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CROSSTALK BETWEEN LYSOPHOSPATIDIC ACID (LPA) AND TRANSFORMING GROWTH FACTOR BETA (TGFβ) IN BREAST AND OVARIAN CANCER CELLS

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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AGK	Acylglycerol kinase
AP2	Activator protein 2
ATF3	Activating transcription factor 3
ATX	Autotaxin
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
C/EBPβ	CCAAT enhancer binding protein β
cAMP	cyclic adenosine monophosphate
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
c-Myc	myelocytomatosis oncogene
CTGF	Connective tissue growth factor
DAG	Diacylglycerol
DMEM	Dulbecco modified Eagle medium
ECM	Extracellular matrix
Edg	Endothelial differentiation gene
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
Erk	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FGFR4	Fibroblast growth factor receptor 4
FOX	Forkhead box

GPCR	G protein coupled receptor
HDAC	Histone deacetylases
Id1	Inhibitor of differentiation 1
Id2	Inhibitor of differentiation 2
IFN-γ	Interferon γ
IP3	Inositol triphosphate
I-Smad	Inhibitory Smad
LAP	TGFβ latency associated peptide
LIP	C/EBPβ liver inhibitory protein
LLC	Large latent complex
LPA	Lysophosphatidic acid
LPAAT	LPA acyl transferase
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPP	Lipid phosphate phosphatase
LPS	Lysophosphatidylserine
LTBP	Latent TGFβ-binding protein
LysoPLD	Lysophospholipase D
MAG	Monoacylglycerol
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblast
MLC	Myosin light chain
MMP	Matrix metalloprotinase
NOE	Normal ovarian epithelium
PA	Phosphatidic acid
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3 kinase

PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA_1	Phospholipase A1
PLC	Phospholipase C
PP1	Protein phosphatase-1
PPARγ	Peroxisome proliferator-activated receptor γ
qPCR	quantitative PCR
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
R-Smad	Receptor-regulated Smad
S1P	Sphingosine 1 phosphate
SBE	Smad binding element
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SnoN	Ski-related novel protein N
sPLA2	secretory phospholipase A2
STAT1	Signaling transducers of activators of transcription 1
TAM	Tumor associated macrophage
TGFβ	Transforming growth factor β
TIE	TGFβ inhibitory element
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TSP-1	Thrombospondin-1
TβR	TGFβ receptor
uPA	urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

ABSTRACT

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By Jinhua Wu, PhD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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ABSTRACT

Lysophosphatidic acid (LPA) and transforming growth factor beta (TGFβ) are platelet-derived intercellular mediators of cell proliferation and motility. LPA is a general growth, survival and motility-stimulating factor in mammalian cells. TGFβ prevents proliferation of normal epithelial cells. However, the growth-inhibitory effect of TGFβ is lost or reduced in most malignant cells. Instead, TGFβ promotes migration and invasion of advanced cancer cells. Since LPA and TGFβ are both present in the blood and tumor microenvironments, we were interested in signal integration and functional outcomes in malignant epithelial cells in an LPA and TGFβ co-stimulatory context. In a subset of breast and ovarian cancer cell lines which remain sensitive to the cytostatic effect of TGFβ, we found that LPA up-regulated expression of the cyclin-dependent kinase inhibitor $p21^{Waf1}$. But this up-regulation was not observed in TGFβ-resistant ones. We examined the possibility that LPA-induced p21 might contribute to the cytostatic response to TGFβ. Indeed, TGFβ alone induced p21 expression weakly in TGFβ-sensitive cells. Serum or serum-borne LPA cooperated with TGFβ to elicit the maximal p21 induction. LPA stimulated p21 via LPA_1 and LPA_2 receptors and Erk-dependent activation of the CCAAT/enhancer-binding protein beta (C/EBPβ) transcription factor independent of p53. Loss or gain of p21 expression led to a shift between TGFβ sensitive and resistant phenotypes in breast and ovarian cancer cells, indicating that LPA-induced

p21 is a key determinant of the growth inhibitory activity of TGFβ. The p21-stimulatory action of LPA is absent from most breast and ovarian cancer cells, leading to their resistance to TGFβ. Therefore we reveal a novel crosstalk between LPA and TGFβ that underlies TGFβ sensitive and resistant phenotypes of breast and ovarian cancer cells.

In the next part of our study, we examined the role of interactions between LPA and TGFβ in regulation of tumor cell motility. LPA and, to a much less extent, TGFβ stimulate chemotactic migration and invasion of breast and ovarian cancer cells. However, when combined together with LPA, TGFβ strongly attenuated LPA-driven migration and invasion of breast and ovarian cancer cells. This inhibitory effect was most likely mediated through TGFβ downregulation of expression of LPA_1, the major receptor subtype responsible for LPA-regulated cell migration. Knockdown of Smad3 or Smad4 with small hairpin RNA (shRNA) eliminated the inhibitory effects of TGFβ on the LPA_1 expression and LPA-dependent cell migration. There are two potential TGFβ inhibitory elements (TIE) (-40 bp and -401 bp) present in the human *LPA1* gene promoter. Deletion or point mutation of the distal TIE at around -401 bp abolished the inhibitory effect of TGFβ on the *LPA1* promoter activity as revealed by luciferase assays. A DNA pull-down assay showed that the -401-TIE-E2F4/5 sequence was capable of binding Samd3, Smad4, and E2F4/5 in TGFβ-treated cells. The binding of the Smad complex to the native TIE-E2F4/5 sequences of the *LPA1* gene promoter was further verified by chromatin immunoprecipitation assay. Our results identify a novel role of TGFβ in the control of LPA_1 expression and LPA_1-coupled biological activities, adding *LPA1* to the list of TGFβ-repressed target genes.

CHAPTER 1

INTRODUCTION

1.0 Overview

Breast cancer and ovarian cancer are leading causes of cancer-related death in women. An estimate of 288,130 new cases of breast cancer and 22,280 new cases of ovarian cancer are expected in the United States during 2012. Most breast cancers are derived from the epithelial cells that line the terminal ducts or lobules (Sainsbury et al 1994). Breast cancer stages are expressed as a number on a scale of 0 through IV (Stage 0 describing non-invasive cancers that remain within the original location and stage IV describing invasive cancers that have spread beyond the breast and near lymph nodes to other organs of the body, such as the lungs, distant lymph nodes, skin, bones, liver or brain). Pleural effusions may occur at any point of the clinical course of breast tumor progression and may be the sole manifestation of metastatic disease (Dieterich et al 1994). Approximately 10-20% breast cancers are triple-negative, the breast tumor type tested negative for estrogen receptors (ER^-), progesterone receptor (PR^-) and HER2 ($HER2^-$). Therefore, the triple-negative breast cancer does not respond to hormonal therapy or target therapy against the HER2 receptor. The most common type of ovarian cancer is

ovarian epithelial carcinoma. Ovarian cancer is classified into four stages (Stages I-IV). At the advanced stages III and IV, it is often characterized by extensive intra-peritoneal distribution of tumors and formation of large volumes of ascites.

Malignant pleural effusions of breast cancer and ascites of ovarian cancer represent tumor microenvironments enriched with a broad spectrum of intercellular mediators including lysophosphatidic acid (LPA) (Mills et al 1990, Moolenaar et al 1992, Xu et al 1995a) and transforming growth factor beta (TGFβ) (Hirte and Clark 1991). Both LPA and TGFβ are elevated in ovarian cancer ascites compared to non-malignant fluids (Ikubo et al 1995, Xu et al 1995b, Xu et al 1998). Previous studies of these coexisting factors have been restricted to analysis of the biological activities of individual ones without consideration of their simultaneous effects (Massague 2008, Mills and Moolenaar 2003). The results from these prior studies may not reflect their physiological roles in vivo. In the present study, we explored the potential crosstalk between LPA and TGFβ signaling in breast and ovarian carcinoma cells. Our results establish that such crosstalk indeed exists and operates to determine ultimate proliferative and migratory responses of breast and ovarian cancer cells.

1.1 LPA

1.1.1 Metabolism of LPA

LPA (1-acyl-2-hydroxy-sn-glycero-3-phosphate) is the simplest natural phospholipid. LPA can be produced by multiple cell types, including activated platelet

(Eichholtz et al 1993), endothelial cells (Siess et al 1999), fibroblasts (van der Bend et al 1992), adipocytes (Valet et al 1998), and cancer cells (Bektas et al 2005, Fang et al 2000a). LPA is present in body fluids including plasma, saliva, hair follicles and malignant effusions (Aoki et al 2008, Sugiura et al 2002) and accounts for many biological properties of serum. In addition to 1-acyl LPA, other LPA species such as cyclic LPA, 1-alkyl and 1-alkenly-LPA are also found in serum and ovarian cancer patient ascites (Kobayashi et al 1999, Xiao et al 2001).

Two main pathways are involved in endogenous generation of LPA from other phospholipids (Aoki et al 2002, Aoki 2004). Activated platelet, which is responsible for a major part of LPA production *in vivo*, generates LPA by sequential actions of phospholipases present in plasma or expressed by blood cells. Two phospholipases, secretory phospholipase A2 ($sPLA_2$) and phospholipase A1 (PLA_1) are involved in the production of lysophospholipids such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS). These lysophospholipids are converted to LPA by action of lysophospholipase D (lysoPLD) (Aoki et al 2002). The lysoPLD responsible for LPA production has been identified as autotaxin (ATX) (Umezu-Goto et al 2002), a previously enigmatic ecto-enzyme involved in tumor invasion, neovascularization and metastasis (Nam et al 2000, Ptaszynska et al 2008, Yang et al 2002). With its intrinsic lysoPLD activity, ATX can hydrolyze LPC, a major phospholipid secreted by hepatocytes and therefore abundant in the blood, into LPA. The recently reported crystal structure of ATX suggests that the binding of ATX to integrins localizes LPA production to platelets and mammalian cells (Fulkerson et al

2011). Another potential pathway of LPA production independent of PLA and ATX is direct phosphorylation of monoacylglycerol (MAG) by acylglycerol kinase (AGK) (Bektas et al 2005). Overexpression of AGK is associated with increased production and secretion of LPA (Bektas et al 2005).

The lipid phosphate phosphotases (LPPs) are a family of enzymes that remove the phosphate group from LPA and convert LPA to MAG (Pilquil et al 2001, Tomsig et al 2009, Xu et al 2000). These LPPs are membrane-associated with extracellularly facing catalytic site for dephosphorylating LPA on the cell membrane. Expression of LPPs reduced endogenous LPA levels and attenuated LPA-mediated cellular functions (Tomsig et al 2009). Other than dephosphorylation, LPA can also be converted to phosphatidic acid (PA) by acylation through the action of LPA acyl transferases (LPAAT) (Eberhardt et al 1997, Leung 2001).

1.1.2 LPA receptors and signal transduction

LPA is a mediator of diverse biological processes including neurogenesis, angiogenesis, wound healing, immunity, and carcinogenesis (Panupinthu et al 2010). These biological functions of LPA are mediated through binding of LPA to its seven-transmembrane G-protein coupled receptors (GPCRs). To date, six GPCRs have been identified as physiological LPA receptors. Based on their amino acid homology, the LPA receptors are classified into two different groups: LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 share more than 50% amino acid identity and belong to the endothelial differentiation gene (Edg) family, whereas GPR23/P2Y9/LPA_4 of the purinergic receptor

family and the related GPR92/LPA_5, and P2Y5/LPA_6 and GPR87/LPA7 are non-Edg LPA receptors or novel subgroup of LPA receptors (Choi et al 2010) (Figure 1.1).

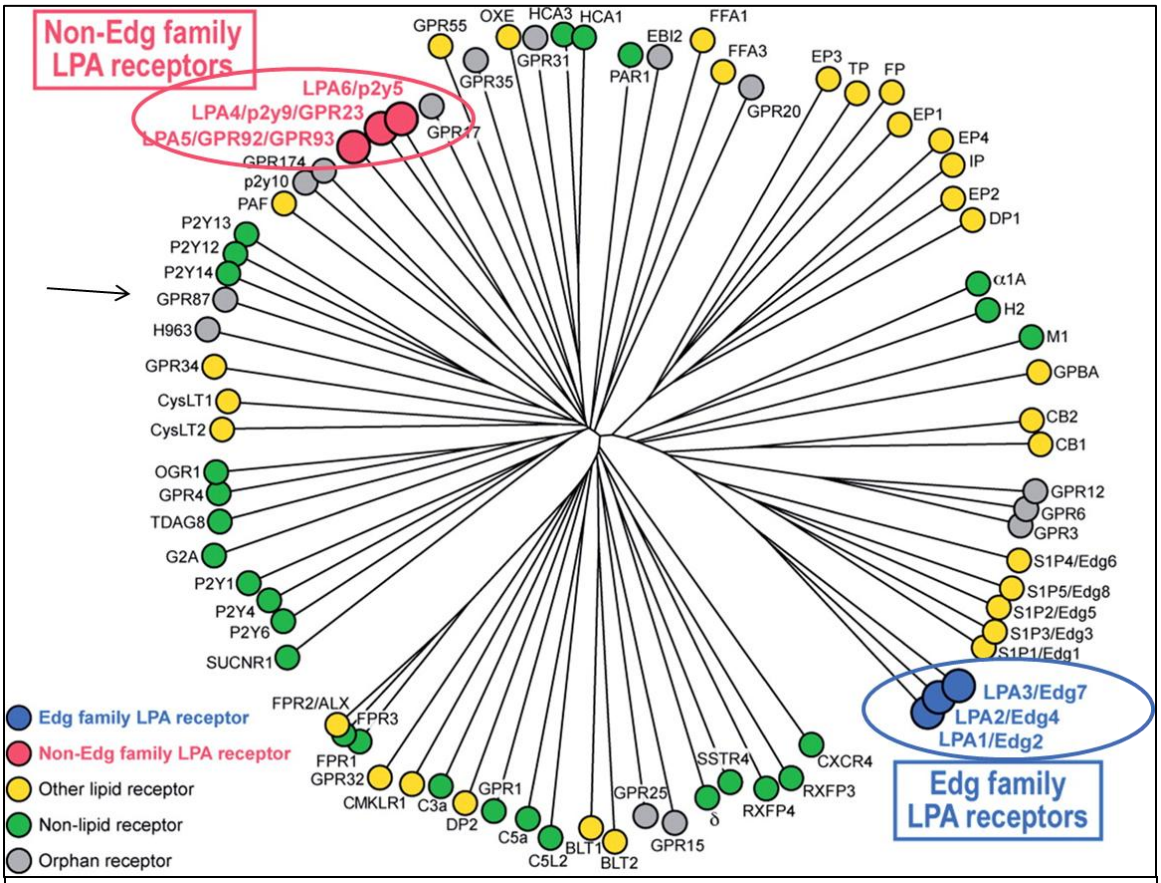

Figure 1.1. A representative phylogenetic tree of human GPCRs. Adapted from Keisuke Yanagida and Satoshi Ishii, "Non-Edg family LPA receptors: the cutting edge of LPA research**",** *J Biochem*, (*2*011) 150 (3): 223-232.

LPA_1 is the first high-affinity receptor identified for LPA (Hecht et al 1996). It is widely expressed in embryonic cells and adult tissues including brain, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, placenta, and skeletal muscle (An et al 1998, Contos et al 2000b). LPA_1 is expressed in neoplastic cells as well. However, gene expression profiling studies failed to show any consensus increase in LPA_1

expression between normal and malignant cells (Hendrix et al 2006, Radvanyi et al 2005, Sanchez-Carbayo et al 2006, Skrzypczak et al 2010, Su et al 2007). Instead, some expression profiling or array analyses suggest decreases in LPA_1 mRNA expression in various malignancies (Hendrix et al 2006, Sanchez-Carbayo et al 2006, Shida et al 2004b, Skrzypczak et al 2010, Su et al 2007).

Only minor abnormalities such as craniofacial dysmorphism and defective sucking behavior were found in *LPA_1*-deficient mice (Contos et al 2000a). However, further analysis of these *LPA_1* knockout mice subjected to pathophysiological conditions has revealed that LPA_1 is required for the initiation of neuropathic pain (Inoue et al 2004) and promotion of pulmonary and renal fibrosis (Pradere et al 2007, Tager et al 2008). More recent studies suggested the importance of LPA_1 in neurogenesis (Matas-Rico et al 2008), hippocampus synaptic function (Musazzi et al 2011), and bone development (Gennero et al 2011).

LPA_2 was identified from GenBank searches of orphan GPCRs. It exhibits about 60% amino acid similarity to LPA_1 (Contos and Chun 2000). The expression of LPA_2 is relatively restricted. High expression of LPA_2 is detected in testis and leukocytes, and moderate expression is found in prostate, spleen, thymus, and pancreas (An et al 1998). Our group is the first to report overexpression of LPA_2 in early and late stages of ovarian cancer (Fang et al 2000a, Fang et al 2002). In breast cancer, LPA_2 overexpression is observed in more than half (57%) of the most common invasive ductal carcinoma (Kitayama et al 2004). LPA_2 is also commonly overexpressed in gastric cancer

(Yamashita et al 2006), differentiated thyroid cancer (Schulte et al 2001) and other human malignancies (Schulte et al 2001). Mice lacking LPA_2 do not show any significant abnormalities in physiology (Contos et al 2002). However, compared to wild type mice, LPA_2-deficient mice are more resistant to intestinal tumor formation induced by colitis or by ApcMin mutation (Lin et al 2009, Lin et al 2010). These studies together are consistent with an oncogenic role for LPA_2.

LPA_3 was isolated as an orphan GPCR gene using degenerate PCR-based cloning and homology searches (Bandoh et al 1999, Im et al 2000). Expression of LPA_3 is observed in heart, testis, prostate, pancreas, lung, ovary, and brain (Bandoh et al 1999, Im et al 2000). LPA_3 is overexpressed in about 45% of ovarian cancers (Fang et al 2002) but not as commonly overexpressed as LPA_2 in many other types of cancer. Homozygous deletion of *LPA_3* leads to identification of a specific function of this LPA receptor in female reproduction. LPA_3 deficient female mice show a delayed implantation and defective embryo spacing, associated with reduced uterine expression of Cox-2 mRNA (Ye et al 2005).

LPA_4 is the first non-Edg LPA receptor identified through ligand screening (Noguchi et al 2003). Its mRNA is expressed in various human tissues with the highest abundance in the ovary (Noguchi et al 2003). Interestingly, LPA_4 expression is undetectable or generally low in most tumor cell lines. Our lab generated *LPA_4* knockout mice. LPA_4 null mice did not show apparent abnormalities compared to the wild type littermates. However, LPA_4 seems to negatively regulate cell motility in that LPA_4-

deficient mouse embryo fibroblasts (MEFs) are more sensitive to LPA-induced migration (Lee et al 2008). Similarly, overexpression of LPA_4 suppresses LPA_1-mediated cell migration in cancer cell lines (Lee et al 2008).

More recently, the orphan GPR92 was identified as another non-Edg LPA receptor, LPA_5. It shares about 35% amino acid homology with LPA_4 (Lee et al 2006). LPA_5 is broadly expressed in murine tissues such as embryonic brain, small intestine, skin, spleen, stomach, thymus, lung, heart, liver and embryonic stem cells (Kotarsky et al 2006, Lee et al 2006). Antagonists study of LPA_5 suggests its role in activation of platelets (Williams et al 2009).

P2Y5/LPA_6 mRNA is expressed in spleen, thymus, leukocyte, prostate, ovary, testis, small intestine, and colon (Pasternack et al 2008). LPA_6 is found to be mutated in hypotrichosis simplex and required for maintenance of human hair growth (Pasternack et al 2008). The LPA_6 knockout mice have not been reported but would be expected to phenocopy the major function of the human receptor in regulating hair growth.

GPR87/LPA_7 is expressed at low levels in most tissues with the exception of prostate, placenta, head and neck. An interesting observation with GPR87 is its overexpression specifically in squamous cell carcinomas of the lung, cervix, skin, urinary bladder, testis, head and neck (Tabata et al 2007). Other orphan GPCRs such as P2Y10 (Murakami et al 2008) and GPR35 (Oka et al 2010) have been also proposed to be additional LPA receptors. However, their identities as bona fide receptors for LPA are yet to be validated through independent studies.

Like other GPCRs, LPA receptor subtypes appear to couple to multiple heterotrimeric G proteins that in turn, activate diverse pathways including stimulation of phospholipase C and D, small GTPases, mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) (Bandoh et al 1999, Contos et al 2002, Fukushima et al 2002, Im et al 2000, Lee et al 2006, Mills and Moolenaar 2003). Activation of these diverse G protein signaling cascades downstream of LPA receptors culminates in cytoskeleton remodeling, cell proliferation, survival and migration (Mills and Moolenaar 2003). As depicted in Fig. 1. 2, a major difference in G protein coupling between the Edg LPA_{1-3} receptors and the novel non-Edg LPA_{4-6} receptors is that LPA_{1-3} couple to Gi but not Gs while LPA_{4-6} couple to Gs instead of Gi. Gs is linked to activation of adenylyl cyclase (AC) and intracellular cAMP. In contrast, Gi inhibits adenylyl AC and decreased cAMP and activates the Ras/MAPK cascade and the PI3K-Akt pathway through subunit released from Gi (Anliker and Chun 2004, Radeff-Huang et al 2004, Van Leeuwen et al 2003). Gq is linked to activation of phospholipase C (PLC), which, in turn, catalyzes hydrolysis of phosphatidylinositol biphosphate (PIP2) to diacylglycerol (DAG) to activate protein kinase C (PKC) and inositol trisphosphate (IP3) to trigger intracellular calcium mobilization (Fukushima and Chun 2001). Activation of G12/13, which directly binds to the Rho-specific guanine nucleotide exchange factor p115RhoGEF, leads to activation of the Rho-ROCK pathway (Kranenburg et al 1999, Ren et al 1999) that regulates actomyosin-driven cytoskeleton contraction and cell shape changes (Bar-Sagi and Hall 2000).

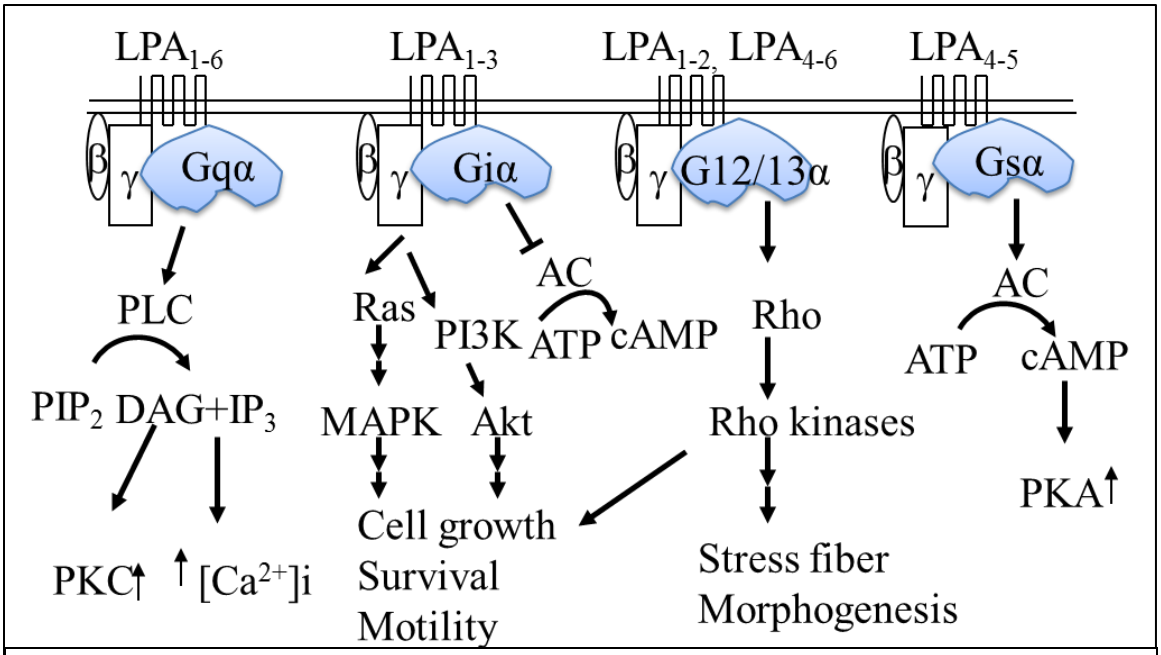

Figure 1.2. LPA receptors and G protein signaling cascades.

1.1.3 LPA and cell proliferation

The role of LPA as a growth factor was initially shown in fibroblasts. The mitogenic activity of LPA has now been demonstrated in several cell lineages including normal and transformed epithelial cells, endothelial cells, smooth muscle cells, astrocytes, and renal mesangial cells (Radeff-Huang et al 2004, Van Leeuwen et al 2003). The mitogenic activity of LPA is sensitive to pertussis toxin (Fang et al 2000b, Van Corven et al 1989, van Corven et al 1992), implicating a Gi-dependent mechanism in LPA-mediated cell proliferation. The Gi-induced inhibition of intracellular cAMP may be a partial but not the sole stimulus of LPA-induced cell proliferation (Van Corven et al 1989). Similar to the mitogenesis driven by peptide growth factors, the Ras/ERK and

PI3K/Akt pathways are essential for LPA-induced cell growth (Cook and McCormick 1996, Fang et al 2000b, Kranenburg and Moolenaar 2001). Active Erk translocates to the nucleus to activate expression of proliferation-associated genes. The dependence on PI3K of LPA-induced cell proliferation may relate to the prominent anti-apoptotic activity of the PI3K-Akt pathway (Kennedy et al 1997).

Due to the critical role of Gi-dependent signals in LPA-driven cell proliferation, the Edg LPA receptors seem to be major receptor subtypes to activate mitogenic program although the possibility of inputs from non-Edg LPA receptors cannot be excluded. However, most cell lines express more than one Edg LPA receptor, making it difficult to link a proliferative response to a specific receptor subtype. In a few earlier studies, ectopic expression of the LPA_1 receptor supports LPA-dependent DNA synthesis in B103 cells (Fukushima et al 1998) and cellular survival in Schwann cells (Li et al 2003, Weiner and Chun 1999). In MEFs, deletion of LPA_1 or LPA_2 does not significantly affect proliferative response to LPA. However, simultaneous disruption of LPA_1 and LPA_2 strongly inhibited LPA-induced cell proliferation (Contos et al 2002), suggesting the functional redundancy between the two LPA receptors in mediating growth response of MEFs to LPA. In malignant cells particularly colon cancer cells, accumulating evidence indicate that LPA_2 mediates mitogenic signals. LPA_2 stimulates expression of a large number of cell cycle promoters or regulators such as c-Myc, cyclin D1, HIF1α, and Krüppel-like factor 5 (Fang et al 2004, Hu et al 2001, Mori et al 2009, Yang et al 2005). The ability of LPA_2 to promote proliferation of malignant cells is consistent with its overexpression in many types of cancer and its recognized function as an oncogene.

The ultimate biological responses to LPA vary from one cell type to another, likely as a result of the types and expression levels of the LPA receptors as well as possible crosstalk with other families of cell surface receptors. LPA transactivation of receptor tyrosine kinases such as EGFR, PDGFR and c-met occurs in a panel of cancer cell lines (Goppelt-Struebe et al 2000, Oyesanya et al 2010, Zhao et al 2007). This cross-communication may provide a permissive signal in combination with other G protein effectors to elicit cellular responses to LPA including cell proliferation.

1.1.4 LPA and cell motility

LPA stimulation of tumor cell invasion was first shown in 1993 (Imamura et al 1993). However, this biological activity of LPA was not well appreciated until the tumor cell motility-stimulating factor ATX was identified to be lysoPLD accounting for LPA production in the blood (Umezu-Goto et al 2002). ATX has long been known as a large protein secreted by melanoma and other cancer cells to enhance tumor cell migration and invasion through a GPCR-mediated mechanism (Stracke et al 1992). It is now well established that ATX stimulates tumor cell motility through formation and action of LPA.

The migratory effect of LPA and the underlying mechanism of action have become a subject of extensive recent studies in the area of LPA biology. It is now well known that LPA activates cell motility through G12/13-mediated activation of RhoA and Gi-PI3K-mediated activation of Rac. These small Rho GTPases regulate cell detachment and forward movement in a coordinate manner (Etienne-Manneville and Hall 2002).

Precisely, RhoA mediates cell contraction through activation of ROCK which phosphorylates myosin light chain (MLC) and thereby induces actomyosin-based contractility and cell rounding (Kimura et al 1996). On the other hand, LPA activates Rac through a Gi, PI3K and guanine nucleotide exchange factor Tiam1 (Van Leeuwen et al 2003) or SOS1/EPS8/ABl1 tri-complex (Chen et al 2010).

Using $LPA_1^{-/-}$ skin fibroblasts, Hama et al provided compelling evidence that LPA_1 is essential for both LPA- and ATX-induced cell migration of fibroblasts (Hama et al 2004). Independent studies in the Rh7777 hepatoma cells (Umezu-Goto et al 2002) and the B103 neuroblastoma cells (Van Leeuwen et al 2003) devoid of functional LPA receptors indicated that ectopic expression of LPA_1 is sufficient to restore migratory responses to LPA. The importance of LPA_1 in LPA-dependent cell migration has been further confirmed in cancer cells of colon, breast (Chen et al 2007, Shida et al 2003), and stomach (Shida et al 2004a). Despite the primary role of LPA_1 in the migratory response to LPA, there has been also evidence for participation or auxiliary role of LPA_2 and even LPA_3 in LPA regulation of cell motility. However, it remains unknown whether LPA_2 or LPA_3 alone in the absence of LPA_1 is sufficient to initiate the migratory response to LPA. It is possible that in certain cellular contexts, these other LPA receptors could cooperate with LPA_1 to enhance or sustain migratory signals such as Rac activation. In contrast to the Edg LPA receptors, the non-Edg LPA receptors do not couple to Gi and Gi-dependent migratory signals. Instead, we have recently shown that using LPA_4 wild type and null MEFs, the LPA4 receptor negatively regulates cell motility, likely through over-activation of the G12/13-Rho pathway to deregulate the ratio of Rac and Rho (Lee et al

2008). Appropriate balance of Rac and Rho activities plays a pivotal role in coordinating cellular migratory responses to various motogens.

1.2 TGFβ

1.2.1 TGFβ production and activation

TGFβ (refers to TGFβ1 in TGFβ family), is a polypeptide member of the TGFβ super family of cytokines. It is ubiquitously expressed in human adult tissues, and dramatically increased in almost all cancer cell lines (Wu et al 2009). TGFβ expression is regulated at multiple levels, including transcription, mRNA stability, and posttranslational processing. Van Obberghen-Schilling et al have shown that TGFβ positively regulates its own expression in normal and transformed cells (Van Obberghen-Schilling et al 1988). In cancer cells, *v-src* was reported to transcriptionally activate TGFβ through AP-1 (Birchenall-Roberts et al 1990). In tumor microenvironment, TGFβ is also produced by infiltrated immune cells including dendritic cells, natural killer cells and natural regulatory T cells (Flavell et al 2010).

TGFβ is synthesized as a precursor molecule containing a propeptide region in addition to the TGFβ monomer (Derynck et al 1985). TGFβ is secreted to the extracellular matrix (ECM) in a latent complex consisting of the TGFβ dimer, latency associated peptide (LAP) dimer and latent TGFβ-binding protein (LTBP) (Rifkin 2005). This complex is called large latent complex (LLC). After its secretion, the LLC remains

in the ECM in the inactivated form. It will be further processed to release the active TGFβ homodimer (Annes et al 2003).

TGFβ can be activated in either integrin-dependent or integrin-independent manner. Matrix metalloproteinases (MMPs), such as MMP9 and MMP2 (Yu and Stamenkovic 2000), acidic pH (Lyons et al 1988), reactive oxygen species (ROS) (Barcellos-Hoff and Dix 1996) and thrombospondin-1 (TSP-1) (Schultz-Cherry and Murphy-Ullrich 1993) have all been shown to stimulate the release of TGFβ from the latent form. The αV integrins can activate TGFβ by binding to the RGD motif of LAP and induce a change in the conformation of the latent complex to liberate/activate TGFβ from its latent complex, thereby allowing access of the mature TGFβ to the TGFβ receptors (TβRs) and induction of classic TGFβ signaling (Munger et al 1999). On the other hand, αV integrins also can create a close connection between the latent TGFβ complex and MMPs (Mu et al 2002), a process similar to the mechanism for integrin interaction with ATX to localize LPA production from LPC in platelets (Fulkerson et al 2011).

1.2.2 TGFβ signaling

After release as a homodimer, TGFβ binds to type II receptor (TβRII), leading to formation of a receptor complex and phosphorylation of the TβRI. After being phosphorylated and activated, the type I receptor subsequently phosphorylates a receptor-regulated SMAD (R-Smad), allowing complex formation of R-Smad with Smad4 (co-Smad) and translocation to the nucleus where the Smad complex associates with a DNA-

binding partner and binds to specific DNA region of TGFβ target genes to activate or repress transcription (Massague 1998).

TGFβ activation of TβRI and TβRII not only triggers activation of the Smad pathway as stated above but also induces activation of other Smad-independent signaling cascades by the kinase activities of the TβRs. Rapid activation of MAPK pathways, including Erk, JNK and p38 MAPK occurs in a Smad-independent manner in TGFβ challenged cells (Derynck and Zhang 2003). In addition, TGFβ treatment rapidly activates Rho GTPases, including RhoA, Cdc42, and Rac. As mentioned earlier, the activation of these small GTPases causes membrane ruffling and formation of stress fibers and lamellipodia. Thus TGFβ could induce changes in cell morphology, promotion of cell motility, and epithelial to mesenchymal transition (EMT) (Zavadil et al 2001).

1.2.3 Complexity and regulation of the TGFβ-Smad pathway

Receptors of ligands of the TGFβ superfamily are characterized with a highly conserved intracellular serine-threonine kinase domain (Liu et al 2001). There are two functional classes of receptors for TGFβ superfamily ligands: type II and type I, which are encoded by five and seven genes in human genome, respectively (Schmierer and Hill 2007). Binding of ligand recruits the pre-formed dimers of TβRII and TβRI to form heterotetrameric receptor complex (Sasaki et al 2003). The TβRII kinase is thought to be constitutively active and phosphorylates the TβRI at several serine and threonine residues in a glycine and serine rich domain (GS domain) which is strictly conserved in all type I TβRs. Phosphorylation of the GS-domain enables recruitment of R-Smads: Smad1,

Smad2, Smad3, Smad5 and Smad8. Smad2 and Smad3 are phosphorylated and activated by TGFβ while Smad1, Smad5 and Smad8 are phosphorylated/activated by bone morphogenetic protein (BMP) signal. R-Smads and Smad4 share two highly conserved domains, MH1 and MH2. Except for Smad2, which cannot bind DNA directly (Yagi et al 1999), the MH1 domains of other R-Smads and Smad4 are capable of DNA binding. The MH2 domain mediates Smad-receptor interactions, Smad-Smad interactions and Smad interactions with other transcription factors, co-activators or co-repressors (Schmierer and Hill 2007).

Negative feedback in TGFβ signaling pathway is mediated by induction of the inhibitory Smads (I-Smads) Smad6 and Smad7. I-Smads also contain the MH2 domain which has been proposed to bind to R-Smads and form heterotrimmers to block their abilities to induce or repress expression of target genes (Zhang et al 2007). Other studies demonstrated the interaction of I-Smads with TβRI, causing competitive inhibition of R-Smad binding (Hayashi et al 1997). As an indirect mechanisms, Smad7 interacts constitutively with the HECT-domain E3 ubiquitin ligases SMURF1 and SMURF2 and targets them to the activated receptors, which leads to degradation of the active TβRs (Ebisawa et al 2001, Kavsak et al 2000, Ogunjimi et al 2005). Smad7 also interacts with GADD34, a regulatory subunit of protein phosphtase-1 (PP1), therefore targeting the catalytic subunit of PP1 to activated TβR for dephosphorylation and inactivation (Shi et al 2004).

The I-Smads are not the only inhibitory mediators of the TGFβ-Smad pathway. The Smad binding elements (SBEs) are not necessarily unoccupied in the absence of TGFβ signal. The proto-oncoproteins SKI and SKI-related novel protein N (SnoN) bind the same repeated elements (GTCTAGAC) as the Smad3/4 complex and are thought to bind in conjunction with Smad4 (Stroschein et al 1999). TGFβ signaling causes rapid degradation of SnoN, and to a lesser extent SKI, allowing the Smad3/4 complex to displace SKI and SnoN from SBEs (Stroschein et al 1999). SKI and SnoN have been shown to be overexpressed in multiple cancers. Therefore, the suppression of TGFβ signal transduction by overexpressed SKI and SnoN may contribute to tumorigenesis (Reed et al 2005).

1.2.4 Cytostatic response genes of TGFβ

The role of TGFβ in inducing cytostasis in normal epithelial cells is well characterized. In TGFβ-treated cells, TGFβ induces rapid and sustained expression of two cyclin-dependent kinase (CDK) inhibitors, $p21^{Waf1}$ and $p15^{Ink4b}$. Moreover, TGFβ downregulates expression of c-Myc, Id1 and Id2, three transcription factors involved in promotion of proliferation and inhibition of differentiation (Siegel and Massague 2003). As a general mode of TGFβ regulation of gene expression, the Smad3-Smad4 complex binds to a DNA-binding cofactor which further lands on the gene promoter and regulates transcription. As illustrated in Figure 1.3, in the case of $p21^{Waf1}$, upon stimulation with TGFβ, phosphorylated Smad3, Smad4 and transcription factor FoxO form an activator complex that binds to a specific region of the *$p21^{Waf1}$* gene promoter to initiate

transcription (Seoane et al 2004). In addition, the Smad complex can interact with the Sp1 transcription factor to activate the *p21*^{Waf1} gene promoter (Pardali et al 2000). Transcription of *p15*^{Ink4b} gene is also induced by binding of a Smad to the *p15*^{Ink4b} gene promoter. However, the co-factor(s) for *p15*^{Ink4b} induction by TGFβ has not been identified yet (Seoane et al 2001). TGFβ downregulation of c-Myc is mediated by a Smads-E2F4/5-p107 complex. Upon binding to the *c-Myc* promoter, the pocket protein p107 within the inhibitory complex recruits HDAC that functions as a transcriptional repressor (Chen et al 2002). The transcriptional repression of Id1 by TGFβ requires a Smads-ATF3 complex binding to the *Id1* promoter (Kang et al 2003). The SBE site and ATF3 element on the *Id1* promoter are not clustered but instead set apart by 18 base pairs. Among the target genes of TGFβ-mediated repression, *Id2* is the only one not directly repressed by a Smad complex. Id2 is transcriptionally activated by c-Myc-Max, hence downregulation of c-Myc expression by TGFβ prevents Id2 transcription (Lasorella et al 2000).

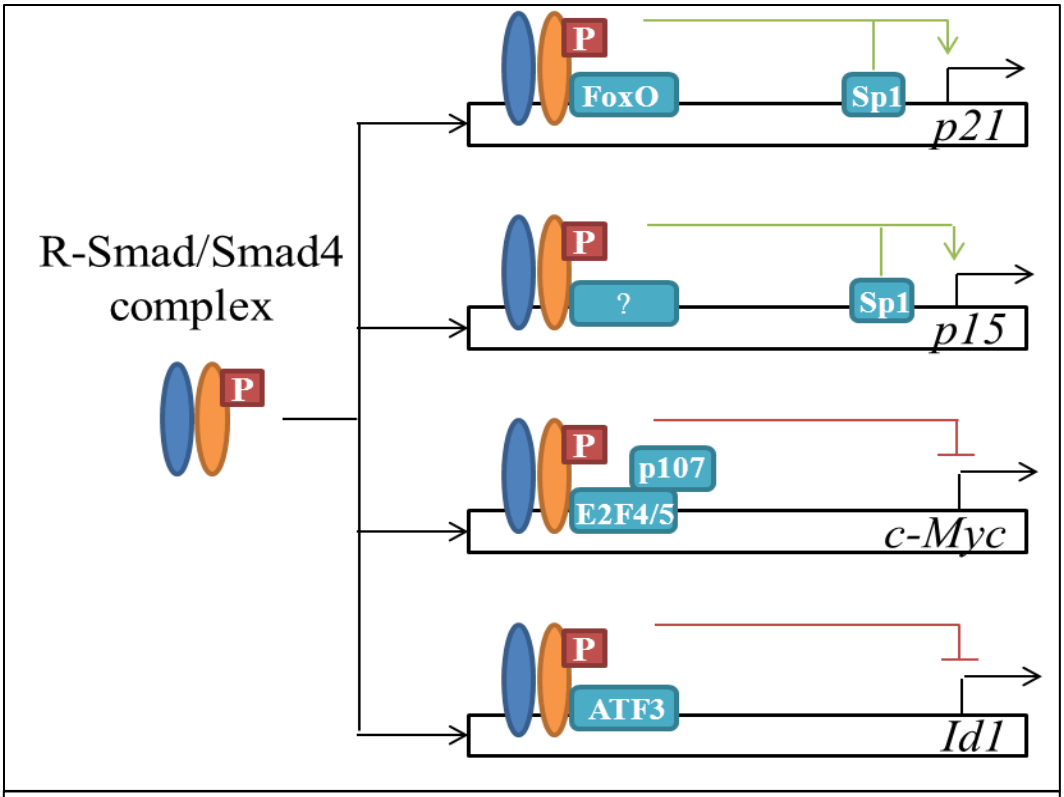

Figure 1.3. The TGFβ cytostatic program in epithelial cells. Adapted from Seoane et al "Escaping from the TGFβ anti-proliferative control" *Carcinogenesis* (2006) 27 (11): 2148-2156.

1.3 Crosstalk between LPA and TGFβ

A number of previous studies suggest that LPA indirectly interacts with TGFβ signaling and functionality. For example, LPA induces expression and secretion of TGFβ in human corneal fibroblasts (Jeon et al 2012). In human mesenchymal stem cells, LPA induces their differentiation into myofibroblast-like cells in an autocrine TβR-Smad-dependent manner (Jeon et al 2008). In keratinocytes, LPA acts as a growth inhibitor instead of stimulator. Sauer et al reported that LPA transactivates Samd3 of the TGFβ signaling pathway to exert such a cytostatic effect (Sauer et al 2004). Activation of latent

TGFβ is the rate-limiting step in TGFβ bioavailability. Recent evidence suggests that LPA could also participate in the process of TGFβ activation from its latent precursor (Xu et al 2009). Xu et al. showed that LPA stimulates αVβ6 integrin dependent activation of TGFβ in epithelial cells via the LPA_2 receptor, RhoA and ROCK (Xu et al 2009). In airway smooth muscle cells, LPA induces TGFβ activation via the integrin αVβ5 (Tatler et al 2011). Studies of genetic mouse models have implicated the LPA_1 receptor in the development of lung and renal fibrosis (Pradere et al 2008, Tager et al 2008). Accumulating evidence suggests that LPA interacts with TGFβ signaling to stimulate expression of the pro-fibrotic connective tissue growth factor (CTGF) (Cabello-Verrugio et al 2011, Gan et al 2011, Pradere et al 2007, Vial et al 2008). Taken together, these investigations indicate that LPA regulates TGFβ expression, activation, secretion or signaling, which may be relevant to diverse physiological and pathophysiological processes in mammalian cells.

On the other hand, there is little information on whether TGFβ may cross-communicate with LPA signal transduction to influence cellular functions, especially in epithelial carcinoma cells. As stated earlier, TGFβ and LPA are co-present at elevated levels in tumor microenvironments. The known biological effects of TGFβ and LPA on carcinoma cells are opposite or overlapping. In this dissertation project, I utilized breast and ovarian cancer cells as model systems to study combinatory effects of TGFβ and LPA on two major cellular processes of cancer: growth and motility. These studies showed novel and interesting biological outcomes of LPA and TGFβ co-treatment of breast and ovarian cancer cells and the underlying mechanisms. As described in chapter 2,

we identified LPA-induced expression of the cyclin dependent kinase inhibitor p21 as an indispensable component of the TGFβ-mediated cytostasis. TGFβ alone induced p21 expression weakly in TGFβ-sensitive cells. LPA cooperated with TGFβ to elicit the maximal p21 induction. Mechanistically, LPA stimulated p21 via LPA_1 and LPA_2 receptors and Erk-dependent activation of the CCAAT/enhancer-binding protein beta (C/EBPβ) transcription factor. When we examined the simultaneous effects of TGFβ and LPA on tumor cell motility in Chapter 3, we found that TGFβ signaling via a Smad-dependent pathway inhibits activity of the LPA_1 gene promoter, LPA_1 mRNA transcription and LPA_1-dependent migration and invasion of breast and ovarian cancer cells. Further characterization using multiple molecular approaches showed that *LPA_1* is a physiological target gene of TGFβ-mediated repression involving a specific TBE element on the LPA_1 promoter. The work represents the first detailed study of transcriptional regulation of an LPA receptor.

CHAPTER 2

LYSOPHOSPHATIDIC ACID–INDUCED P21^{WAF1} EXPRESSION MEDIATES THE CYTOSTATIC RESPONSE OF BREAST AND OVARIAN CANCER CELLS TO TRANSFORMING GROWTH FACTOR BETA

Part of the work presented in this Chapter has been published in Molecular Cancer Research 9(11): 1562-70, 2011

2.0 Abstract

Lysophosphatidic acid (LPA) is a multifunctional intercellular phospholipid mediator presents in blood and other biological fluids. In cancer cells, LPA stimulates expression or activity of inflammatory cytokines, angiogenic factors, matrix metalloproteinases, and other oncogenic proteins. In this study, we showed that LPA upregulated expression of the cyclin-dependent kinase inhibitor p21^{Waf1} in TGFβ-sensitive breast and ovarian cancer cells, but not in TGFβ-resistant cells. We examined the possibility that LPA-induced p21 might contribute to the cytostatic response to TGFβ. In serum-free conditions, TGFβ alone induced p21 expression weakly in TGFβ-sensitive cells. Serum or serum-borne LPA cooperated with TGFβ to elicit the maximal p21 induction. LPA stimulated p21 via LPA_1 and LPA_2 receptors and Erk-dependent

activation of the CCAAT/enhancer binding protein beta transcription factor independent of p53. Loss or gain of p21 expression led to a shift between TGFβ-sensitive and -resistant phenotypes in breast and ovarian cancer cells, indicating that p21 is a key determinant of the growth inhibitory activity of TGFβ. Our results reveal a novel cross-talk between LPA and TGFβ that underlies TGFβ-sensitive and -resistant phenotypes of breast and ovarian cancer cells.

2.1 Introduction

LPA is a naturally occurring intercellular mediator of diverse biological processes including neurogenesis, angiogenesis, would healing, immunity, and carcinogenesis (Panupinthu et al 2010). LPA is produced by activated platelets during coagulation and thus is a normal constituent of serum (Eichholtz et al 1993, Sano et al 2002). LPA is a ligand of at least six G protein-coupled receptors (GPCRs) (Choi et al 2010). The LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 receptors are members of the endothelial differentiation gene (Edg) family, sharing 50-57% homology in their amino acid sequences. GPR23/P2Y9/LPA_4 of the purinergic receptor family and the related GPR92/LPA_5 and P2Y5/LPA_6 have been identified as additional LPA receptors, which are structurally distinct from the LPA_{1-3} receptors (Choi et al 2010). The LPA receptors couple to multiple G proteins, Gq, Gi, G12/13 and Gs, which, in turn, activate diverse pathways including Gq-mediated stimulation of phospholipase C (Fukushima and Chun 2001), Gi-mediated activation of the Ras-MAPK and PI3K (Takeda et al 1999), and

G12/13-mediated activation of RhoA (Kranenburg et al 1999). Activation and integration of these signaling events downstream of LPA receptors leads to cytoskeleton remodeling, cell proliferation, survival, and migration (Mills and Moolenaar 2003). Recent studies demonstrated that LPA exerts its biological actions through transcriptional activation of multiple target genes involved in a wide range of physiological and pathophysiological processes (Teo et al 2009).

TGFβ is also a platelet-derived factor that controls a multitude of biological activities including cell proliferation (Huang and Huang 2005), differentiation (Fei and Chen 2010), and apoptosis (Perlman et al 2001). The complicated role of TGFβ is mediated through the heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors (TβRI and TβRII), and the Smad family of transcription factors and non-Smad signaling pathways (de Caestecker et al 2000, Derynck and Zhang 2003). TGFβ inhibits proliferation of epithelial cells and thus plays a role in early tumor suppression. However, TGFβ frequently fails to induce growth arrest in transformed epithelial cells. On the other hand, TGFβ stimulates migration and invasion of neoplastic cells, thereby promoting the metastatic potentials of advanced cancer (Bierie and Moses 2006, Massague 2008).

The anti-proliferative effect of TGFβ is mediated by a complex signaling network involving TβRI and TβRII activation of Smad2/3 and ultimately transcriptional modulation of growth control genes such as induction of the cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1}$ and $p15^{Ink4b}$, and suppression of the *c-Myc*, *Id1* and *Id2*

transcription factors (Siegel and Massague 2003). Cancer cells tend to escape from the anti-proliferative effect of TGFβ through acquisition of mutations in components of the TGFβ signal transduction pathway or through deregulation of other signaling cascades interconnecting with the TGFβ pathway (Derynck et al 2001). Mutations in the TβRII receptor gene (Siegel and Massague 2003) as well as mis-sense mutation or deletion of Smad2 and Samd4 (Sjoblom et al 2006, Yang and Yang 2010) have been identified in different types of cancer. There is also evidence for overexpression of oncoproteins in inactivation of the cytostatic effect of TGFβ in cancer, such as Myc-Miz-1 complex (Seoane et al 2001), Evi-1 (Kim and Letterio 2003), FoxG1 (Seoane et al 2004), CDK (Matsuura et al 2004) and Ski and/or SnoN (Zhang et al 2003). However, these aberrations seen in only fractions of human tumors do not explain the generally altered responses to TGFβ in a wide spectrum of cancers.

In the present study, we examined the potential crosstalk between LPA signaling and TGFβ in growth regulation of breast and ovarian cancer cells. We report that LPA up-regulates expression of the CDK inhibitor p21 in breast and ovarian cancer cells sensitive to TGFβ- induced growth arrest but not in TGFβ-resistant cancer cells. In TGFβ-sensitive cells, LPA cooperates with TGFβ to elicit the maximal induction of p21 to mediate the cytostatic response to TGFβ. Loss or gain of p21 expression led to a shift between TGFβ sensitive and resistant phenotypes in these cells. Our results reveal a novel mechanism underlying the cytostatic program of TGFβ in breast and ovarian cancer cells.

2.2 Materials and Methods

Materials Anti-phospho C/EBPβ, phospho-Erk1/2, tubulin α/β antibodies and PD98059 were obtained from Cell Signaling (Danvers, MA). Anti-C/EBPβ, p21, and Erk antibodies were from Santa Cruz Biotech (Santa Cruz, CA). LPA (1-oleoly, 18:1) and S1P were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, LPA and S1P were dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA) obtained from Roche (Indianapolis, IN). TGFβ was obtained from PeproTech Inc (Rocky Hill, NJ). TPA was from Sigma (St Louis, MI). Fetal bovine serum (FBS) was obtained from Atlanta Biological (Atlanta, GA). Oligonucleotides were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). TRIzol and cell culture reagents were obtained from Invitrogen Inc. (Carlsbad, CA). The transfection reagent Dharmafect 1 was obtained from Dharmacon (Lafayette, CO). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA).

Cell Culture MDA-MB-231 was provided by S Spiegel (Virginia Commonwealth University). SK-BR-3 and BT-549 were obtained from Dr. G. Mills (MD Anderson Cancer Center). MDA-MB-231 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Other breast and ovarian cancer cell lines used in the study were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics as we described previously (Fang et al 2004).

Western blotting Cells were lysed in SDS sample buffer or in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM Na PPi, and protease inhibitor cocktail]. Total cellular proteins were resolved by SDS-PAGE, transferred to Immun-Blot membrane [poly(vinylidene difluoride)] from BIO-RAD (Hercules, CA), and immunoblotted with antibodies following the protocols of manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit from Amersham (Piscataway, NJ).

siRNA knockdown The siRNA oligos for LPA receptors (LPA_1 #4050, LPA_2 #44997, LPA_3 #136436 and LPA_5 #s32725), p21 (#S415) and C/EBPβ (#s2891) were obtained from Applied Biosystems (Carlsbad, CA). siRNA oligos for Erk (Erk1 #L-003592-00 and Erk2 #L-03555-00) were obtained from Dharmacon (Lafayette, CO). They were transfected into cells using Dharmafect 1 following the manufacturers' protocol. In brief, cells were plated in 6-well plates to reach 50% confluence before transfection for 12-16 hours with specific siRNA (100 pmole) and Dhamafect 1 (4 μL). The transfected cells were cultured in complete medium for approximately 48 hours before experiments.

Quantitative PCR Total cellular RNA was isolated using Trizol (Invitrogen). Complementary DNA (cDNA) was synthesized from RNA (1 μg, random primers) using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The relative levels of individual LPA receptors were determined using gene specific probes, the TaqMan Universal PCR Master Mix and the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Statistics All numerical data were presented as mean ± SD from at least 3 independent experiments. The statistical significance of differences was analyzed using Student's *t* test where $p<0.05$ was considered statistically significant.

2.3 Results

2.3.1 Induction of p21 by LPA in TGFβ-sensitive breast and ovarian cancer cells

It has been well documented that LPA regulates expression of cytokines, angiogenic factors and many other proteins involved in tumorigenesis and cancer metastasis (Fang et al 2004, Oyesanya et al 2008, Song et al 2009). In contrast to these oncogenic mediators, we also found that in a subset of breast and ovarian cancer cell lines, LPA up-regulates expression of p21, an inducible inhibitor of CDKs. As shown in Fig. 2.1A, in the MDA-MB-231 breast carcinoma cells and the Caov-3 ovarian carcinoma cells, LPA stimulated p21 expression in a time-dependent manner. Following addition of

10 μM LPA to serum-starved cells, p21 protein was induced at 1 hour. The p21 protein levels reached the maximum by 4 hours. Similarly, LPA also induced upregulation of p21 mRNA in these two cell lines (Fig. 2.1B).

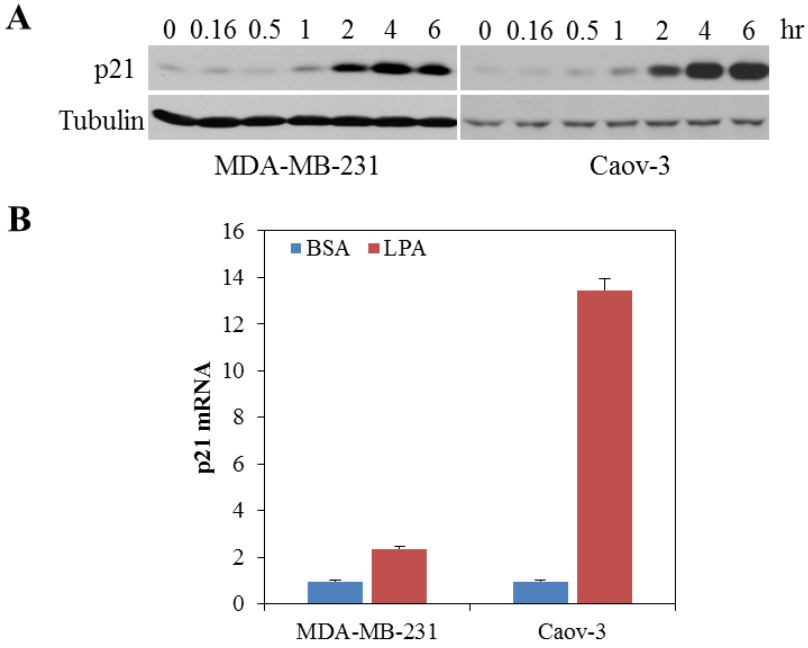

Figure 2.1. LPA-induction of p21 in TGFβ-sensitive breast and ovarian cancer cells. ***A***. MDA-MB-231 cells and Caov-3 cells were starved in serum-free medium and treated with LPA (10 μM) for the indicated periods of time. The cells were lysed with SDS sample buffer and expression of p21 protein was examined by immunoblotting. ***B***. MDA-MB-231 cells and Caov-3 cells were treated with LPA (10 μM) for 1 hour. Cells were lysed with TRIzol and RNA was isolated as described in Materials and Methods. The p21 mRNA levels were normalized to the untreated sample (untreated sample as 1). In this and following figures, data is presented as mean ± SD, the statistical significances of the data were indicated with * if $p<0.05$, or ** if $p<0.01$.

The p21 protein inhibits activity of cyclin-CDK2 or cyclin-CDK4 complexes to block cell cycle progression at G1 phase (Brugarolas et al 1999). In addition, p21 can bind to proliferating cell nuclear antigen thereby blocking DNA synthesis (Waga et al

1994). Paradoxically, p21 has been also reported to stabilize interactions between CDK4/CKD6 and D-cyclins thus promoting the formation of active complexes in a concentration-dependent manner (Giacinti and Giordano 2006). Although modest levels of p21 may promote assembly of active cyclin-CDK complex (Cheng et al 1999), excessive expression of p21 generally causes cell cycle arrest. In our hands, the induction of p21 by LPA was not associated with growth inhibition. Instead, LPA treatment led to increased proliferation in MDA-MB-231 and Caov-3 cells (see Fig. 2.8) as well as in other breast and ovarian cancer cell lines in which LPA did not trigger p21 expression (Fig. 2.2).

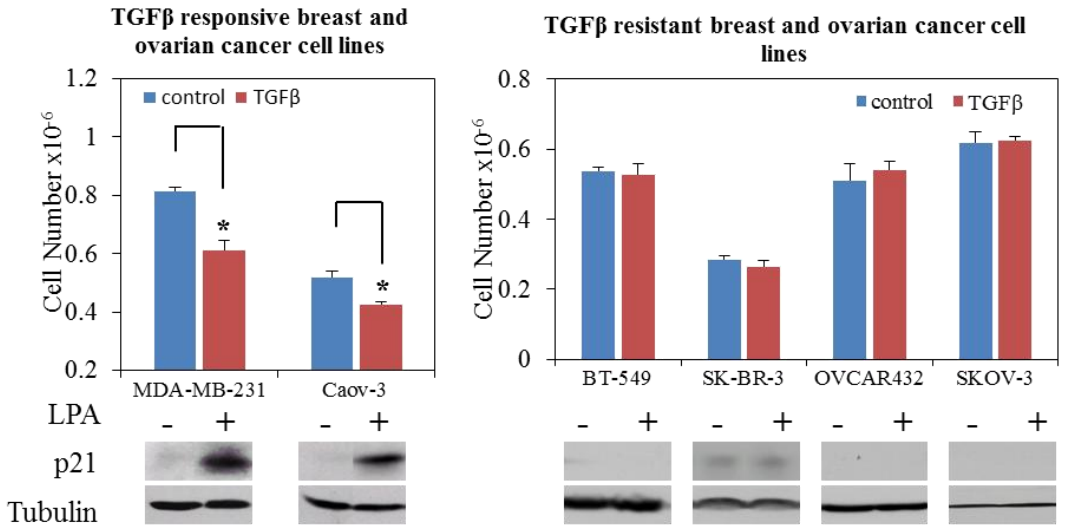

Figure 2.2. Correlation between LPA-mediated p21 induction and sensitivity to TGFβ mediated cytostasis. Various breast and ovarian cancer cell lines in 6-well plates were incubated for 48 hours in complete medium with or without TGFβ (2.5 ng/ml). Cell numbers were quantified with a Coulter counter. The induction of p21 protein by LPA in these cell lines was examined by immunoblotting as described in ***Fig. 2.1.***

In an effort to understand the biological significance of LPA-mediated p21 induction, we noticed surprisingly that LPA stimulated p21 expression only in cell lines sensitive to the TGFβ-induced growth arrest but not in cells refractory to TGFβ. As demonstrated in Fig. 2.2, treatment of MDA-MB-231 and Caov-3 cells with TGFβ (2.5 ng/ml) for 48 hours resulted in a significant decrease in cell numbers compared to control cells cultured in the absence of TGFβ. In contrast, TGFβ did not inhibit the growth of cell lines such as BT-549, SK-BR-3, OVCA-432 and SKOV-3 in which LPA did not induce p21 (Fig. 2.2).

2.3.2 Correlation of LPA and TGFβ induction of p21

We next explored the possibility that LPA-driven p21 expression modulates the sensitivity of breast and ovarian cancer cells to TGFβ. Coincidently, the effect of TGFβ on p21 expression was identical to that of LPA in these breast and ovarian cancer cells. As shown in Fig. 2.3, TGFβ induced p21 expression at significant levels only in MDA-MB-231 and Caov-3 cells but not in TGFβ-resistant lines in which LPA failed to induce p21 (Fig. 2.3).

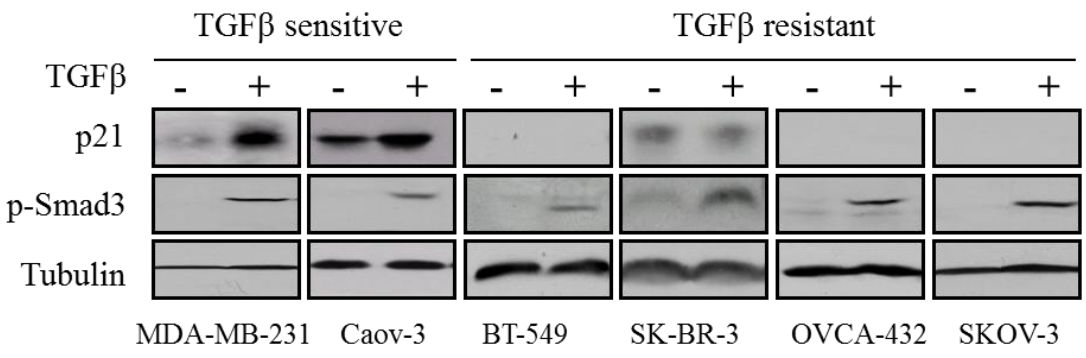

Figure 2.3. p21 induction was only found in TGFβ sensitive cells. Breast and ovarian cancer cell lines in complete medium were treated for 6 hours with or without TGFβ (2.5 ng/ml) before lysis with SDS sample buffer. Expression of p21 protein and phosphorylated Smad3 was analyzed by immunoblotting.

The loss of p21 inducibility by TGFβ could be due to abnormalities in TGFβ receptors or the TGFβ intracellular signaling through Smads. It is well known that TGFβ superfamily ligands bind to a TβRII, which recruits and phosphorylates a TβRI. TβRI then phosphorylates receptor-regulated Smads (R-Smad) such as Smad2 and Smad3, which then bind to the common mediator Smad (co-Smad). R-Smad forms heterodimeric complexes with co-Smads and accumulates in the nucleus where the complexes participate in regulation of TGFβ target genes involved in growth control (Siegel and Massague 2003). As shown in Fig. 2.3, TGFβ induced phosphorylation of Smad3 in all breast and ovarian cancer cell lines examined, irrespective of their status of TGFβ sensitivity. To further confirm the presence of the intact TGFβ signaling in these cells, we examined TGFβ induction of another TGFβ target gene Plasminogen activator inhibitor-1 (PAI-1). Upon treatment of TGFβ, all cell lines showed increased expression of PAI-1

mRNA (Fig. 2.4). This suggests that both TGFβ-sensitive and resistant cells maintain functional TGFβ receptors and the Smad3 signal transducer.

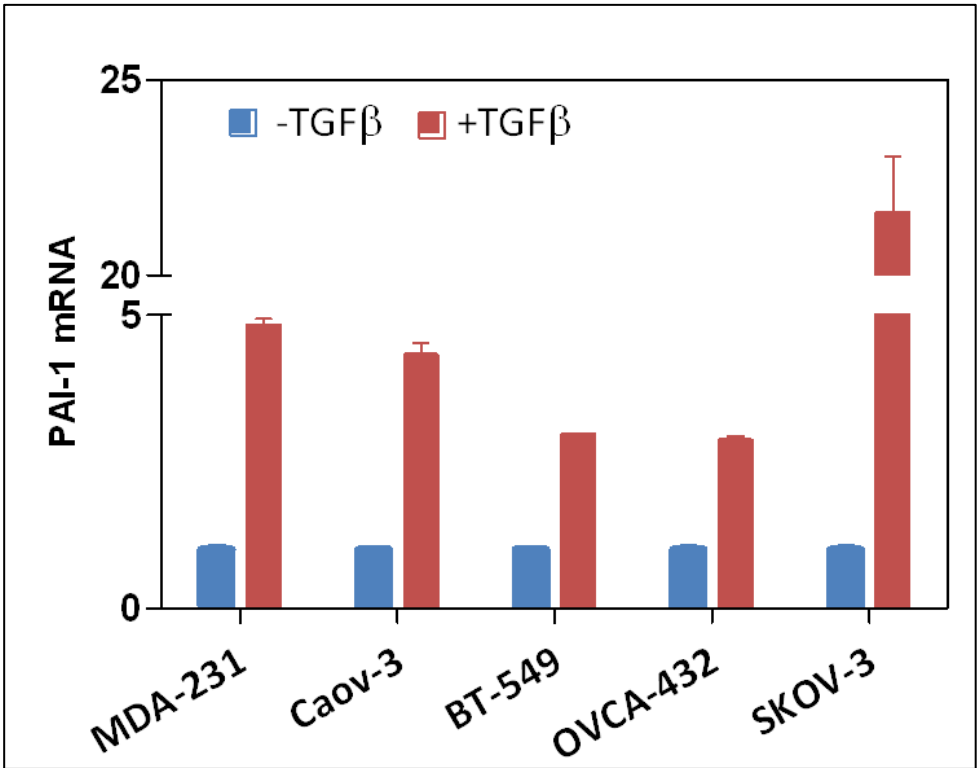

Figure 2.4. Induction of TGFβ target gene PAI-1 in resistant cells. Breast and ovarian cancer cell lines in complete medium were treated for 2 hours with or without TGFβ (2.5 ng/ml) before lysis with TRIzol. PAI-1 mRNA level was determined by qPCR using TaqMan probe.

2.3.3 Input of LPA signaling in TGFβ-induced p21 expression

Because phosphorylation of Smad3 by TGFβ was observed in both TGFβ-sensitive and resistant cells, p21 induction by TGFβ seems to involve signaling routes other than the canonical Smad pathway in these cells. In addition, both MDA-MB-231

and Caov-3 carry mutant p53 (Hui et al 2006, Yaginuma and Westphal 1992). TGFβ-induced p21 expression in these cells is apparently mediated by a p53-independent process. We therefore examined the possibility that LPA contributes to TGFβ-induced p21 expression in the TGFβ-sensitive MDA-MB-231 and Caov-3 cells. When these cells were cultured in serum-free medium, TGFβ stimulated only weak to modest levels of p21 (Fig. 2.5A). The maximal p21 induction by TGFβ was seen when the cells were incubated in complete medium containing FBS (Fig. 2.5A), a condition in which the effects of TGFβ on cell proliferation and p21 expression were assessed in earlier experiments (Fig. 2.2). Serum itself induced p21 expression in MDA-MB-231 and Caov-3 cells. This suggests that induction of p21 by TGFβ that we had observed resulted from a combined action of TGFβ and a co-factor present in serum.

LPA is a prominent serum-borne factor responsible for many biological activities of serum (Moolenaar 1999). To determine whether LPA reproduces the action of serum in concert with TGFβ to maximize p21 induction, we examined the effect of LPA and TGFβ on p21 expression in MDA-MB-231 and Caov-3 cells. Indeed, p21 induction was maximized when both LPA and TGFβ were present (Fig. 2.5B). We also assessed other serum factors such as sphingosine 1 phosphate (S1P) and insulin for their ability to regulate p21 expression (Hiromura et al 2002, Kim et al 2004). In contrast to LPA, S1P and insulin did not increase p21 expression. Nor did S1P and insulin potentiate the effect of TGFβ on p21. Taken together, these results suggest that a significant input of TGFβ-induced p21 is attributable to the action of LPA, which likely underlies the sensitivity of breast and ovarian cancer cells to TGFβ.

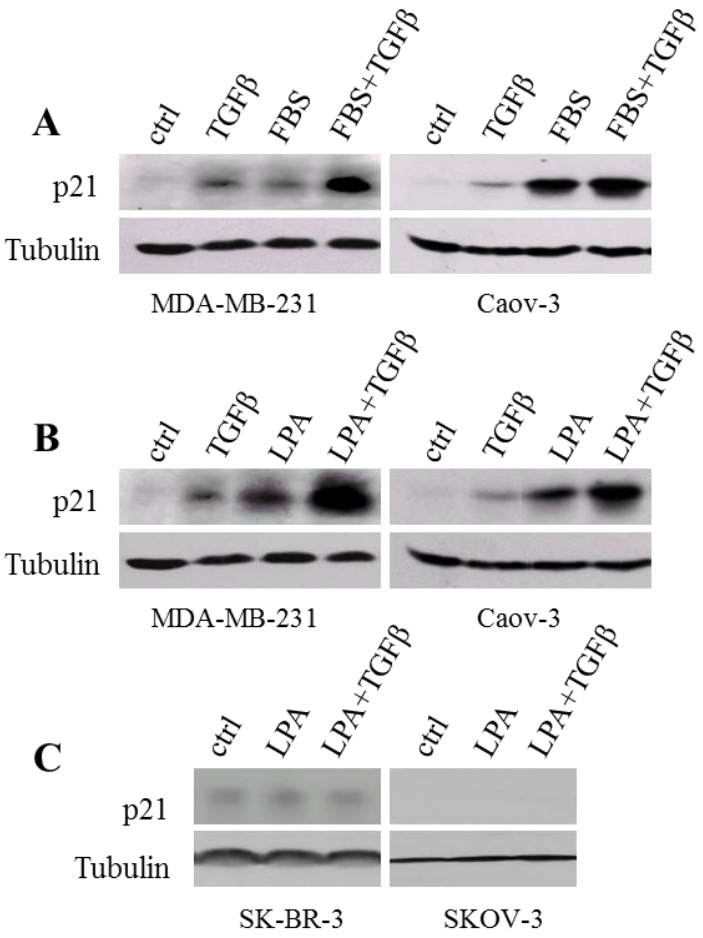

Figure 2.5. Input of LPA action in TGFβ-mediated p21 induction. ***A***. MDA-MB-231 and Caov-3 cells were serum starved and stimulated for 6 hours with TGFβ (2.5 ng/ml), FBS (5%), or TGFβ+FBS. Expression of p21 in these cells was assessed by immunoblotting. ***B.*** MDA-MB-231 and Caov-3 cells were treated and analyzed as described in ***A*** except that FBS was replaced with LPA (10 μM).

2.3.4 Role of p21 in mediating the cytostatic response to TGFβ

To confirm an essential role for p21 in mediating the TGFβ response, we used siRNA to knockdown p21 expression in the TGFβ sensitive MDA-MB-231 and Caov-3 cells. As shown in Fig. 2.6A, suppression of p21 induction by siRNA converted these

cells into a resistant phenotype. The p21 knockdown cells became insensitive to the inhibitory effect of TGFβ, confirming that p21 induction is indeed a key component of TGFβ-induced cytostasis in breast and ovarian cancer cells.

If the p21 inducibility distinguishes TGFβ-sensitive cells from resistant ones, we assume that the resistant cells could be rendered sensitive to TGFβ when p21 is induced somehow by other p21 stimuli. To test this possibility, we took advantage of the fact that phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate, TPA) induces expression of p21 in cancer cells (Salabat et al 2006). We treated the TGFβ-resistant cell lines with TGFβ alone or TGFβ and TPA (0.1 μM). The presence of TPA led to induction of high and sustained expression of p21 while the cells treated with TGFβ alone did not show p21 expression (Fig.2.6B). Treatment with TPA was not associated with inhibition of cell proliferation as shown in Fig. 2.6B, suggesting that p21 induced by TPA was not sufficient to affect cell growth without TGFβ. However, the presence of TPA-induced p21 expression enables TGFβ to suppress growth of these otherwise TGFβ-resistant cells, consistent with the importance of p21 in mediating TGFβ sensitivity (Fig. 2.6B). To confirm that the growth inhibitory effect was due to the presence of p21 not the apoptotic effect of TPA, we performed annexin V staining on TGFβ or TGFβ+TPA treated BT-549 and OVCA-432 cells. No significant increase of apoptosis was observed with the presence of TPA (Fig. 2.7).

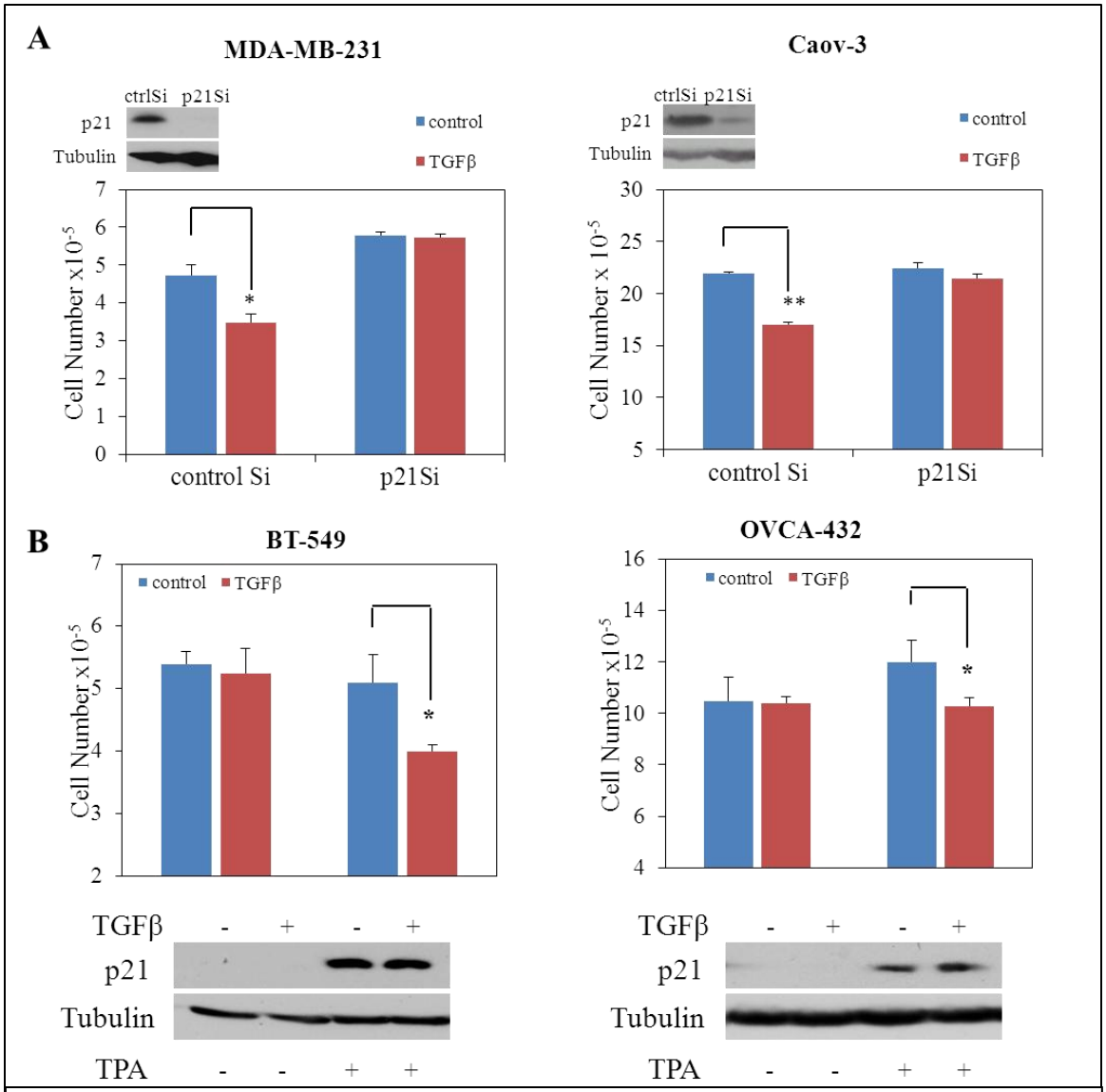

Figure 2.6. Essential role of p21 in the cytostatic response to TGFβ. ***A***. The TGFβ-sensitive cells lost sensitivity to TGFβ following siRNA knockdown of p21 expression. MDA-MB-231 and Caov-3 cells in 6-well plates were transfected with control or p21 siRNA. The cells were treated for 48 hours with or without TGFβ (2.5 ng/ml) before quantification of cell numbers with a Coulter counter. Efficiency of p21 siRNA knockdown was confirmed by immunoblotting. ***B***. TGFβ resistant cell lines gained sensitivity to TGFβ following TPA induction of p21. BT-549 and OVCA-432 in 6-well plates were treated for 48 hours with or without TGFβ in the presence of TPA (0.1 μM) or vehicle before quantification of cell numbers. Expression of p21 in these cells treated with TGFβ, TPA or TGFβ+TPA was analyzed by immunoblotting.

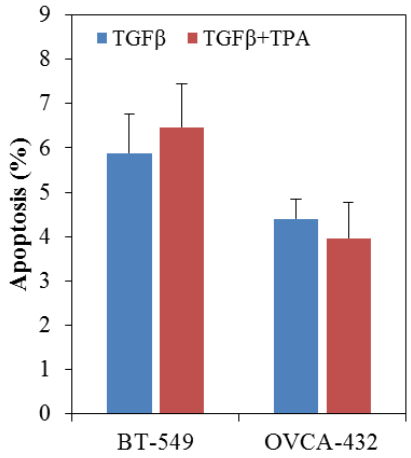

Figure 2.7. TPA treatment has no effect in cell apoptosis. BT-549 and OVCA-432 cells treated with TGFβ or TPA+TGFβ for 48 hours. Cells were analyzed for apoptosis using vibrant Apoptosis Kit #3 (Invitrogen) to stain apoptotic cells with fluorescence-conjugated Annexin V.

2.3.5 p21-dependent inhibition of LPA-driven cell proliferation by TGFβ

LPA stimulated p21 expression in MDA-MB-231 and Caov-3 cells (Fig. 2.5B). However, in spite of the robust and sustained induction of p21, LPA is mitogenic towards these cells. To determine whether TGFβ is able to block the mitogenic effect of LPA, we compared the growth of MDA-MB-231 and Caov-3 cells incubated with LPA in the absence or presence of TGFβ. Fig. 2.8A shows that TGFβ effectively inhibits cell number increases stimulated by LPA. Moreover, siRNA knockdown of p21 expression resulted in resistance of these cells to TGFβ (Fig. 2.8B), confirming an essential role for p21 in TGFβ repression of LPA-induced cell proliferation. In TGFβ-resistant breast and ovarian

cancer cell lines, LPA also acted as a mitogen. The mitogenic activity of LPA, however, was not affected by TGFβ, consistent with the lack of induction of p21 by LPA, TGFβ or LPA and TGFβ in these cells.

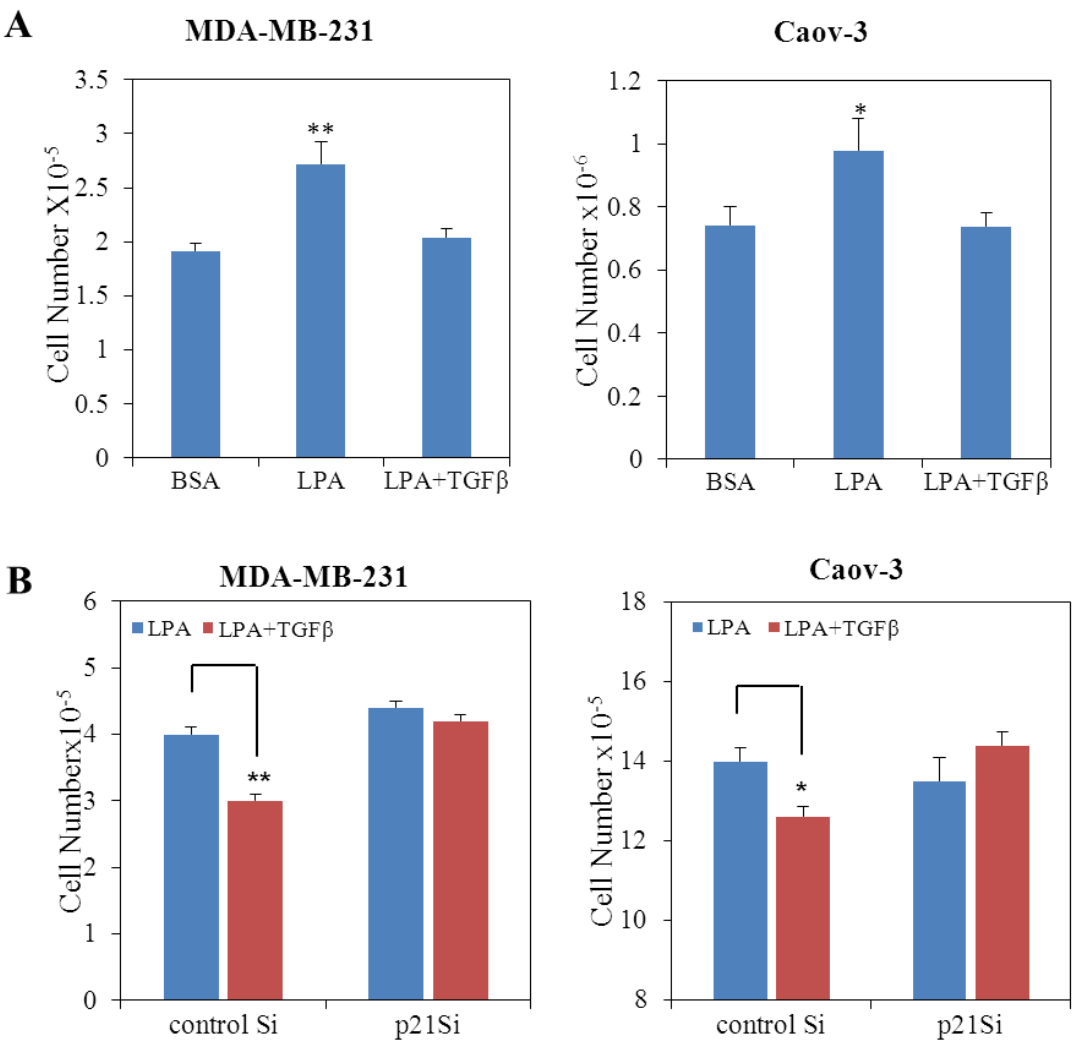

Figure 2.8. p21-dependent inhibition of LPA-induced cell proliferation by TGFβ. ***A***. TGFβ inhibits LPA-afforded cell proliferation. MDA-MB-231 and Caov-3 cells in 6-well plates were incubated with LPA (10 μM) or vehicle (BSA) in the presence or absence of TGFβ (2.5 ng/ml). ***B***. The growth inhibitory effect of TGFβ depends on p21 induction. MDA-MB-231 and Caov-3 cells were transfected with control or p21 siRNA as described in Figure 4. The cells were treated with LPA in the presence or absence of TGFβ. The cell numbers presented in both panels were determined after 48 hours. Efficiency of p21 siRNA knockdown in transfected cells was analyzed by immunoblotting as shown in ***Fig. 2.5A.***

2.3.6 Mechanisms for LPA induction of p21

Ovarian and breast cancer cells express multiple LPA receptors including LPA_1, LPA_2, LPA_3 and LPA_5 as described previously (Fang et al 2002, Finak et al 2008, Liu et al 2009). Expression of the LPA_4 and LPA_6 receptors was very low in the breast and ovarian cancer lines. We thus used siRNA to knockdown expression of LPA_1, LPA_2, LPA_3 or LPA_5. The cells treated with LPA were then examined for p21 protein expression. LPA-induced p21 was drastically reduced by downregulation of LPA_1 or LPA_2 (Fig. 2.9). Knockdown of LPA_3 or LPA_5 did not attenuate the effect of LPA on p21 expression. Therefore, we conclude that LPA-stimulated p21 expression in MDA-MB-231 and Caov-3 cells occurs through the LPA_1 and LPA_2 receptors.

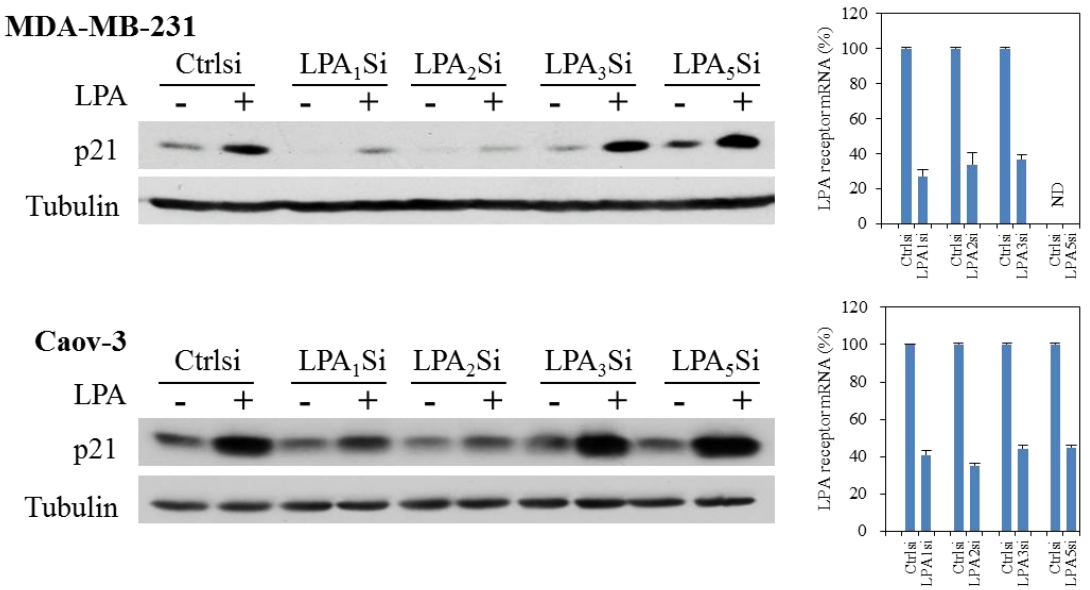

Figure 2.9. LPA_1 and LPA_2 are responsible for LPA stimulation of p21. MDA-MB-231 and Caov-3 cells were transfected with specific siRNA for LPA_{1-3}, LPA_5 or non-target control siRNA. The cells were serum starved and stimulated with LPA (10 μM) before immunoblotting analysis of p21 protein expression. The efficiency of knockdown of individual LPA receptors was examined by RT and quantitative PCR as described in Materials and Methods. The mRNA levels of LPA receptors were presented as percentages relative to those in the control siRNA-transfected cells (defined as 100%).

LPA induced strong and sustained activation of Erk in MDA-MB-231 and Caov-3 cells (Fig. 2.10) (Du et al 2010, Oyesanya et al 2010). When Erk1 and Erk2 were silenced by siRNAs, LPA induction of p21 was blocked (Fig. 2.10A), indicating that the Erk pathway is linked to activation of p21 expression in response to LPA. In contrast to Erk, PI3K was dispensable for LPA-induced p21 induction because its inhibitor LY-294002 did not attenuate the effect of LPA on p21 expression.

Erk couples directly or indirectly to diverse downstream effectors and transcription factors that could culminate in p21 expression. We used siRNA to screen for transcription factors required for LPA-induced p21 expression including AP-1, Sp1, NF-κB and C/EBPβ. In this group of transcription factors, C/EBPβ was found to be critical to the p21 induction. Knockdown of C/EBPβ expression prevented LPA-induced p21 expression (Fig. 2.10B). Finally, inhibition of Erk activity with the MEK inhibitor PD98059 prevented C/EBPβ phosphorylation and the subsequent p21 induction in LPA-treated MDA-MB-231 and Caov-3 cells (Fig. 2.10C). These findings demonstrate that LPA stimulates p21 expression through the $LPA_{1/2}$-Erk-C/EBPβ signaling network.

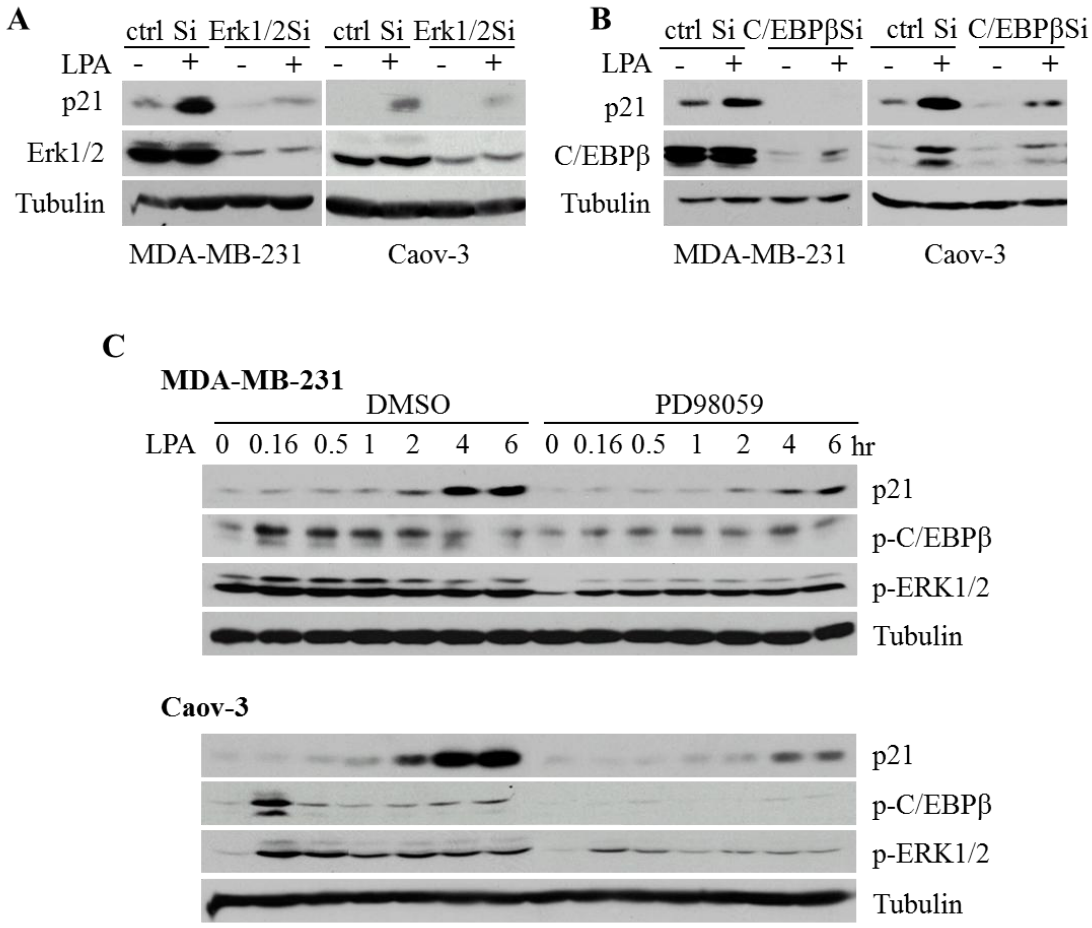

Figure 2.10. Induction of p21 by LPA through the LPA_1/LPA_2-Erk-C/EBPβ pathway. ***A.*** Knockdown of Erk1/2 blocks LPA induction of p21. MDA-MB-231 and Caov-3 cells were transfected with Erk siRNAs or non-target control siRNA. The cells were stimulated for 6 hours with LPA (10 μM) followed by immunoblotting analysis of p21, Erk and tubulin proteins. ***B***. C/EBPβ was critical for LPA induction of p21. MDA-MB-231 and Caov-3 cells were transfected with control or C/EBPβ siRNA. The transfected cells were treated for 6 hours with LPA and analyzed for p21 and C/EBPβ protein expression. ***C***. MDA-MB-231 and Caov-3 cells were serum starved and then treated with LPA (10 μM) in the presence of PD98059 (30 μM) or vehicle (DMSO). PD98059 was added 1 hour before LPA. Cells were then lysed at indicated time points. Levels of phospho-Erk1/2, p21 and phospho-C/EBPβ were examined by immunoblotting.

2.4 Discussion

TGFβ-mediated cytostasis is induced, at least in part by Smad-dependent activation of TGFβ target genes involved in cell cycle control, primarily CDK inhibitors p15, p21 and p27. In addition, TGFβ activation of Smad represses expression of proteins that promotes cell cycle progression including c-Myc, Id1, Id2, E2F, and Sp-1 (Feng et al 2000, Massague 2004). These TGFβ-induced cytostatic transcriptional programs, however, are subverted in a majority of cancers, leading to cytostatic resistance to TGFβ (Barcellos-Hoff and Ewan 2000). In addition to genetic and epigenetic aberrations in TGFβ receptors or Smad proteins, emerging data suggests that in most malignancies, abrogation of TGFβ-induced growth arrest is mediated by abnormal expression or function of intracellular proteins implicated in Smad regulation of its target genes (Massague 2008). In theory, environmental cues that influence expression or activity of Smad, Smad regulatory circuits or Smad responsive genes could also alter cellular responses to TGFβ. However, there have been few studies to analyze potential crosstalk between extracellular factors such as LPA and TGFβ-Smad to regulate the responsiveness of cancer cells to TGFβ.

Using breast and ovarian cancer cells as a model system, we demonstrated that LPA upregulates expression of the prototype Smad target gene p21, contributing to the TGFβ-mediated growth inhibition. The conclusions were summarized as a model shown in Fig. 2.11. In these cells, the ability of LPA to stimulate p21 expression correlated well with TGFβ induction of p21 and the cytostatic effect of TGFβ. By means of induction and suppression of p21 expression in TGFβ-resistant and sensitive cells, we could reverse

the cellular responses to TGFβ confirming an essential role of p21 in mediating the cytostatic response to TGFβ. Previous studies in breast and ovarian cancer cells also supported the involvement of p21 as a key mediator of TGFβ-induced growth inhibition (Massague 2004).

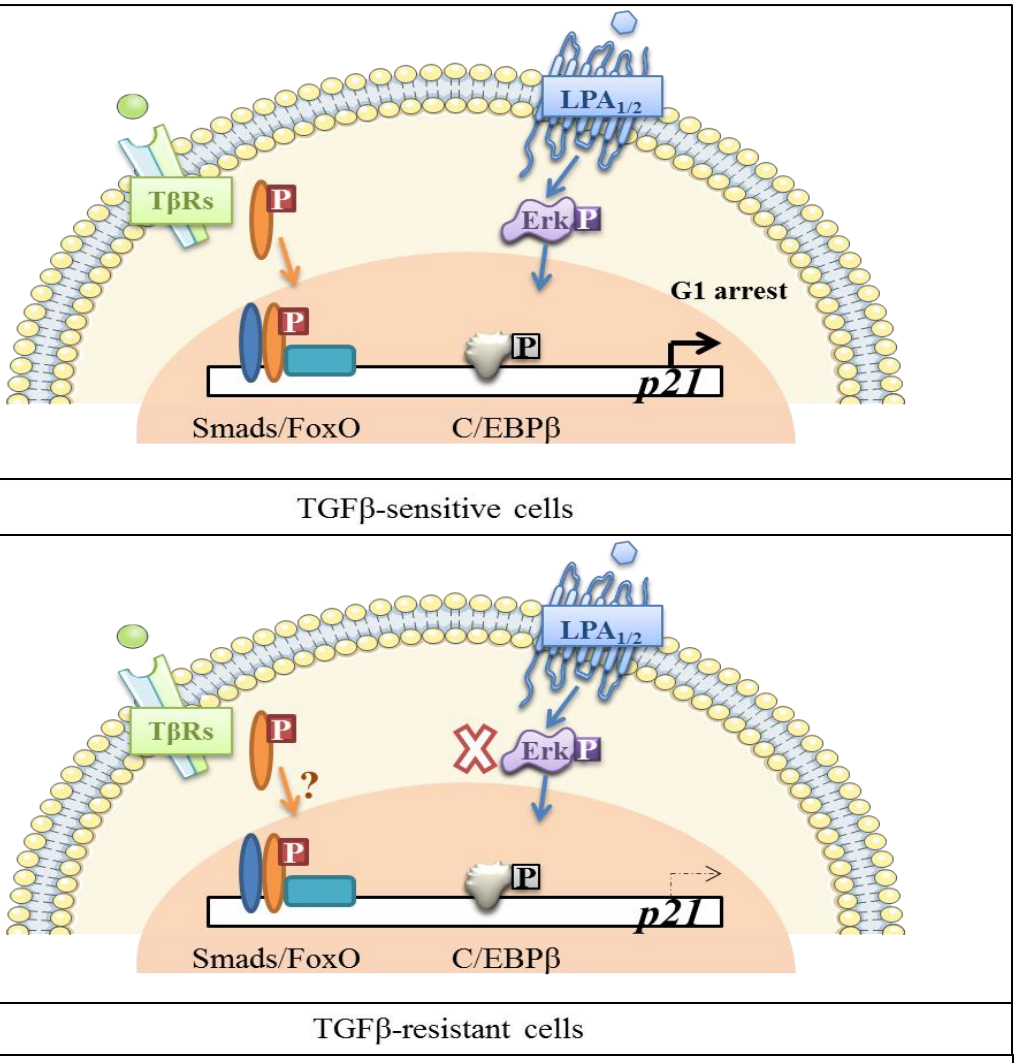

Figure 2.11. Working model: LPA induced p21 mediates response of breast and ovarian cancer cells to TGFβ. Induction of p21 from the $LPA_{1/2}$-ERK-C/EBPβ pathway is required for cells to respond to TGFβ mediated cytostasis. P: phosphate, yellow oval: Smad3, blue oval: Smad4.

Another observation in ovarian cancer indicates that abrogation of TGFβ induced growth arrest is associated with overexpression of FoxG1, a negative regulator of p21 expression (Chan et al 2008). Therefore, p21 seems to be a general mediator of TGFβ-induced growth arrest in multiple types of cancer cells. The findings of the present work highlight the possibility that the sensitivity to TGFβ in breast and ovarian cancer cells could be reconstituted through upregulation of p21 expression. It will be of interest to develop and test agents that can specifically activate p21 expression or stabilize p21 protein in cancer cells.

An interesting finding in the current study is that p21 induction in TGFβ sensitive cells is accomplished through cooperative effects of TGFβ and the serum-borne factor LPA. A significant input of p21 expression is evoked from LPA activation of its receptors, namely LPA_1 and LPA_2. Using molecular and pharmacological approaches, we further demonstrated that LPA upregulates p21 expression in TGFβ responsive cells through the Erk-C/EBPβ signaling pathway. We have previously shown that C/EBPβ is a transcription factor activated by LPA which accounts for LPA-induced expression of Cox-2 and sphingosine kinase 1 in various cancer cells (Oyesanya et al 2008, Ramachandran et al 2010). The results in the present work links C/EBPβ to the induction of p21 by LPA in TGFβ growth arrest program in breast and ovarian cancer cells, suggesting a general role for this transcription factor in regulation of LPA target genes. Consistent with their resistance to TGFβ, the stimulatory effect of LPA on p21 was not seen in most breast and ovarian cancer cell lines. The differential effects of LPA on p21 in different cell lines are not fully understood but could be due to distinct expression

patterns of LPA receptors in these cells. The receptor knockdown experiments in the TGFβ-sensitive MDA-MB-231 and Caov-3 cells indicated that both LPA_1 and LPA_2 receptors are required for induction of p21 by LPA. Among the TGFβ-resistant cell lines, SKOV-3 and BT-549 express low levels of LPA_2 (Chen et al 2007, Fang et al 2004) and OVCA-432 exhibits elevated LPA_3 (Fang et al 2004). It is conceivable that co-expression of two or more receptors at appropriate levels is important for optimal induction of p21 by LPA. Alternatively, it is also possible that certain LPA receptors including the conventional LPA_3, novel LPA receptor subtypes and other unknown LPA receptors could be present in the resistant cells and function as negative regulators of certain biological functions of LPA.

CHAPTER 3

THE LYSOPHOSPHATIDIC ACID RECEPTOR 1 IS A NOVEL TARGET GENE OF TRANSFORMING GROWTH FACTOR BETA

3.0 Abstract

LPA_1/Edg2 is the first identified LPA receptor. Although its wide tissue distribution and biological functions have been well studied, little is known about how *LPA_1* is transcriptionally regulated. In this part of my dissertation, I showed that *LPA_1* is a physiological target of transforming growth factor beta (TGFβ)-mediated repression. In both normal and neoplastic cells, TGFβ inhibits *LPA_1* promoter activity, LPA_1 mRNA expression, and LPA_1-dependent chemotaxis and tumor cell invasion. Knockdown of the TGFβ intracellular effector Smad3 or Smad4 with lentivirally transduced shRNA relieved these inhibitory effects of TGFβ. Interestingly, the *LPA_1* promoter contains two potential TGFβ inhibitory elements (TIEs), each consisting of a Smad binding site and an adjacent E2F4/5 element, structurally similar to the TIE found on the promoter of the well-defined TGFβ target gene *c-Myc*. Deletion and point mutation analysis indicate that the distal TIE

located at 401 bp from the transcription initiation site, is required for TGFβ repression of the *LPA$_1$* promoter. A DNA pull-down assay showed that the -401 TIE was capable of binding Samd3 and E2F4/5 in TGFβ-treated cells. The binding of the Smad complex to the native -401 TIE sequence of the *LPA$_1$* gene promoter was further verified by chromatin immunoprecipitation assays. We therefore identified a novel role of TGFβ in the control of *LPA$_1$* expression and LPA_1-coupled biological functions, adding *LPA$_1$* to the list of TGFβ-repressed target genes.

3.1 Introduction

LPA is a naturally occurring intercellular mediator of diverse biological processes including neurogenesis, angiogenesis, wound healing, immunity, and carcinogenesis (Panupinthu et al 2010). LPA is produced by activated platelets during coagulation and thus is a normal constituent of serum (Sano et al 2002). LPA is a ligand of multiple G protein-coupled receptors (GPCRs) (Fukushima and Chun 2001). The LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 receptors are members of the endothelial differentiation gene (Edg) family, sharing 50-57% homology in their amino acid sequences. In addition to the Edg LPA_{1-3} receptors, GPR23/P2Y9/LPA_4 of the purinergic receptor family, the related GPR92/LPA_5, and P2Y5/LPA_6 have been reported to be additional LPA receptors (Fukushima and Chun 2001).

LPA_1 is expressed in most adult tissues and in embryonic cells. Only minor abnormalities such as craniofacial dysmorphism and defective sucking behavior were

found in *lpa$_1$*-deficient mice (Contos et al 2000a). However, more recent studies of *lpa$_1$* null mice subjected to various pathophysiological conditions revealed that LPA_1 is involved in initiation of neuropathic pain (Inoue et al 2004), embryonic and adult neurogenesis and promotion of pulmonary and renal fibrosis (Pradere et al 2007, Tager et al 2008). Some of these biological functions of LPA_1 are attributed to the motility-stimulating activity of LPA in mammalian cells. Substantial evidence indicates that LPA_1 is the primary LPA receptor subtype to mediate LPA-dependent chemotaxis and tumor cell invasion. In contrast to the LPA_2 receptor that is commonly overexpressed in various cancers, gene expression profiling studies failed to show any consensus increase in LPA_1 expression between normal and malignant cells (Hendrix et al 2006, Radvanyi et al 2005, Sanchez-Carbayo et al 2006, Skrzypczak et al 2010, Su et al 2007). Instead, some expression profiling or array analyses suggest decreases in LPA_1 mRNA expression in various malignancies (Hendrix et al 2006, Sanchez-Carbayo et al 2006, Shida et al 2004b, Skrzypczak et al 2010, Su et al 2007).

More intriguingly, several groups recently reported that *LPA$_1$* expression is repressed by Nm23 (Horak et al 2007b, Marshall et al 2010). Nm23 is the first identified metastasis suppressor gene that, by definition, inhibits the process of metastasis but not growth of primary tumors (Marshall et al 2010). In human breast carcinomas, LPA_1 expression inversely correlated with that of Nm23 (Horak et al 2007b). However, little is known about how Nm23 represses LPA_1. Furthermore, there is no evidence that LPA_1 expression is elevated in metastatic cancer compared to primary tumors. Thus, LPA_1 expression is apparently controlled by complex regulatory mechanisms involving other

unrecognized activators or repressors. In the present study, we showed for the first time that TGFβ, a platelet-derived cytokine co-present with LPA in the circulation and tumor microenvironments, represses *LPA$_1$* gene transcription and LPA$_1$-dependent motility-stimulating activity via a TGFβ inhibitory element (TIE) containing both Smad and E2F4/5 binding sites on the *LPA$_1$* gene promoter. These results represent a novel form of crosstalk between TGFβ and LPA signaling.

3.2 Materials and Methods

Materials LPA (1-oleoly, 18:1) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, LPA was dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA) obtained from Roche (Indianapolis, IN). TGFβ was obtained from PeproTech Inc (Rocky Hill, NJ). Anti-Smad3 and Smad4 antibodies were from Abcam (Cambridge, MA). Tubulin α/β antibody was obtained from Cell Signaling (Danvers, MA). Anti-E2F4 antibody was from Santa Cruz Biotech (Santa Cruz, CA). FBS was obtained from Atlanta Biological (Atlanta, GA). All primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). Biotinylated dsDNA were synthesized by IDT (Coralville, IA). TRIzol and cell culture reagents were obtained from Invitrogen Inc. (Carlsbad, CA). Reverse transcription kit, TaqMan gene expression assays, SYBR Green PCR mix and QPCR master mix were obtained from Applied Biosystems (Carlsbad, CA). The transfection reagent LT1 was obtained from Mirus (Madison, WI). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA).

Cell Culture MDA-MB-231 was provided by Dr. S Spiegel (Virginia Commonwealth University) and was maintained in Dulbecco modified Eagle medium (DMEM) with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin). IOSE-29 was originally obtained from Dr. N. Auersperg (University of British Columbia, Canada) and cultured as described previously (Auersperg et al 1994). Primary Mammary epithelial cells (1001-8) and primary ovarian epithelial cells (NOE-71) were provided by Dr. Y. Yu (MD Anderson Cancer Center) and were cultured in HuMEC Ready Medium (Invitrogen) and 50:50 M199/F12 medium with 10% FBS, 20 ng/ml EGF and gentamicin (10 μg/ml), respectively. MCF-10A was provided by Dr. D. Gewirtz (Virginia Commonwealth University) and kept in DMEM/F12 medium with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin and 0.5 μg/ml hydrocortisone. Other cancer cell lines used in the study were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics as we described previously (Fang et al 2004).

Migration and invasion assays Cell migration was measured using the Transwell chambers (Costar, Corning, NY). Transwell were coated with 10 μg/ml Type I collagen and placed in the lower chamber containing serum-free medium supplemented with vehicle, TGFβ, LPA or LPA+TGFβ. Cells suspended in serum-free medium containing 0.01% fatty acid-free BSA were added to the upper chamber at 2×10^4 cells/well. Cells were allowed to migrate for 6 hours at 37℃. Non-migrated cells were removed from the top filter surface with a cotton swab. Migrated cells attached to the underside of the Transwell were washed with PBS and stained with crystal violet and counted under a

microscope. The invasion of tumor cell lines was measured using the growth factor–reduced Matrigel invasion chambers (BD Biosciences, San Jose, CA). The assays were performed as migration assays except that the cells were incubated for 20 hours.

shRNA short hairpin RNA (shRNA)-expressing lentivirus constructs were generated using pLV-RNAi vector (Biosettia, San Diego, CA). The Smad3 target sequences (Smad3sh1 GTGA-CCACCAGATGAACCA (Blount et al 2008), Smad3sh2 GGATTGAGCTGCACCTGAATG (Jazag et al 2005) and Smad4 target sequences (Smad4sh1 GCAGGTGGCTGGTCGGAAA (Giampieri et al 2009), Smad4sh2 GCCAGCTACTTACCATCATA (Deckers et al 2006)) were inserted to the pLV-RNAi plasmid following the manufacturer's protocol. The LPA_1 shRNA plasmids were obtained from Dr. S. Huang (Medical College of Georgia). The shRNA lentiviruses were propagated in 293FT cells. The culture supernatants were used to infect cancer cell lines. The GFP-positive cells were sorted out using flow cytometer proximately a week after virus infection.

Quantitative PCR (qPCR) Total cellular RNA was isolated using Trizol (Invitrogen). Complementary DNA (cDNA) was synthesized from RNA (1 μg, random primers) using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The relative levels of individual LPA receptors were determined using gene specific probes, the TaqMan Universal PCR Master Mix and the 7900HT Prism Real-Time PCR System

(Applied Biosystems).

Luciferase vectors, deletion, and site-directed mutagenesis The luciferase reporter vector pGL2-LPA_1-Luc containing -1156 to +86 was generated by PCR amplification of the *LPA_1* promoter sequence (forward 5'-GCACTCGAGTGCAAAGCT-ACACTGGGAAA-3', reverse 5'-GCAAAGCTTCACACTCTCACTGGCACTCG-3'). The PCR product was inserted into pGL2-Basic-Luc at *XhoI* and *HindIII* sites. The deletion mutant (-366 to +86) was made by PCR amplification of the fragment from pGL2-LPA_1-Luc (forward 5'-GCACTCGAGCTGACGCTCCCTGAGTGG-3', reverse 5'-GCAAAGCTTCACACTCTC-ACTGGCACTCG-3') and re-inserted into the pGL2-Basic-Luc at the *XhoI* and *HindIII* sites. The promoter sequences in these plasmids were verified by automatic sequencing. The -401 and -40 TIE consensus sites within pGL-LPA_1-Luc were converted into inactive sequences by site-directed mutagenesis. The wild type -401 TIE 5'-GGCTTTGGCGCG and wide type -40 TIE 5'-GGCTTCGCGC were converted into 5'-GGCT*AATT*CGCGC and 5'-GGC*AATT*CGCC, respectively. For luciferase assays, MDA-MB-231 and SKOV-3 were transfected with luciferase vectors using TransIT-LT1 (Mirus Bio). About 48 to 60 hours after transfection, the cells were treated with TGFβ or vehicle for 16-20 hours. Cell extracts were prepared and assayed for luciferase activity using the luciferase assay kit from Promega.

DNA pull-down assay Lysates of MDA-MB-231 and SKOV-3 cells were prepared by

brief sonication in the HKMG buffer (10 mM, Tris-HCl, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.1% NP40 and protease inhibitors) using the Fisher Scientific Sonic Dismembrator Model 100, followed by 10 minutes of centrifugation at 12,000 $\times g$ at 4 ℃. Cellular proteins (400 μg) were incubated with 4 μg of biotinylated double-stranded oligonucleotides (5'-CCCTACTGCCCGGCTTTGGCGCGCTGG-CAGGAGGAG–biotin) for 16 hours at 4 ℃. The M-280 Streptavidin Dynabeads (Invitrogen) (30 μl) were added to each sample and incubated for another hour at 4 ℃. The Dynabeads were washed three times with PBS before western analysis of Smad3 or E2F4.

Chromatin immunoprecipitation (ChIP) assay TGFβ or vehicle-treated MDA-MB-231 and SKOV-3 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The cells were lysed for 10 minutes in ice-cold lysis buffer (5 mM HEPES, pH 8.0, 80 mM KCl, 1% NP40 and protease inhibitors). The nuclear fraction that was recovered by centrifugation (5 minutes at 5000 $\times g$) was resuspended in a ChIP assay buffer (50 mM HEPES, pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors) and sonicated on ice to achieve an average chromatin length of 200-1000 bp. The sonicated samples were pre-cleared by incubation with Protein G Dynabeads (Invitrogen). The material recovered from the equivalent of 10^6 cells was incubated for 16 hours at 4 ℃ with 2 μg of either normal rabbit IgG (Santa Cruz), anti-Smad3, Smad4 or E2F4 antibodies. Protein G Dynabeads were added and incubated for 2 hours. The DNA-

protein-beads mixes were washed sequentially once with a low salt buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton 100), once with a high salt buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton 100), once with LiCl buffer (10 mM Tris-HCl, pH 8.0, 0,25 M LiCl, 1 mM EDTA, 1% deoxycholate, 1% NP-40), and finally twice with TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The specifically bound complexes were eluted from the Protein G Dynabeads by incubation twice for 15 minutes at 65 ℃ with TE elution buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 1% SDS). The immunoprecipitated complexes and the starting material (input) were incubated overnight at 65 ℃ to reverse crosslinking, then treated with RNase A followed by proteinase K and purified using the QIAquick Spin Columns (Qiagen, Valencia, CA). The DNA samples were recovered in 100 µL H_2O, and analyzed by qPCR using SYBR Green. Details of the primer used for qPCR were listed in Table 3.1.

Table 3.1. Primers used in ChIP assays

-401 Forward	5'-GTGCTACGTGGAACAAGCAG-3'
-401 Reverse	5'-GGCGGGACAGTGTGAGC-3'
-40 Forward	5'-AGCGAGCGCAGGTAAGG-3'
-40 Reverse	5'-GCACCCACACTCTCACTGG-3'
c-Myc TIE Forward	5'-TTATAATGCGAGGGTCTGGA-3'
c-Myc TIE Reverse	5'-TGCCTCTCGCTGGAATTACT-3'

Statistics All numerical data were presented as mean ± SD from at least 3 independent experiments. The statistical significance of differences was analyzed using Student's *t* test where $p<0.05$ was considered statistically significant.

3.3 Results

3.3.1 TGFβ inhibits expression of LPA_1

Previous studies showed that LPA stimulates production and release of TGFβ (Xu et al 2009), transactivates the intracellular effectors of TGFβ (Sauer et al 2004) or cooperates with TGFβ to regulate gene expression (Wu et al 2011). However, little is known about whether TGFβ communicates with LPA signal transduction to modify cellular responses to the multi-functional LPA. To explore this possibility, we treated the MDA-MB-231 breast carcinoma cell line and the SKOV-3 ovarian carcinoma cell line with TGFβ for 3 or 6 hours, and monitored changes in mRNA expression of LPA signaling molecules including various LPA receptors. Interestingly, the treatment resulted in 67% and 48% decreases in LPA_1 mRNA levels in MDA-MB-231 and SKOV-3, respectively, as analyzed by RT and qPCR (Fig. 3.1A). TGFβ did not decrease expression of other known LPA receptors in these cells (Fig. 3.1B)

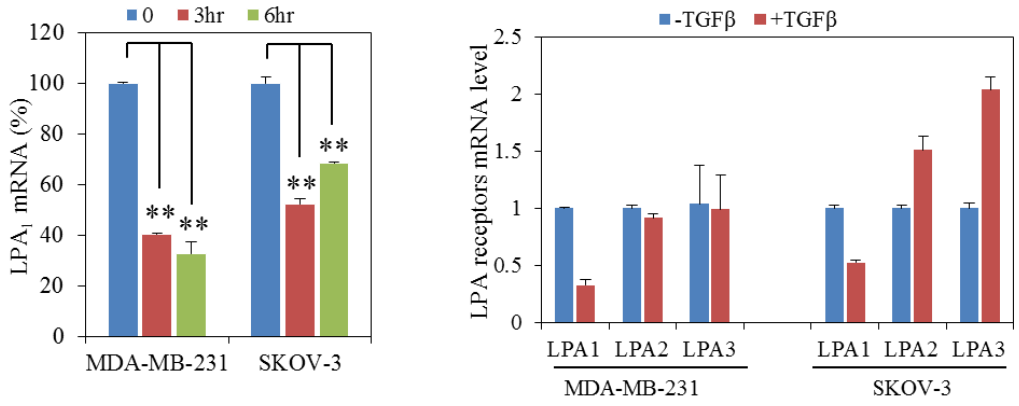

Figure 3.1. TGFβ inhibits expression of LPA_1 mRNA**. *A.*** MDA-MB-231 and SKOV-3 cells were cultured with TGFβ (2.5 ng/ml) for 3 and 6 hours. LPA_1 mRNA levels were examined by RT and qPCR as described in Materials and Methods. The mRNA levels of LPA_1 in TGFβ treated cells were presented as percentages relative to those in untreated control cells (defined as 100%). ***B.*** MDA-MB-231 and SKOV-3 cells were treated with TGFβ (2.5 ng/ml) for 6 hours, LPA_1, LPA_2 and LPA_3 mRNA levels were examined using specific TaqMan probes as described in ***A.***

To generalize the observation of the inhibitory effect of TGFβ on LPA_1, we examined a panel of breast, ovarian and other cancer cell lines, including BT-549, Caov-3 and DOV-13. Treatment with TGFβ induced 30-67% decreases in LPA_1 mRNA levels in these cell lines (Fig. 3.2A). Most of the cancer cell lines such as SK-BR-3, BT-549, SKOV-3 and DOV-13 were resistant to the growth inhibitory effect of TGFβ as we reported recently (Wu et al 2011). Thus, the inhibition of LPA_1 expression by TGFβ was independent of the cytostatic program of TGFβ. In addition, TGFβ also downregulated expression of LPA_1 in normal primary and immortalized epithelial cells such as primary

mammary epithelial cells (1001-8), primary ovarian epithelial cells (NOE-71), immortalized breast epithelial cell line MCF-10A, and immortalized ovarian surface epithelial cell line IOSE-29 (Fig. 3.2A). The only exception to the negative regulation by TGFβ was the DLD1 colon cell line. DLD1 was deficient in TβRII as reported previously (Markowitz et al 1995) and as evidenced by the inability of TGFβ to induce Smad3 phosphorylation in this particular line (Fig. 3.2B). It is also worth noting that LPA_1 was highly expressed in DLD1 cells (Lee et al 2008, Shida et al 2003), likely as a result of the absence of TGFβ-mediated repression.

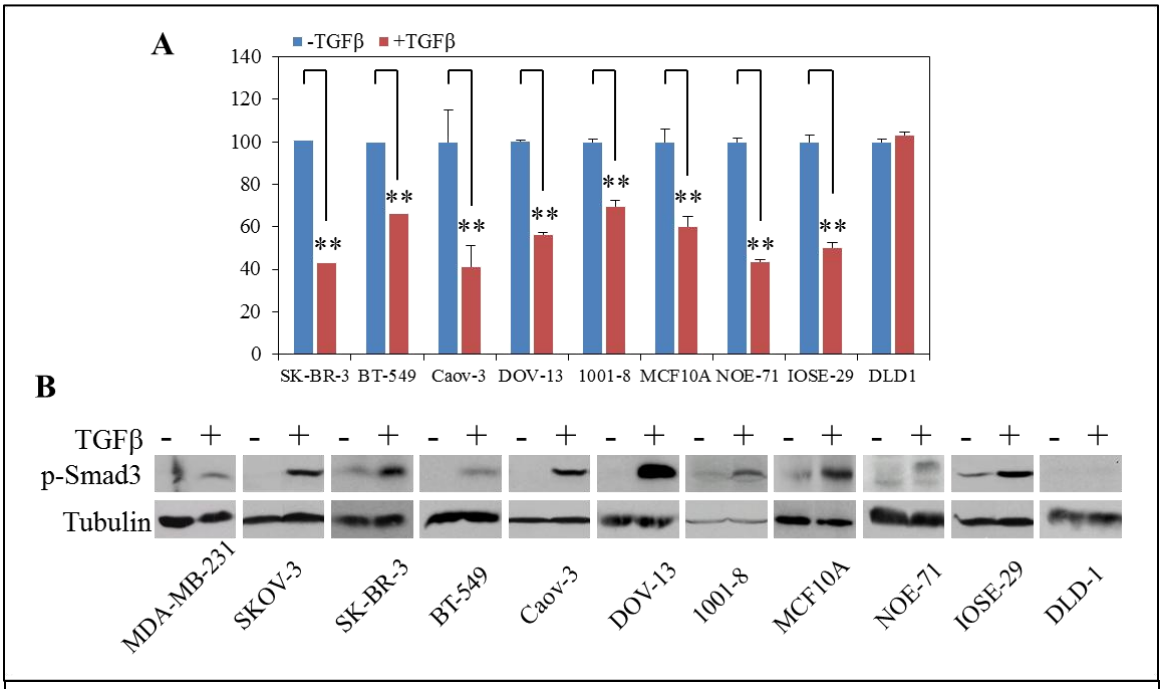

Figure 3.2. TGFβ inhibits expression of LPA_1 mRNA in all cancer cells with intact TGFβ pathway. ***A.*** Multiple cancer cell lines, immortalized breast (MCF-10A) and ovarian (IOSE-29) epithelial cell lines, primary mammary (1001-8) and ovarian (NOE71) epithelial cells were treated for 6 hours with TGFβ (2.5 ng/ml) and analyzed for LPA_1 mRNA expression as in ***Fig.3.1***. ***C***. All cell lines and primary cells were treated with TGFβ (2.5 ng/ml) for 1 hour before lysis with SDS sample buffer and immunoblotting analysis of Smad3 phosphorylated at Ser423/425.

3.3.2 TGFβ attenuates LPA_1-dependent cell migration and invasion

Since TGFβ represses LPA_1 expression, we anticipated that TGFβ would attenuate LPA_1-dependent actions of LPA. Although each of the Edg-family LPA receptors may contribute to promoting cell motility in certain cellular contexts, substantial evidence supports an essential and probably sufficient role for LPA_1 in driving random migration, chemotaxis and tumor cell invasion (Hama et al 2004, Shida et al 2003, Van Leeuwen et al 2003). In breast and ovarian cancer cell lines that we examined (MDA-MB-231, SKOV-3, and DOV-13), LPA stimulated a robust chemotactic response as analyzed by the transwell assay (Fig. 3.3A). LPA also promoted invasion of these cells through Matrigel (Fig. 3.3B).

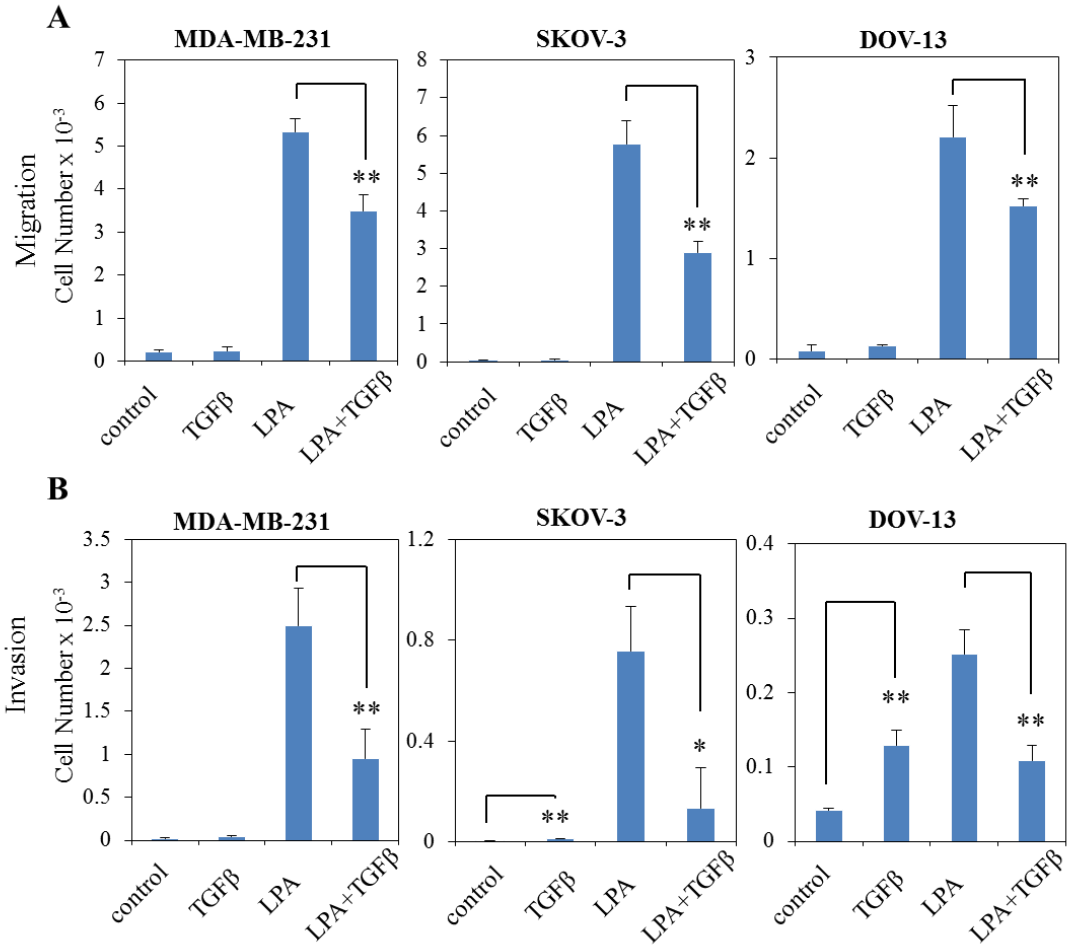

Figure 3.3. TGFβ attenuates LPA_1-dependent cell migration and invasion. ***A***. The chemotactic responses to TGFβ (2.5 ng/ml), LPA (5 μM), or LPA+TGFβ in the breast cancer cell line MDA-MB-231, and the ovarian cancer cell lines SKOV-3 and DOV-13 were measured by transwell chambers. The cells (2×10^4 cells/well) were loaded to the upper wells and allowed to migrate for 6 hours. The migrated cells on the underside of the Transwell were stained with crystal violet, counted under a microscope and presented as numbers of cells/well. ***B***. Cell invasion induced by TGFβ (2.5 ng/ml), LPA (5 μM), or LPA+TGFβ in MDA-MB-231, SKOV-3 and DOV-13 cells was measured with the growth factor–reduced Matrigel invasion chambers. The experiment was performed as the migration assay in ***A*** except that the cells were allowed to invade for 20 hours.

In agreement with the crucial role of LPA_1 in stimulation of cell motility, shRNA knockdown of the LPA_1 receptor expression or pharmacological inhibition of LPA_1 with Ki16425 blocked LPA-induced chemotaxis (Fig. 3.4A and B). On the other hand, TGFβ only weakly increased migration of MDA-MB-231, SKOV-3 and DOV-13 cells (Fig. 3.3). This trend of increase in chemotactic migration towards TGFβ was not statistically significant. However, TGFβ was capable of stimulating modest but significant increases in invasion of SKOV-3 and DOV-13 cells (Fig. 3.3B). The ability of TGFβ to stimulate invasion of breast and ovarian cancer cell lines was much weaker than that of the potent motogen LPA.

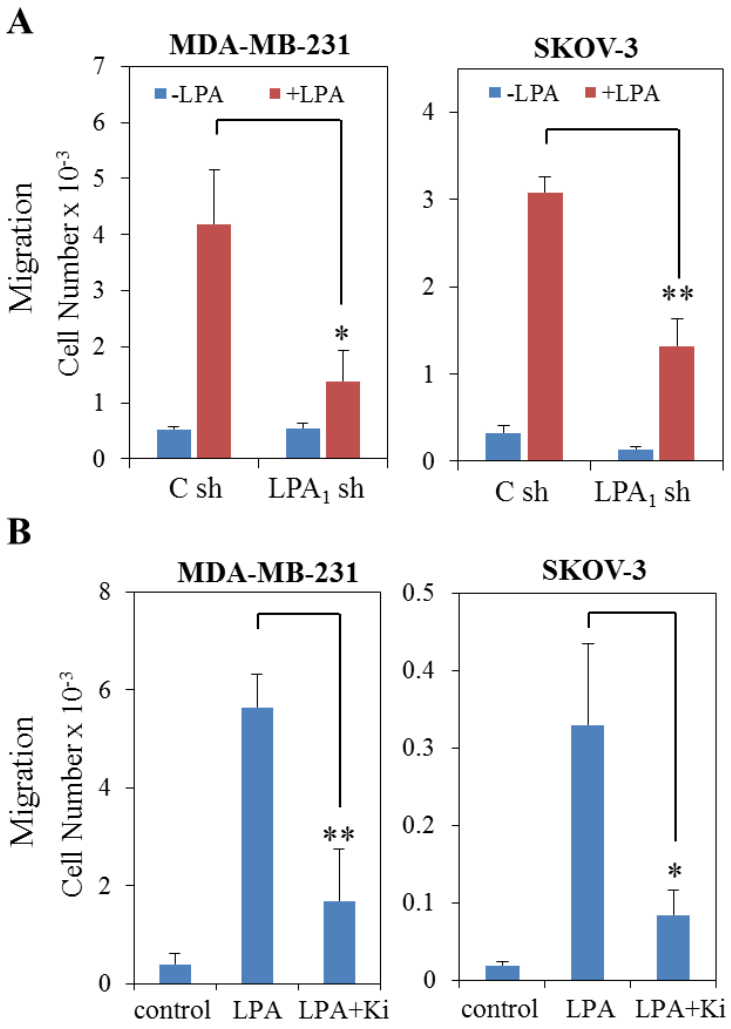

Figure 3.4. Important role of LPA_1 in mediating LPA induced cell migration. ***A***. Expression of LPA_1 in MDA-MB-231 and SKOV-3 cells was silenced with lentivirally transduced shRNA. The chemotactic migration of these stable knockdown cells and control cells induced by LPA (5 μM) was analyzed as described in ***Figure 3.3A***. ***D***. LPA-induced migration of MDA-MB-231 and SKOV-3 cells was analyzed in the presence or absence of the LPA_1/LPA_3 specific antagonist Ki16425 (Ki) (10 μM).

When MDA-MB-231, SKOV-3 and Dov-13 cells were co-stimulated with both LPA and TGFβ, TGFβ significantly inhibited LPA stimulation of migration and invasion. We observed 30-50% decreases in migration and 60-80% decreases in invasion in the presence of LPA and TGFβ compared to the effects of LPA alone. Moreover, when pre-treated MDA-MB-231 and SKOV-3 cells with 2.5 ng/ml TGFβ for 6 hours to achieve the downregulation of LPA_1, followed by migration (Fig. 3.5A) and invasion (Fig. 3.5B) assay only using LPA as motogen, we observed similar reductions in cell migration and invasion comparing with vehicle treated control cells. In all cell lines we examined, the TGFβ mediated inhibition of invasion was more prominent than the effect of TGFβ on migration. This was likely due to the longer incubation of the cells with TGFβ during the invasion experiments. These data demonstrated that TGFβ repression of LPA_1 expression was sufficient to impair LPA_1-dependent cell migration and invasion.

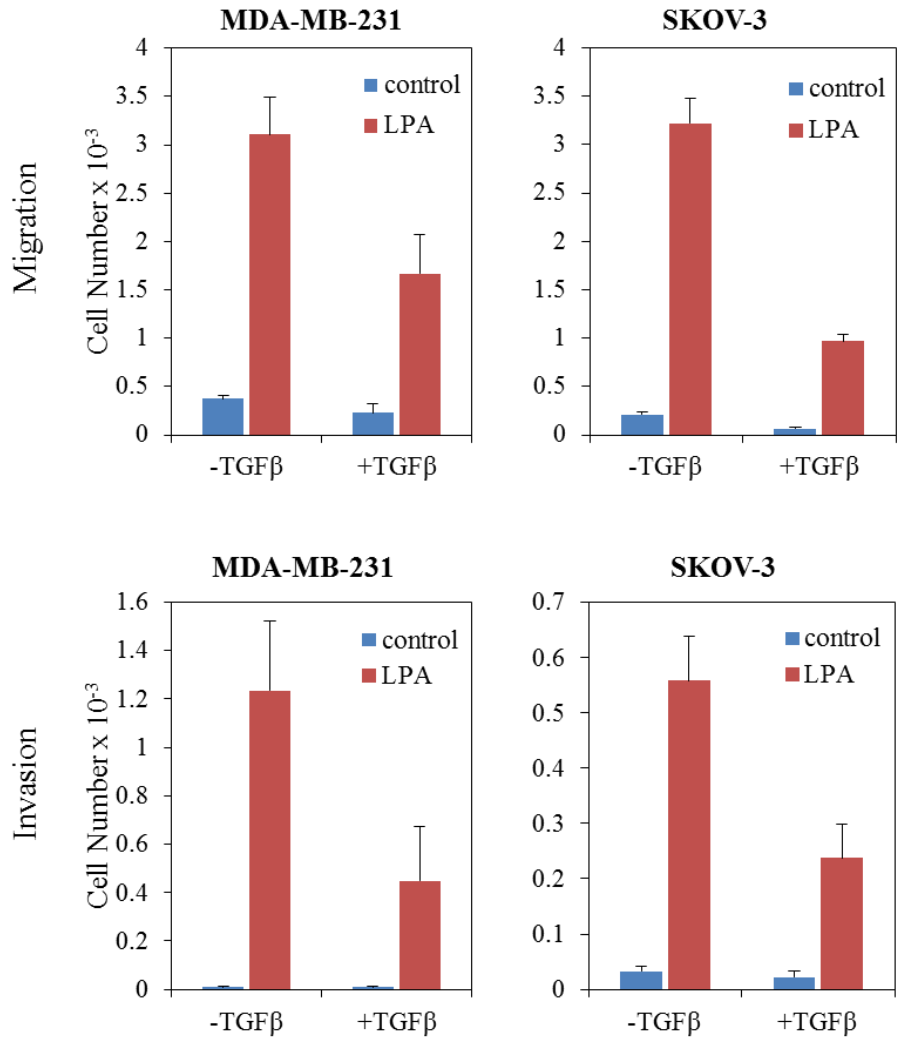

Figure 3.5. TGFβ pre-treatment attenuates LPA_1-dependent cell migration and invasion. ***A***. MDA-MB-231 and SKOV-3 cells were treated with TGFβ (2.5 ng/ml) for 6 hours. LPA (5 μM) was used to induce migration. Migration assays were performed as described in ***Figure 3.3. B***. TGFβ (2.5 ng/ml) pre-treated cell invasion induced by LPA (5 μM) in MDA-MB-231 and SKOV-3 was measured with the growth factor–reduced Matrigel invasion chambers. The experiment was performed as the migration assay in ***A*** except that the cells were allowed to invade for 20 hours.

3.3.3 TGFβ represses LPA_1 expression and LPA_1-dependent cell migration and invasion in a Smad-dependent manner

Upon binding of TGFβ to its receptors, both Smad-dependent and Smad-independent pathways are activated by the kinase activity of TβRs (Derynck and Zhang 2003). Regulatory Smads (RSmads), such as Smad2 and Smad3, are phosphorylated by TβRs, and form heterodimers with the co-Smad, Smad4, to translocate to the nucleus where the Smad complex regulates transcription of target genes (Massague et al 2005). In addition, TGFβ activates TβR-associated proteins and other intracellular signaling pathways such as MAPK, PP2A/p70S6K, RhoA and TAK1/MEKK1 to elicit Smad-independent responses to TGFβ (Seoane 2006). To elucidate the mechanism underlying TGFβ repression of LPA_1, we examined the possibility for the participation of the Smad-dependent pathway in the process. To this end, we first knocked down Smad3 expression in MDA-MB-231 and SKOV-3 cells using lentivirally transduced shRNA. Smad3, but not Smad2, was reported to be the R-Smad involved in binding to TIE to downregulate TGFβ target genes, most notably *c-Myc* (Chen et al 2002). Expression of Smad3 protein was efficiently silenced by Smad3 shRNA in these cell lines (Fig. 3.6A). The silencing of Smad3 expression eliminated the inhibitory effect of TGFβ on expression of LPA_1 (Fig. 3.6B).

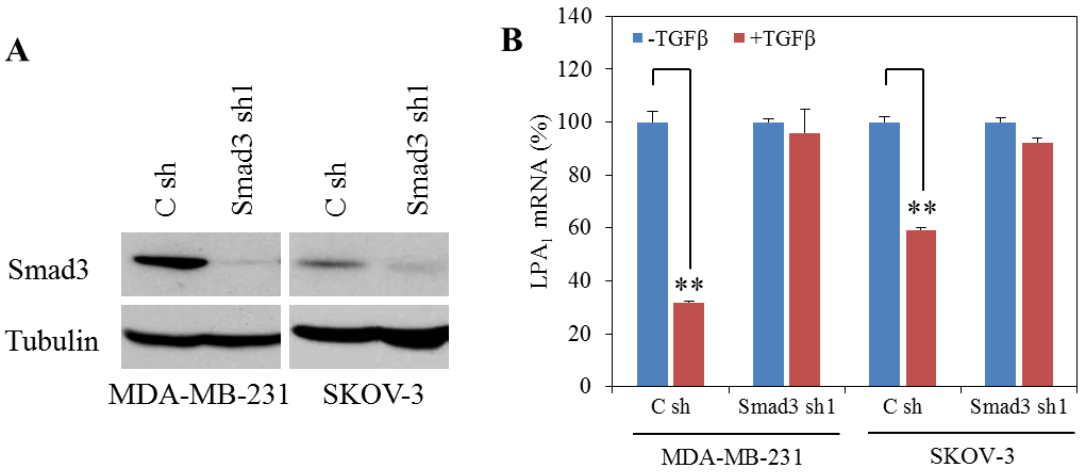

Figure 3.6. TGFβ represses LPA_1 in a Smad3-dependent manner. ***A***. Expression of Smad3 in MDA-MB-231 and SKOV-3 cells was silenced with lentivirally transduced shRNA. The Smad3 protein levels in shRNA knockdown cells and control shRNA transduced cells were examined by immunoblotting. ***B***. Smad3 shRNA and control shRNA-transduced MDA-MB-231 and SKOV-3 cells were treated with or without TGFβ (2.5 ng/ml) for 6 hours prior to RT and qPCR analysis of LPA_1 mRNA levels.

To determine the role of Smad3 in TGFβ repression of LPA-driven cell motility, we performed migration and invasion assays in these cells. In Smad3 knockdown cells, TGFβ no longer inhibited LPA-driven cell migration (Fig. 3.7A) or invasion (Fig. 3.7B). These results suggest a Smad3-dependent mechanism to control LPA_1 expression and LPA_1-linked migration and invasion. In further support of this, Smad3 knockdown was accompanied by considerable increases in basal LPA_1 mRNA levels (Fig. 3.6B) as well as in basal and LPA-induced cell migration and invasion (Fig. 3.7A and B). Likewise, shRNA knockdown of the co-Smad, Smad4 in these cells abrogated the inhibitory effects of TGFβ on LPA_1 mRNA expression and LPA_1-dependent cell migration (Fig. 3.8).

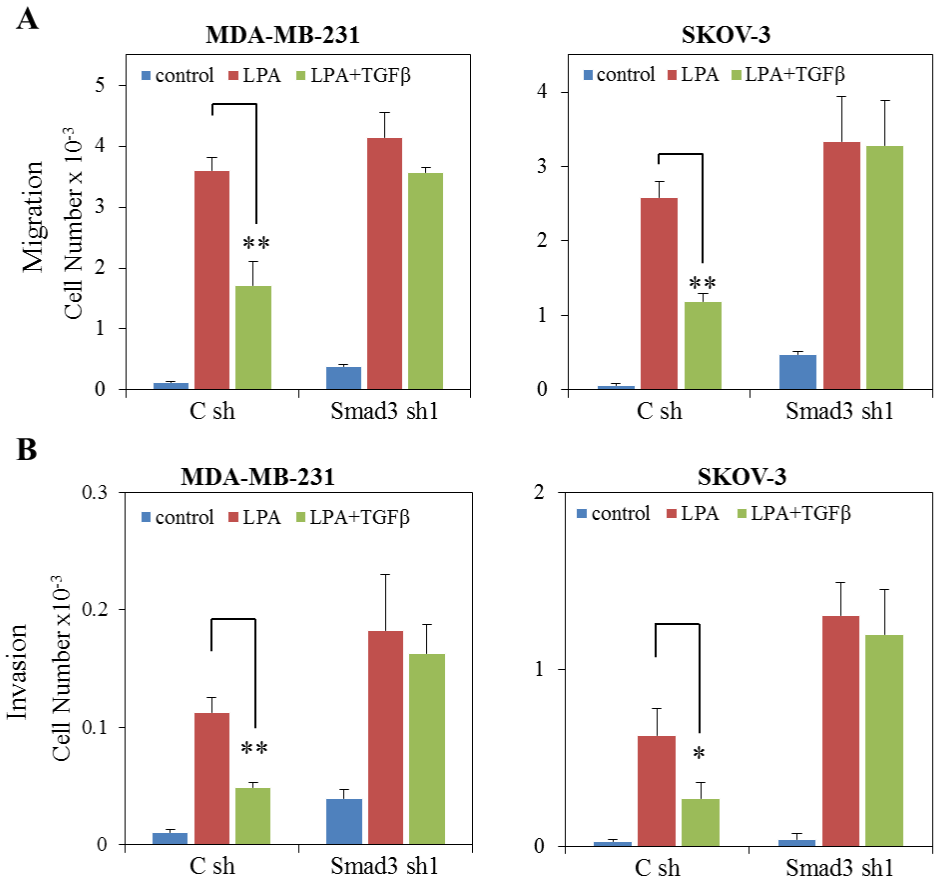

Figure 3.7. Knockdown of Smad3 abolished the inhibitory effect of TGFβ in LPA-induced cell motility. LPA-mediated chemotactic migration (***A***) and invasion (***B***) of control and Smad3 knockdown MDA-MB-231 and SKOV-3 cells were analyzed in the absence or presence of TGFβ (2.5 ng/ml). Data was presented as described in ***Fig 3.3.***

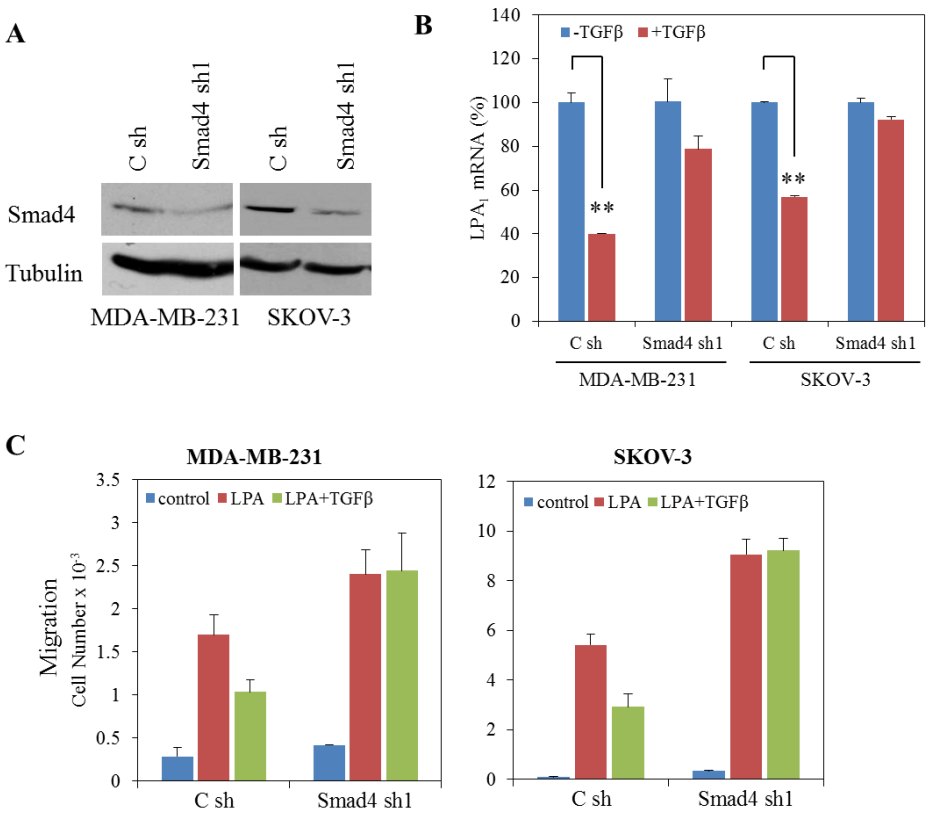

Figure 3.8. Knockdown of Smad4 abolished the inhibitory effect of TGFβ in LPA-induced cell motility. ***A.*** Smad4 knockdown efficiency was analyzed with immunoblotting. ***B***. Smad4 shRNA and control shRNA-transduced cells were treated with or without TGFβ (2.5 ng/ml) for 6 hours prior to RT and qPCR analysis of LPA_1 mRNA levels. ***C.*** LPA-mediated chemotactic migration of control and Smad4 knockdown cells were analyzed in the absence or presence of TGFβ (2.5 ng/ml). Data was presented as described in Fig 3.3.

3.3.4 The LPA_1 gene promoter contains two potential TIEs

The TGFβ-Smad pathway both activates and represses gene transcription. There is a long list of TGFβ activated targets such as type I collagen and the cyclin-dependent kinase inhibitors $p21^{Waf1}$ and $p15^{Ink4b}$. Conversely, only a few TGFβ-repressed genes have been well defined with c-Myc and Id1 being the best characterized. As mentioned in

Chapter 1, the co-repressors for two TGFβ target genes *c-Myc* and *Id1* have been identified. In the case of c-Myc, Smad3 forms a complex with E2F4/5-p107 in the cytosol and translocates into nucleus with Smad4 when Smad3 is phosphorylated by the active TβRs (Chen et al 2002). The complex binds to a Smad binding element right next to an E2F binding site. For inhibition of Id1, the co-repressor ATF3 binds with Smads in the nucleus, consequently the repressor complex binds to a Smad binding element adjacent to a CREB binding site (Kang et al 2003).

Analysis of the human *LPA$_1$* gene promoter sequences revealed the presence of two potential TIEs, one located at -401 (designated -401 TIE) and the other at -40 (designated -40 TIE) from the transcription initiation site (see sequence details in Fig. 3.9A). The composite TIE consisting of Smad and E2F4/5 binding sites was present only in the *LPA$_1$* gene promoter but not in the promoters of other LPA receptors (LPA$_{2\text{-}6}$). Between these two TIEs, there are also an SBE (-324 GTCT -321) and a possible ATF site (-348 TGACGCTC -341) with 5 out of 8 nucleotides matching with the ATF consensus sequence (TGACGTCA).

3.3.4 TGFβ represses the transcriptional activity of the LPA$_1$ gene promoter

In an effort to identify the co-repressor for the TGFβ-Smad pathway to control LPA$_1$ expression, we cloned a 1242-bp fragment of the *LPA$_1$* gene promoter (-1156 to +86) into pGL2-Basic-Luc vector to construct pGL2-LPA$_1$-Luc. MDA-MB-231 and SKOV-3 cells were transfected with pGL2-LPA$_1$-Luc and cultured with or without TGFβ for 16 hours before measurement of luciferase activity in cell lysates. TGFβ treatment

resulted in a modest but consistent decrease in luciferase activity (Fig. 3.9B). Deletion of the proximal -401 TIE (named del in Fig. 3.9B) at -366 abolished the negative effect of TGFβ on the *LPA$_1$* promoter-driven luciferase activity (Fig. 3.9B), suggesting that the deleted sequence containing the -401 TIE rather than the potential SBE-ATF3 or the further downstream -40 TIE is the major site for TGFβ repression of *LPA$_1$* transcription. Indeed, similar to the deletion mutant, point mutation of the -401 TIE (GGCTTTGGCGCG to GGCTAATTCGCGC) also eliminated the repressive effect of TGFβ on the *LPA$_1$* promoter activity. However, mutation of the -40 TIE (GGCTTCGCGCC to GGCAATTCGCC) only slightly reduced the effect of TGFβ, which was statistically insignificant. Taken together, these experiments indicate that the -401 TIE site is required for TGFβ-Smad mediated repression of the *LPA$_1$* gene.

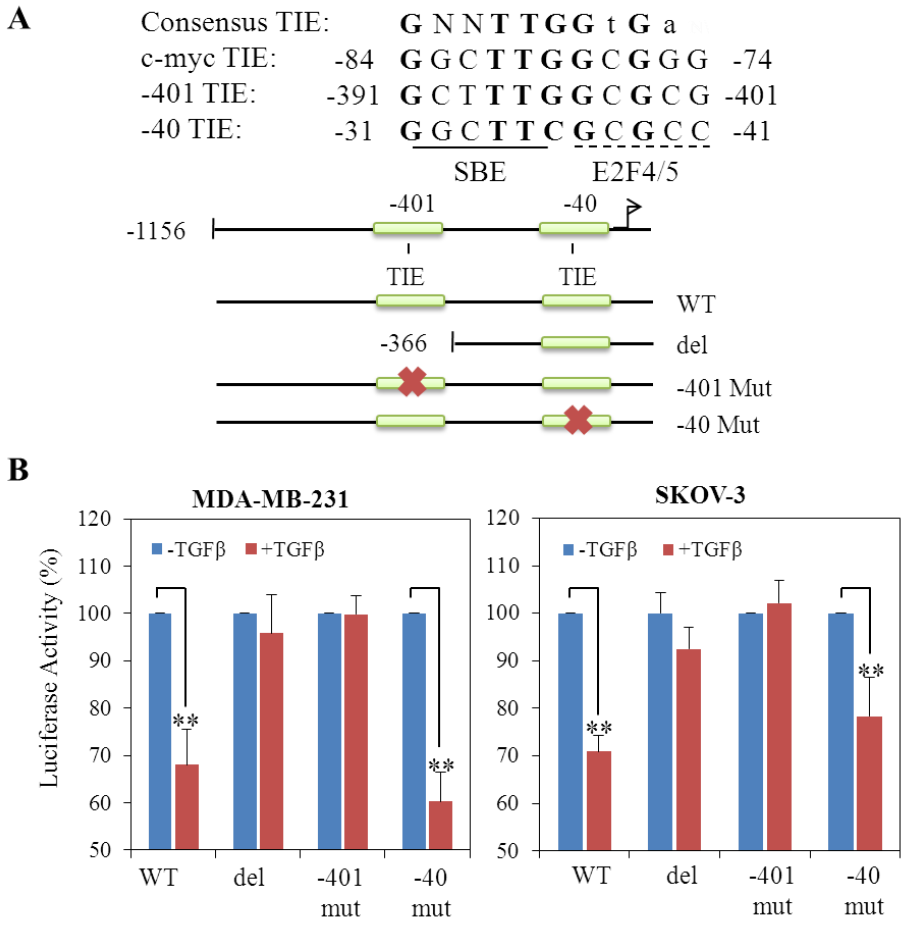

Figure 3.9. TGFβ represses the transcriptional activity of the *LPA$_1$* gene promoter containing TIEs. ***A.*** DNA sequences of two potential TIEs of the human *LPA$_1$* promoter were compared with that of the *c-myc* TIE (***A, upper panel***). The potential Smad and E2F4/5 binding sites are underlined. Lower case in the TIE consensus sequence suggests for preferred nucleotide. The *LPA$_1$* promoter (-1156 to +86) was cloned into pGL2-Basic-Luc to constructed the pGL2-LPA$_1$-Luc luciferase reporter (WT) (***A, lower panel***). The deletion (del) and point mutations of each TIE (-401 Mut and -40 Mut) were made as described in Materials and Methods. ***B***. MDA-MB-231 and SKOV-3 cells were transfected with the indicated plasmids and cultured with or without TGFβ for 16 hours before luciferase activities were determined. The results were presented as percentages relative to the values of the cells cultured without TGFβ (defined as 100%).

3.3.5 Smad complex binds to the -401 TIE of the *LPA$_1$* promoter

To determine whether the Smad complex binds to the *LPA$_1$* promoter at the -401 TIE, we performed a DNA pull-down assay using biotinylated double-stranded oligonucleotides corresponding to the sequences between -413 and -378 that included the -401 TIE of the *LPA$_1$* promoter. MDA-MB-231 and SKOV-3 cells were treated for 1 hour with TGFβ or vehicle. The 36-bp DNA fragment was incubated with cell lysates to allow binding and precipitating Smad3, Smad4 and E2F4 as detailed in Materials and Methods. As demonstrated in Fig. 3.10, co-precipitated Smad3, Smad4 and E2F4 were detected from TGFβ-treated cells but not from vehicle-treated control cells, suggesting that the 36-bp DNA fragment is capable of binding active Smad3 and E2F4.

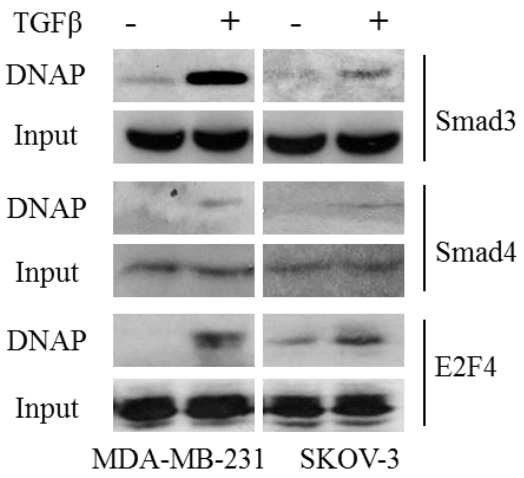

Figure 3.10. TGFβ induces occupancy of the Smad complex to the LPA_1 gene promoter. Cell extracts from MDA-MB-231 and SKOV-3 cells untreated or treated with TGFβ (2.5 ng/ml) for 1 hour were incubated with biotinylated DNA fragment containing the -401 TIE and strepatavidin beads. The DNA precipitates (DNAP) were subjected to western blot analysis for Smad3 and E2F4. Whole cell lysates was included in western blots (WCL) as input.

To determine if TGFβ induces Smad3, Smad4 and E2F4 binding to the native -401 TIE region of the *LPA$_1$* promoter, we performed ChIP assays in MDA-MB-231 and SKOV-3 cells. The efficiency of anti-Smad3, anti-Smad4 or anti-E2F4 antibody to precipitate cellular Smad3, Smad4 or E2F4 was illustrated in Fig. 3.11A. qPCR analysis of Smad3 immunoprecipitates from MDA-MB-231 and SKOV-3 cells showed that TGFβ induced 3.8- and 3.7-fold increases in Smad3 binding as well as 21.3-fold and 3.9-fold increase inSmad4 binding to the -401 TIE (Fig. 3.11B). We also observed increases (2.0 and 1.8 fold) in Smad3 binding to the -40 TIE in MDA-MB-231 and SKOV-3, respectively, however there was no significant increase of Smad4 binding to -40 TIE. Thus TGFβ induced physical binding of activated Smad3 and Smad4 to the -401 TIE and to a lesser extent, to the -40 TIE of the *LPA$_1$* promoter. The binding of E2F4, another partner of the Smad complex, to the -401 TIE also increased to 2.5 and 2.7 fold following TGFβ treatment of MDA-MB-231 and SKOV-3 cells. However, no significant increase in binding of E2F4 to the -40 TIE in TGFβ-treated MDA-MB-231 cells was observed. In these ChIP experiments, the binding of Smad3, Smad4 and E2F4 to the *c-Myc* TIE sequences in SKOV-3 cells was included as internal positive controls. It has been reported no TGFβ-mediated repression of c-Myc was found in MDA-MB-231 cells (Chen et al 2001); therefore, we used binding of Smad3 and Smad4 to PAI-1 SBE as the positive control. In summary, these experiments provide mechanistic insight into the TGFβ-mediated repression of *LPA$_1$* transcription and LPA$_1$-linked biological activities.

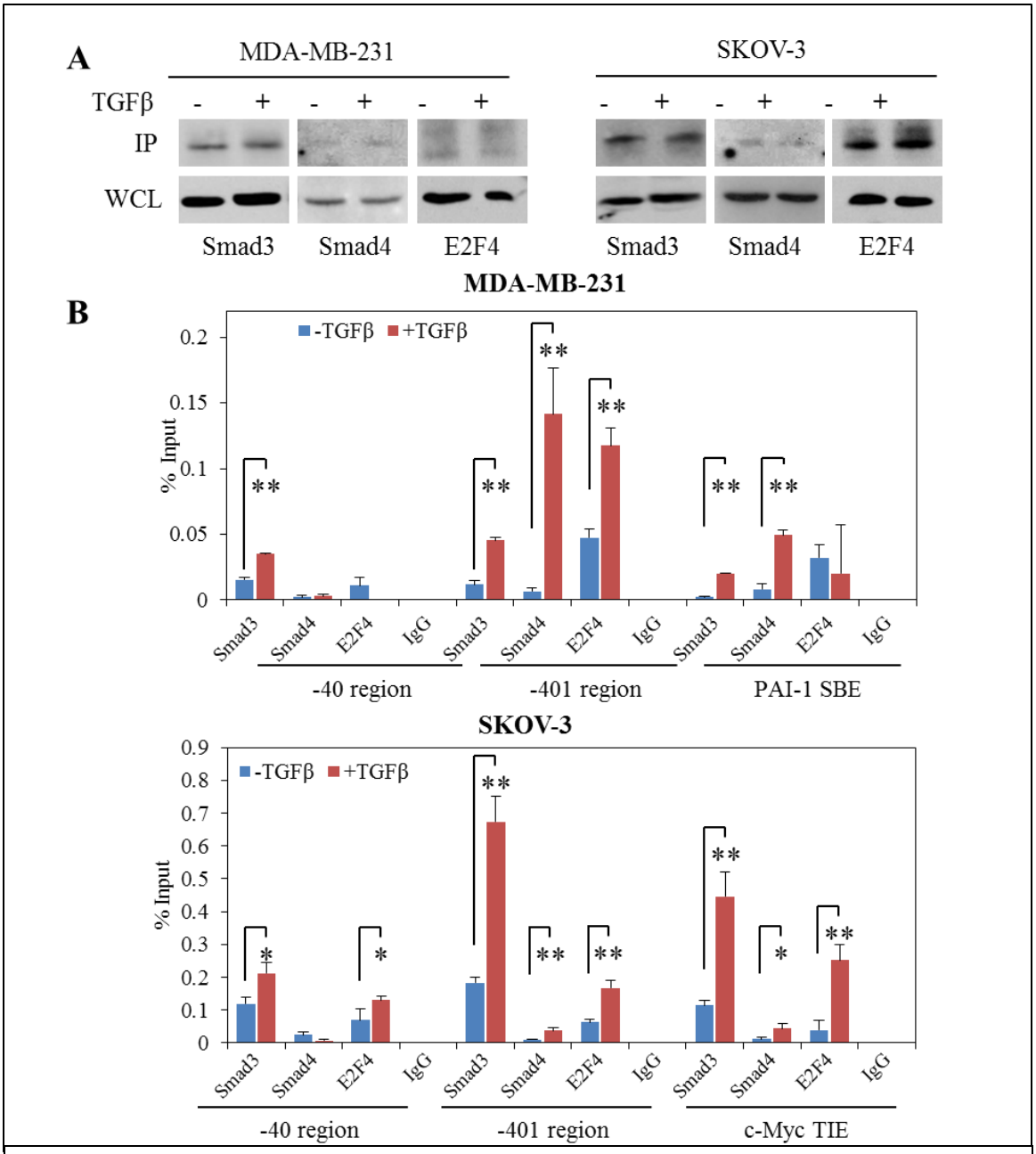

Figure 3.11. TGFβ induces occupancy of the Smad complex to the LPA_1 gene promoter. ChIP assays were performed to examine the binding of Smad3 and E2F4 to the -40 and -401 TIEs of the *LPA_1* promoter and to the *c-myc* TIE (positive controls). The immunoprecipitation of Smad3 and E2F4 was verified by western blotting analysis of immunoprecipitates (IP) and cell lysates (WCL). The binding was quantitated by qPCR using SYBR Green and the specific primers listed in Table 1. The results were normalized to the Ct values of inputs and presented as percentages of inputs. Blank: not detectable.

3.4 Discussion

In the present study, we showed that the *LPA$_1$* gene is a target of TGFβ-mediated repression. This inhibitory effect of TGFβ on LPA_1 expression is detected in both normal and neoplastic cells with intact TβRs and Smad signaling. Importantly, the inhibition of LPA_1 by TGFβ is sufficient to suppress the LPA_1-dependent migratory response to LPA. The detailed analysis of the underlying mechanism indicates that TGFβ triggers downregulation of LPA_1 through activation of Smad and binding of the Smads-E2F4 complex to the -401 TIE of the *LPA$_1$* gene promoter, a process analogous to the well-defined mode of repression of c-Myc by TGFβ (Chen et al 2002).

Among the multiple LPA receptors, LPA_1 is the only receptor subtype transcriptionally repressed by the TGFβ-Smad signaling. In TGFβ-challenged cells, Smad3 forms a large complex with E2F4/5-p107 and Smad4 in the cytoplasm, translocates to the nucleus and binds to the TIE motif where the complex recruits HDAC and silences gene expression (Li et al 1997). Hence both Smad binding site and the conjugated E2F4/5 element are instrumental to TGFβ repression of target genes (Chen et al 2002). Extensive analysis of the promoter sequences of other LPA receptors does not reveal TIE consensus sequence or SBEs in the *LPA$_4$* and *LPA$_5$* promoters. There are putative SBEs in the *LPA$_2$*, *LPA$_3$*, and *LPA$_6$* promoter sequences. However, none of these SBEs is closely linked to a nearby E2F4/5 binding site or to an ATF3 site. It is intriguing that the two TIE sites of the *LPA$_1$* gene promoter do not function equally. The -401 TIE was identified to be the major one for Smad-mediated repression of LPA_1 while the contribution of the -40 TIE was negligible. This difference could be attributed to the fact

that only 4 out of 11 nucleotides match with the consensus E2F4/5 sequence at the -40 TIE while the -401 TIE matches the consensus at 9 out of 11 nucleotides. Alternatively, the TIE location relative to the transcriptional initiation site or other regulatory sequences beyond the TIE sites could influence the interaction with the Smad complex and the transcriptional repression. These possibilities could also explain the irrelevance of the possible SBE-ATF3 between the two TIEs.

The biological function of LPA_1 has been a subject of extensive studies in both *in vitro* cell culture and genetic animal models (Santin et al 2009, Shida et al 2003). Compared to other LPA receptors, LPA_1 is most widely expressed (Matas-Rico et al 2008). The nearly ubiquitous distribution of LPA_1 has led to the assumption that LPA_1 is constitutively expressed. However, a few recent studies have hinted at the regulation of LPA_1 by intracellular and extracellular cues (Horak et al 2007a, Stadler et al 2006, Xing et al 2004). The most exciting observation is that LPA_1 is one of the target genes repressed by the metastatic tumor suppressor Nm23 (Marshall et al 2010). Another study showed that germline polymorphism of fibroblast growth factor receptor 4 (FGFR4) at residue 388 (G388R) correlates with enhancement of LPA_1 expression and more aggressive migratory and invasive responses to LPA in tumors carrying R388 FGFR4 (Sugiyama et al 2010). Although LPA_1 expression may indeed be regulated by Nm23 and FGFR4, it is not known whether or how these modulators affect transcription, stability or translation of *LPA_1*. The results from the current study represent the first example that an endogenous factor transcriptionally restrains expression of LPA_1 and LPA_1-dependent cellular effects.

The roles of LPA and LPA receptors in cancer have drawn considerable attention in recent years. The LPA_2 receptor is overexpressed in ovarian, breast, thyroid and rectal colon cancers (Hendrix et al 2006, Radvanyi et al 2005, Sanchez-Carbayo et al 2006, Skrzypczak et al 2010, Su et al 2007). The transgenic and knockout mouse models further support an oncogenic role of LPA_2 (Huang et al 2004, Lin et al 2009). Expression of LPA_1, on the other hand, does not show consensus increases from normal to malignant phenotypes. Instead, several independent groups have reported a tendency of downregulation of LPA_1 in diverse cancers (Hendrix et al 2006, Murph et al 2008, Sanchez-Carbayo et al 2006, Shida et al 2004b, Skrzypczak et al 2010, Su et al 2007) in sharp contrast to the upregulation of LPA_2 in malignant diseases. The findings of the current study offer a plausible explanation to this phenomenon. The enhanced TGFβ signaling during cancer development and progression may serve as a repressor of expression of LPA_1 but not other LPA receptors.

TGFβ controls a multitude of biological activities in mammalian cells. It inhibits proliferation of epithelial cells and thus plays a part in early tumor suppression. However, TGFβ frequently fails to induce growth arrest in transformed epithelial cells. Instead, TGFβ stimulates migration and invasion of cancer cells, thereby promoting the metastatic potential in advanced cancer (Bierie and Moses 2006). This presumed effect of TGFβ on tumor cell invasion and metastasis is largely based on *in vitro* assays involving only TGFβ as a motogen (Seton-Rogers et al 2004, Yang et al 2008). The conclusion may not truly reflect the physiological role of TGFβ in *in vivo* conditions where tumor cells are exposed to a complex mix of multiple chemokines, cytokines, nutrients and growth

factors. We found in the current study that the effects of TGFβ on cell motility could be opposite under different conditions. In the cancer cell lines we tested, TGFβ itself was a weak stimulus of tumor cell invasion. In the presence of LPA, however, the role of TGFβ was reversed, counteracting the strong motogenic activity of LPA. Since both TGFβ and LPA are present in the circulation and malignant effusions, TGFβ probably acts as a negative regulator of cell motility in physiological and pathophysiological conditions. By extension, the findings of the current work underscore the importance of crosstalk between LPA and other coexisting factors in coordination of the overall cellular responses.

CHAPTER 4

GENERAL DISCCUSION

Uncontrolled cell proliferation is a hallmark of cancer. A major drive behind the uncontrolled proliferation of cancer cells is the activation of growth factor pathways. Activation of oncogenes and inactivation of tumor suppressors could also sensitize cells to growth stimuli. A prototype growth factor pathway involved in promotion of mammalian cell growth is the EGF-EGFR system (Paez et al 2004). Substantial evidence suggests overexpression or genetic mutations of EGFR in significant percentages of human malignancies, 69% in nonsmall cell lung cancer NSCLC (da Cunha Santos et al 2011), 50% in breast cancer (Teng et al 2011) and 55%-98% in advanced ovarian carcinoma (Mustea 2006). In breast and ovarian cancers, HER2, another member of the EGFR family, is also abnormally overexpressed or activated in up to 30% breast and ovarian cancers, resulting in more aggressive tumor behaviors and poor prognosis (Tan and Yu 2007). Recently, the anti-EGFR or HER2 small inhibitors and antibodies have been used alone or in combination with chemotherapies for treatment of a variety of solid tumors with significant improvement of patient survivals, confirming the importance of

the EGFR family in maintaining cancer cell growth and survival (Ciardiello and Tortora 2008).

In contrast to these receptor tyrosine kinases (RTKs), the significance of GPCR, the largest family of cell surface receptors, in regulation of cancer cells has not been as well appreciated although significant evidence exists that many GPCR/ligand systems stimulate proliferation of normal and neoplastic cells. The lysophospholipid mediators LPA and S1P represent the most characterized and important growth-promoting ligands acting through GPCRs. Our group was the first to describe overexpression of the LPA_2 and LPA_3 receptors in ovarian cancer (Fang et al 2004). The overexpression of LPA_2 has since been extended by independent studies to many other types of malignant diseases including breast cancer, colorectal cancer, gastric cancer, pancreatic cancer, and differentiated thyroid cancer (Hendrix et al 2006, Radvanyi et al 2005, Sanchez-Carbayo et al 2006, Skrzypczak et al 2010, Su et al 2007). However, it remains unknown how LPA mediates oncogenic processes. Substantial studies suggest that crosstalk between LPA and EGFR or other RTKs plays a role in eliciting the proliferative effect of LPA. Specifically, LPA transactivates EGFR through production or release of EGFR ligands or through EGFR ligand-independent interactions between EGFR and GPCR signals. Thus activation of LPA receptor signal transduction could partially contribute to the constitutive activity of EGFR to promote proliferation and aggressiveness of cancer cells. However, this possibility does not exclude EGFR-independent contribution of LPA as many oncogenic actions of LPA are more robust than those emanating from direct ligand activation of EGFR as we reported recently (Oyesanya et al 2010, Wu et al 2011).

In addition to the highly proliferative potential driven by signals from RTKs or GPCRs, cancer cells may evade growth inhibitory action of TGFβ and the TGFβ family members (e.g. BMPs, activin). In normal epithelial cells, TGFβ exerts its cytostatic action through TβR and TβR-activated Smad pathways to induce expression of CDK inhibitors such as p21, p15 and p27 and to suppress expression of the growth-promoting c-Myc. However, the cytostatic response to TGFβ is reduced or lost in most malignancies. Previous studies have identified partial mechanisms for the aberrant TGFβ responses in cancer, such as genetic mutation or deletion of components of the TGFβ-Smad pathway, and abnormal expression or functionality of negative regulators of the TGFβ-Smad pathway and critical transcription factors involved in TGFβ regulation of gene expression. However, these genetic and epigenetic defects are not common in diverse human tumors and cannot explain the general compromise of TGFβ growth inhibitory response, particularly in breast and ovarian cancer cells.

In order to identify the common mechanism underlying the defective TGFβ responses in breast and ovarian cancer cells, the first part of this dissertation study was designed to determine whether the cellular response to TGFβ was influenced by other extracellular factors co-present with TGFβ in tumor microenvironments. LPA is a ubiquitous growth factor present in serum, could be replenished in serum through serum-borne enzyme autotaxin or produced by tumor cells in culture. Our original hypothesis was that the abnormally active LPA signaling might confer overwhelmingly strong proliferative cues beyond the capacity of the TGFβ to overcome. However, our data demonstrates that the mitogenic activity of LPA does not contribute to the resistance of

breast and ovarian cancer cells to TGFβ. LPA shows a general mitogenic activity towards both TGFβ-sensitive and resistant breast and ovarian cancer cells. Instead, we observed a correlation of TGFβ responsiveness with LPA induction of p21 in breast and ovarian cancer cell lines. The CDK inhibitor p21 is a well-known TGFβ cytostatic response gene. Surprisingly, TGFβ alone only weakly induces p21 expression even in the TGFβ-responsive breast and ovarian cancer cell lines. Only in the presence of serum or serum-borne LPA, a full-magnitude elevation of p21 expression is observed. We further confirmed that LPA acts in concert with TGFβ in inducing p21 expression. Downregulation of p21 induction by p21 siRNA in TGFβ-responsive cells or induction of p21 with other known stimuli such as TPA in TGFβ-resistant cells leads to reversal of responsiveness to TGFβ. Hence, the combined effect of LPA and TGFβ on p21 induction is critical to TGFβ-mediated growth inhibition in TGFβ-sensitive breast and ovarian cancer cells. On the contrary, in the TGFβ-resistant cells, LPA does not stimulate p21 expression or cooperates with TGFβ to induce p21. Therefore, lack of p21 inducibility by LPA is potentially linked to the loss or impairment of the cytostatic response to TGFβ seen in many breast and ovarian cancers.

It remains to be determined how LPA induction of p21 is differentially regulated between TGFβ-sensitive and resistant cells. In our experiments, LPA induces p21 expression through LPA_1 and LPA_2 receptors. Knockdown of LPA_1 or LPA_2 partially compromises p21 expression induced by LPA. Since both of these receptors are present in most breast and ovarian cancer cell lines irrespective of their TGFβ response statuses, it is unlikely LPA_1 and LPA_2 levels are primary determinants of the differential p21

induction in these cells. The Erk activation is also a generic response to LPA among breast and ovarian cancer cell lines. In our study, we further demonstrate that the C/EBPβ transcription factor downstream of Erk mediates p21 induction by LPA. Coincidently, C/EBPβ is a transcription factor crucial to induction of another TGFβ cytostatic gene p15 (Gartel and Tyner 1999). In metastatic breast cancer cells, the transcriptional activity of C/EBPβ is inhibited by an excess of LIP (liver inhibitory protein), an inhibitory, truncated form of C/EBPβ. It is possible that the presence of high levels of endogenous LIP could prevent LPA-induced p21 expression and TGFβ cytostatic response. It will be of interest to examine expression of LIP in TGFβ-resistant versus sensitive breast and ovarian cancer cells.

Another important observation made from the first part of my study is that p21 is essential but not sufficient to render breast and ovarian cancer cells sensitive to TGFβ. LPA alone induces significant expression of p21 in TGFβ-sensitive MBA-MB-231 and Caov-3 cells, which is associated with growth promotion rather than inhibition. In TGFβ-resistant breast and ovarian cancer cell lines, TPA by itself induces p21 expression but does not affect proliferation of these cells. These results suggest that another TGFβ-mediated effector cooperates with increased p21 to halt cell cycle progression. This is consistent with the fact that the TGFβ-resistant breast and ovarian cancer cell lines we examined also possess functional TβRs and Smad3 signaling. In these otherwise TGFβ-resistant cells, the cytostatic sensitivity to TGFβ could be reconstituted through TGFβ-independent induction or activation of p21. Therefore, in the future, p21 activating or

inducing agents could be pursued in conjunction with TGFβ to develop new therapies against breast and ovarian cancer.

In the second part of this study, we extended to examine the crosstalk between LPA and TGFβ in modulation of cell migration and invasion. Activation of tumor cell motility is one of the critical steps leading to tumor invasion and distant metastasis. Different from the cooperative effects of LPA and TGFβ on p21 induction to control cell proliferation, TGFβ dramatically antagonizes LPA-induced migration and invasion of breast and ovarian cancer cells. A 30-50% decrease in migration and 60-80% reduction in invasion are observed in the presence of TGFβ although TGFβ alone weakly promotes cell migration and invasion of breast and ovarian cancer cell lines. The inhibitory effect of TGFβ on LPA-dependent cell migration and invasion is manifest in all cancer cell lines with functional TβRs and Smad3 including those resistant to cytostatic effect of TGFβ, suggesting that TGFβ down-regulates the motogenic activity of LPA via a mechanism independent of the cytostatic effectors of TGFβ.

LPA stimulates cell motility through activation of Rac and Rho in a coordinate manner. Rac promotes lamellipodia protrusion and forward movement, whereas RhoA regulates actomyosin-driven cytoskeleton contraction and detachment of the rear of migrating cells. Since LPA_1 plays a primary role in LPA stimulation of Rac and Rho and the subsequent migration and invasion (Van Leeuwen et al 2003), we tested the possibility that TGFβ may repress expression of LPA_1 to inhibit LPA-induced cell motility. The qPCR analysis of multiple breast and ovarian cancer cell lines as well as

their normal and immortalized epithelial counterparts show that TGFβ treatment induces 30-70% decreases in LPA_1 mRNA. The TGFβ-induced downregulation of LPA_1 is further supported by a coincident finding of microarray analysis from our collaborator Dr. Deborah Lebman that LPA_1 mRNA is inhibited by TGFβ treatment of the OE33 esophageal cancer cell line. In the literature, a microarray study of TGFβ transcriptome in human pituitary cells also revealed that LPA_1 is one of 109 genes repressed by TGFβ although the finding was not validated or pursued by individual gene expression and function analyses (Ruebel et al 2008).

We next explored how TGFβ represses LPA_1 expression. In contrast to well-studied SBE-dependent gene activation by TGFβ, the molecular mechanism for TGFβ-mediated gene repression is only partially understood through studies of two prototype genes repressed by TGFβ, namely c-Myc and Id1. The *c-Myc* gene p1 promoter contains TIE element made of an SBE and a closely linked E2F4/5 cis element that allows binding of the Smad3/4-E2F4/5-p107 complex to limit transcription of *c-Myc*. TGFβ inhibition of *Id1* is executed through binding of the Smad-ATF3 complex to an SBE site and a nearby CREB element on the *Id1* promoter. Interestingly, the LPA_1 gene promoter contains two putative TIE sites (-401 TIE and -40 TIE), each consisting of an SBE site and a flanking E2F4/5 element, analogous to TIE present on the *c-Myc* gene promoter. As described in detail in Chapter 3, we demonstrated that TGFβ represses LPA_1 transcription through binding of the Smad3/4-E2F4/5-p107 complex to the -401 TIE of the LPA_1 gene promoter. The proximal -40 TIE does not seem to be involved in TGFβ repression of LPA_1 transcription. The conclusion is drawn from substantial evidence from multiple

molecular approaches including luciferase reporter assay, site-directed mutagenesis of the TIEs, TIE DNA precipitation of cellular Smads and E2F4 protein, ChIP assays of TGFβ-induced recruitment of the Smad3/4-E2F4 complex to the native -401 TIE sequence of the LPA_1 promoter, and shRNA knockdown of Smads.

The identification of LPA_1 as a novel physiological target of TGFβ-mediated repression is a significant advance in LPA biology. Little is known about how LPA_1 is transcriptionally regulated. Our results provide the first mechanistic insight into transcriptional control of LPA_1. Different from LPA_2 that is generally overexpressed in cancer, LPA_1 is down-regulated in a variety of cancer lineages including ovarian, bladder, colorectal and breast cancers (Hendrix et al 2006, Murph et al 2008, Sanchez-Carbayo et al 2006, Shida et al 2004b, Skrzypczak et al 2010, Su et al 2007). The TGFβ repression of LPA_1 may offer a plausible explanation of the cancer-associated downregulation of LPA_1. Despite the common loss of the cytostatic response to TGFβ, most cancer cells exhibit intact TβRs and Smad signaling, which could lead to transcriptional repression of LPA_1, in particular when TGFβ levels are elevated in advanced cancers.

Furthermore, TGFβ repression of LPA_1 is functionally sufficient to inhibit LPA_1-dependent cell migration and invasion. This observation sheds new light on the physiological role of TGFβ in regulation of tumor cell invasion and metastasis. TGFβ has been shown to positively regulate tumor cell motility. In our hands, we detected a weak and sometimes statistically insignificant migratory activity of TGFβ in breast and ovarian cancer cell lines. However, the activity of TGFβ is minimal compared to that of LPA.

Given the fact that these two factors are elevated and co-present in tumor microenvironments, TGFβ most likely neutralizes the robust migratory activity of LPA and therefore may function to prevent tumor cell invasion and metastasis in vivo. This argument, however, is contrary to the current dogma in the field of TGFβ biology. The evidence for the pro-metastatic role of TGFβ has been largely derived from analysis of TGFβ alone in *in vitro* migration and invasion assays. The results of the present study underscore the need to revisit these effects of TGFβ in more physiologically relevant conditions or using *in vivo* models. As a general rule, the interactions among coexisting chemokines, cytokines, growth factors and nutrients should be taken into consideration during functional analysis of biological factors.

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PUBLICATIONS

Wu, J., Mukherjee, A., and Fang, X. The lysophosphatidic acid receptor 1 is a novel target of transforming growth factor beta-smad pathway. Submitted to Oncogene. Feb. 2012, under revision

Mukherjee, A., **Wu, J.**, Barbour, E. S., and Fang, X. Lysophosphatidic acid activates lipogenic pathways and de novo lipid synthesis in ovarian cancer cells. Submitted to J Bio Chem. Jan. 2012, under revision

Wu, J., Mukherjee, A., Lebman, D.A., and Fang, X. Lysophosphatidic acid-induced p21Waf1 expression mediates the cytostatic response of breast and ovarian cancer cells to TGFβ. Mol Cancer Res. Nov;9(11):1562-70, 2011.

Oyesanya, R. A., Greenbaum, S., Dang, D., Lee, Z., Mukherjee, A., **Wu, J.**, Dent, P., and Fang, X. Differential requirement of the epidermal growth factor receptor for G protein-mediated activation of transcription factors by lysophosphatidic acid. Mol Cancer, 9: 8, 2010.

Song, Y., **Wu, J.,** Oyesanya, R. A., Lee, Z., Mukherjee, A., and Fang, X. Sp-1 and c-Myc mediate lysophosphatidic acid-induced expression of vascular endothelial growth factor in ovarian cancer cells via a hypoxia-inducible factor-1-independent mechanism. Clin Cancer Res, 15: 492-501, 2009.

Oyesanya, R. A., Lee, Z. P., **Wu, J.**, Chen, J., Song, Y., Mukherjee, A., Dent, P., Kordula, T., Zhou, H., and Fang, X. Transcriptional and post-transcriptional mechanisms for lysophosphatidic acid-induced cyclooxygenase-2 expression in ovarian cancer cells. Faseb J, 22: 2639-2651, 2008.

Lee, Z., Cheng, C. T., Zhang, H., Subler, M. A., **Wu, J.**, Mukherjee, A., Windle, J. J., Chen, C. K., and Fang, X. Role of LPA4/p2y9/GPR23 in negative regulation of cell motility. Mol Biol Cell, 19: 5435-5445, 2008.

Song, Y., Wilkins, P., Hu, W., Murthy, K. S., Chen, J., Lee, Z., Oyesanya, R., **Wu, J.**, Barbour, S. E., and Fang, X. Inhibition of calcium-independent phospholipase A2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells. Biochem J, 406: 427-436, 2007.

ABSTRACTS AND PRESENTATIONS

Wu, J., Mukherjee, A., Lebman, D.A., and Fang, X. The 39th Annual John C. Forbes Research Colloquium, Virginia Commonwealth University, Richmond, VA, USA 2011

Wu, J., Mukherjee, A., and Fang, X. The 45th Annual Southeastern Regional Lipid Conference Cashiers, NC,USA 2010

Wu, J., Mukherjee, A., and Fang, X. The 44th Annual Southeastern Regional Lipid Conference Cashiers, NC,USA 2009

Wu, J., Song, Y., and Fang, X. Massey Cancer Center Cancer Research Retreat, Virginia Commonwealth University, Richmond, VA, USA 2008

Wu, J., Song, Y., Lee, P,. Oyesanya, R., Mukherjee, A., and Fang, X. The 42nd Annual Southeastern Regional Lipid Conference, Cashiers, NC, USA 2007

AWARDS AND HONORS

Travel Award, The 45th Annual Southeastern Regional Lipid Conference, Cashiers, NC,USA Nov. 2010

Travel Award, The 42nd Annual Southeastern Regional Lipid Conference, Cashiers, NC, USA Nov. 2007

Graduate Fellowship, Virginia Commonwealth University, Richmond, VA, USA 2006-present

Postgraduate recommendation, Huazhong University of Science and Technology, Wuhan, Hubei, PRC 2006

3rd class scholarship, Huazhong University of Science and Technology, Wuhan, Hubei, PRC 2004

2nd class scholarship, Huazhong University of Science and Technology, Wuhan, Hubei, PRC 2003