in TGF-β or PKCδ signaling were killed by rapamycin in the presence of serum. We propose here that rapamycin, by suppressing mTOR, leads to the activation of TGF-β signals and G1 arrest. However, if TGF-β signaling is either defective or suppressed (common in cancer), the cells are allowed to progress into S-phase where the lack of mTOR signals result in apoptosis. These data indicate that rapamycin could be made a more effective anti-cancer drug option in combination with the suppression of TGF-β signals that cause G1 arrest. The data also point out that tumors with defective TGF-β signaling may be selectively killed by rapamycin.

As the era of molecular medicine and pathology evolves and individual tumors are examined at the molecular level, elevated PLD activity could be easily determined and the signals generated by PLD activity could then be targeted specifically. The involvement of mTOR in PLD signaling indicates that rapamycin-

based treatment strategies could be developed to target tumors with elevated PLD activity.

CHAPTER VI:

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