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ROLE OF LYSOPHOSPHATIDIC ACID IN REGULATION OF CANCER CELL METABOLISM

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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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BRCA1	Breast cancer type 1 susceptibility protein
AC	Adenyl cyclase
ACC	Acetyl CoA carboxylase
ACL	ATP citrate lyase
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
ATP	Adenosine-5'-triphosphate
ATX	Autotaxin
CAMKKβ	Calmodulin-dependent protein kinase kinase-beta
cAMP	cyclic-adenosine monophosphate
cDNA	Complementary DNA
cox-2	Cyclooxygenase-2
CREB	cAMP-response element binding protein
DAG	Diacylglycerol
Edg	Endothelial differentiation gene
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FDG-PET	Fluorodeoxyglucose positron emission tomography
FH	Fumarate hydratase
Fru-2,6-BP	Fructose-2, 6-bisphosphate
GDP	Guanosine diphosphate
GLS1	Glutaminase 1
GLUT1	Glucose transporter 1
GPAT	Glycerol-3-phosphate acyltransferase
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HER2	Human Epidermal Growth Factor Receptor 2
Hif-1	Hypoxia inducible factor -1
HK-2	Hexokinase 2
IL-6	Interleukin-6
IL-8	Interleukin-8
INSIG	Insulin-induced gene
IP3	Inositol triphosphate
KLF7	Kruppel-like factor 7
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LKB1	Liver kinase B1

LOH	Loss of heterozygosity
LPA	Lysophosphatidic acid
LPAAT	LPA acyl transferase
LPC	lysophosphocholine
LPPs	Lipid phosphate phosphatases
LysoPLD	lysophospholipase D
MAG	Monoacylglycerol
MAPK	Mitogen-activated protein kinase
MDH1B	Malate dehydrogenase 1B
MEFs	Mouse embryonic fibroblasts
MO25	Mouse protein 25
mTOR	mammalian target of rapamycin
NF1	Nuclear factor 1
NHBE	Normal human bronchial-epithelial
NPP	Nucleotide pyrophosphatase/phosphodiesterase
NSCLC	Non-small cell lung cancer
PA	Phosphatidic acid
PDGFR	Platelet derived growth factor receptor
PDK	Pyruvate dehydrogenase kinase
PEP	Phosphoenolpyruvate
PFK1	Phosphofructokinase 1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzyme 3

PGAM1	Phosphoglyceric acid mutase 1
PGK1	Phosphoglycerate kinase-1
PHD2	Prolyl hydroxylase domain protein 2
PI3KCA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIP2	Phosphoinositol biphosphate
PK	Pyruvate kinase
PKC	Protein kinase C
PKM2	Pyruvate kinase muscle isozyme
PLA	Phospholipase A
PLC	Phospholipase C
PPP	Pentose phosphate pathway
PRPS1L1	Phosphoribosyl pyrophosphate synthetase 1-like 1
PTEN	Phosphatase and tensin homolog
PTX	Pertussis toxin
qPCR	Quantitative PCR
RNAi	RNA interference
Rock	Rho-associated protein kinase
RPIA	Ribose-5-phosphate isomerase
RT-qPCR	Real time- quantitative PCR
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
S1P	Site 1 protease

S2P	Site 2 protease
SCAP	Sterol regulatory element binding protein cleavage activating protein
SDH	Succinate dehydrogenase
SMB	Somatomedin B
SP1	Specificity Protein 1
SREBPs	Sterol regulatory element binding proteins
SREs	Sterol regulator elements
STRAD	Ste20-related adaptor
TCA	Tricarboxylic acid cycle
TIGAR	TP53-induced glycolysis and apoptosis regulator
TP53	Tumor protein 53
UDP	Uridine diphosphate
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
VHL	von Hippel–Lindau tumor suppressor protein
VZG-1	Ventricular zone gene-1

ABSTRACT

ROLE OF LYSOPHOSPHATIDIC ACID IN REGULATION OF CANCER CELL METABOLISM

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

Virginia Commonwealth University School of Medicine, 2012

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The simplest phospholipid, lysophosphatidic acid (LPA), is a heat stable component of serum known for its proliferative and migratory activities in cancer cells. Strong evidence suggests that LPA production and expression of its receptors are dysregulated in multiple human malignancies. The mechanism behind LPA-mediated tumor cell growth and oncogenesis remains poorly understood. In this thesis project I used ovarian and other cancer cells as a model system to examine the hypothesis that LPA present in the tumor microenvironment is a pathophysiological determinant of hyperactive d*e novo* lipogenesis and aerobic glycolysis, two hallmarks of cancer cells.

We demonstrated that LPA induced proteolytic activation of sterol regulatory element binding proteins (SREBPs) in a cancer specific manner, leading to activation of the SREBP-FAS (fatty acid synthase) lipogenic pathway. Treatment of cancer cell lines with LPA also led to dephosphorylation and inhibition of AMP-activated kinase (AMPK), thereby activating acetyl CoA carboxylase (ACC). Moreover, these effects of LPA were mediated by LPA_2, a receptor subtype overexpressed in multiple cancers, providing an explanation for the cancer specific regulation of FAS and ACC by LPA. Downstream of the LPA_2 receptor, we identified the $G_{\alpha 12}$-Rho-Rock pathway to activate SREBPs and the $G_{\alpha q}$-PLC (phospholipase C) pathway to inactivate AMPK. Consistent with LPA mediated activation of the key lipogenic enzymes FAS and ACC, LPA stimulated *de novo* lipid synthesis via LPA_2, leading to accumulation of intracellular triacylglycerol and phospholipids. Pharmacological and molecular inhibition of LPA_2, FAS or ACC

attenuated LPA-dependent cell proliferation, indicating that upregulation of lipid synthesis is an integral component of the proliferative response to LPA. In further support of this, downregulation of LPA_2 expression led to dramatic inhibition of anchorage-dependent and –independent growth of ovarian cancer cells.

To support increased biomass generation, rapidly proliferating cancer cells enhance carbon influx by activating glycolysis. In the next part of the study, we investigated if LPA signaling was also involved in activating aerobic glycolysis in cancer cells. LPA indeed activated glycolysis in ovarian and other cancer cells but failed to elicit this response in non-transformed cells, suggesting a cancer specific role of LPA in regulation of glucose metabolism. While LPA had no effect on glucose uptake, we found that LPA altered expression of multiple genes involved in glucose metabolism. The most significant observation was that LPA treatment dramatically upregulated expression of HK-2, one of the rate-limiting glycolytic enzymes. We explored the underlying mechanism and found that LPA activates HK-2 transcription through LPA_2-mediated activation of SREBP-1. Two sterol regulator elements (SREs) on the human HK-2 promoter were identified to be responsible for LPA activation of the promoter. DNA pulldown and chromatin immunoprecipitation assays confirmed that SREBP-1 bound to these SREs in LPA-treated cells. Although in ovarian cancer cells, LPA treatment also stabilized Hif-1α protein, an established activator of HK-2 and glycolysis, LPA-regulated HK-2 expression and glycolysis was largely independent of Hif-1α. These results established that LPA stimulates glycolysis via the LPA_2-SREBP-HK-2 cascade in neoplastic cells.

Taken together, this dissertation provides the first evidence for regulation of cancer cell metabolism by LPA. The results indicate that LPA signaling is causally linked to lipogenic and glycolytic phenotypes of cancer cells. Therefore, targeting the key LPA_2 receptor could offer a novel and innovative approach to blocking tumor-specific metabolism.

CHAPTER 1

GENERAL INTRODUCTION

1.0 OVERVIEW

Ovarian cancer is the most lethal gynecological malignancy. It is estimated that in the Unites States, 22,280 women will be diagnosed with ovarian cancer and 15,500 of them will die of the disease in this year alone (1). Ovarian cancer is a heterogeneous neoplastic group primarily originating from the ovarian surface epithelium. Based on the microscopic morphologies, ovarian cancer can be classified as serous, endometrioid, clear cell and mucinous subtypes (2). At Stage I, when the tumor is found only within the ovary, or Stage II, when the tumor has spread only to pelvic organs, ovarian cancer is highly curable, with an overall 5-year survival rate of greater than 80%. However, ovarian cancer is usually diagnosed at advanced stages when malignant tumor cells have spread to the abdomen (Stage III) or beyond the peritoneal cavity (Stage IV), where the survival rate drops to 26.9%. The poor prognosis of ovarian cancer is primarily due to lack of early detection and effective therapies for late stages of ovarian cancer. Thus it is imperative to identify early markers for ovarian cancer in order to diagnose the disease at curable stages.

Less than 10% of ovarian cancers are linked to germline mutations of BRCA1 (breast cancer type 1 susceptibility protein) and BRCA2 genes (3). Most ovarian cancers are sporadic, and like other epithelial cancers, are clonal in nature (4), accumulating series of mutations during disease progression. Mutations in a number of tumor suppressors and oncogenes have been implicated in ovarian cancer development and progression. The tumor suppressor gene tumor protein 53 (TP53) has been found to be mutated in 10-15% of low grade and 40-50% of high grade ovarian cancers (5) and its expression is directly correlated with therapeutic responsiveness (6). Somatic mutations in other tumor suppressors such as PTEN (Phosphatase and tensin homolog) and BRCA1/2 have a low incidence. However, additional genes that have been suggested to act as tumor suppressors exhibit reduced expression in ovarian cancer cancers by epigenetic and other mechanisms (Table 1.1). Along with loss of tumor suppressor genes, several oncogenes have also been found to be mutated, amplified or overexpressed in ovarian cancers (Table 1.2). Thus based on the mutational profiling, ovarian cancers can be divided into either low grade tumors, with mutations in *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), *BRAF* (v-Raf murine sarcoma viral oncogene homolog B1) and *PIK3CA (*phosphoinositide-3-kinase, catalytic, alpha polypeptide), and LOH (loss of heterozygosity) on Xq, or high grade tumors with mutations in *TP53, BRCA1, BRCA2* and LOH on 7q and 9p (2,5).

Table 1.1 Altered expressions of tumor suppressor genes in ovarian cancer

Gene	Percent alteration	Mechanism of altered expression	Reference
TP53	10-50%	Mutation, loss of function	(5)
ARHI	60-75%	Imprinting; LOH; promoter methylation	(5,7,8)
RASSF1A	50-70%	Promoter methylation	(9,10)
PEG3	75%	LOH; promoter methylation	(8,11)

Table 1.2 Altered expressions of oncogenes in ovarian cancer

Gene	Percent alteration	Mechanism of altered expression	Reference
RAB25	54-89%	Overexpression	(12,13)
MYC	29%	Overexpression	(14,15)
EGFR	28-62%	Overexpressed,activation mutations	
ERBB2	8-11%	Overexpression, activation mutation	(16,17)
KRAS	30-52%	Overexpression, activation mutation	(18,19)

In addition to abnormal expression of oncogenes and tumor suppressor genes, a variety of autocrine and paracrine growth factors influence ovarian cancer progression. A prototype growth factor pathway involved in promotion of ovarian cancer progression is the EGF-EGFR (Epidermal growth factor- Epidermal growth factor receptor) system (20). Substantial evidence suggests that overexpression or mutations of EGFR is seen in a

significant percentage of ovarian cancer 28-62% (21,22). HER2 (Human Epidermal Growth Factor Receptor 2), another member of the EGFR family, is also abnormally overexpressed (38-52%) or activated in ovarian cancers, resulting in more aggressive tumor behavior and a poor prognosis (23,24). 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 survival, confirming the importance of the EGFR family in maintaining cancer cell growth and survival (25).

In contrast to these receptor tyrosine kinases (RTKs), the significance of G-protein coupled receptor (GPCR), the largest family of cell surface receptors, in regulation of cancer cells has not been as well appreciated, although numerous publications suggest that many GPCR/ligand systems stimulate proliferation of normal and neoplastic cells. The most important GPCR/ligand system in ovarian oncogenesis is lysophosphatidic acid (LPA) and its GPCRs. Many ovarian cancers exhibit aberrant LPA production, receptor expression or signal transduction (5). In spite of ample evidence for LPA to promote proliferation, migration and invasion of ovarian tumor cells, the molecular players involved in LPA-mediated regulation of these processes and ovarian oncogenesis remains poorly understood. We have undertaken this study to test a novel hypothesis that LPA is a pathophysiological factor present in the tumor microenvironment to drive hyperactive lipogenesis and glycolysis, which are hallmarks of malignant cells. Our results presented in Chapter 1 and Chapter 2 indeed provide strong evidence to support this previously unrecognized role of LPA in ovarian and other

cancer cells. We have also gained evidence that LPA promotion of lipogenesis and glycolysis is an integral component of the cellular proliferative program. Thus this thesis study provides a link from LPA signaling to regulation of cellular metabolic processes, proliferation and malignant phenotypes.

1.1 LYSOPHOSPHATIDIC ACID

LPA (*1-acyl*-2-hydroxy-sn-glycero-3-phosphate) is the simplest naturally occurring phospholipid. It is comprised of a glycerol backbone with one phosphate group at sn-3 position and a fatty acyl chain at either the sn-1 or sn-2 position. Fatty acyl chains found in LPA are either saturated (C16:0, C18:0) or unsaturated (C18:1, C20:4) long chain fatty acids which are linked to the glycerol backbone by acyl or alky linkages. LPA is a component of serum, reaching concentrations of 1-5 μM (26), and is found attached to albumin with a stoichiometry of 3 mole of LPA/mole of albumin (27). Binding with albumin is necessary for LPA to elicit its activity and albumin-bound LPA is often regarded as the heat stable and lysophospholipase sensitive component of serum's mitogenic activity (28,29). Apart from serum, LPA is also found in other body fluids such as plasma, saliva, hair follicles and malignant effusions (30).

1.1.1 LPA METABOLISM

LPA production is an enzyme-catalyzed process and, depending on the site of production, can be catalyzed by different cascades of enzymes. LPA is primarily produced and secreted in extracellular fluids. However, LPA in small amounts can also be produced intracellularly. LPA is an intermediate product of triacyl glyceride synthesis

and can be generated by glycerol-3-phosphate acyltransfeases (GPAT) in the mitochondria and endoplasmic reticulum by acylation of glyceraldehyde-3-phosphate. LPA produced by this route is rapidly converted to phosphatidic acid (PA) which serves as the precursor for synthesis of other glycerol phospholipids (31). Till date there has been no direct evidence that this intracellular pool of LPA is secreted out of the cell and acts as a ligand of cell surface LPA receptors.

There are two major pathways that contribute to extracellular LPA production depending on the starting substrate (Figure 1.1). One route is mediated by the action of phospholipase A_2 (PLA_2) or phospholipase A_1 (PLA_1) on phospholipids, followed by conversion of resulting lysophospholipids to LPA by lysophospholipase D (lysoPLD). The identity of this mysterious lyso PLD enzyme remained elusive for a long time, even though several observations suggested the presence of such an enzyme. Extended incubation of rat plasma (32) or human follicular fluid (33) lead to generation of LPA with a concomitant decrease of lysophosphocholine (LPC). Moreover, incubation of fibroblasts with phospholipase D from *Streptomyces chromofuscus* lead to rapid release of LPA and a reduction of LPC (34). These studies provided evidence for the presence of a secreted enzyme that could use LPC either in circulation or from the outer leaflet of cell membranes as its substrate to generate LPA. This enzyme was later discovered as autotaxin (ATX) (35), an eco-enzyme of the nucleotide pyrophosphatase/ phosphodiesterase (NPP) family. This route involving ATX is generally believed to be the primary source of LPA production in cancer and by activated platelets in blood circulation (36). Subsequently, mice heterozygous for ATX have been shown to have 50%

less LPA plasma levels as compared to their wild type counterparts, suggesting the importance of ATX in physiological production of LPA (37). ATX is an enigmatic enzyme which is known for its role in tumor invasion, neovascularization and metastasis (38-40). The crystal structure of ATX has recently been determined which offers new understanding about the substrate recognition and its mechanism of action. ATX has multiple domains, one of which is an atypical phosphodiesterase catalytic domain that is responsible for LPA production, while two N-terminal somatomedin B (SMB) like domains and the C-terminal nuclease-like (NUC) domain aid in substrate specificity and presentation (41,42). Moreover, it was also suggested that ATX could attach itself to β3-integrins and deliver LPA directly to its receptors via a hydrophobic channel (42). It could be thus speculated that the actual concentrations of LPA around the tumor cells could be more than the serum or plasma LPA levels in cancer patients.

The second less studied route of LPA generation is by the action of monoacylglycerol kinase (MAG-kinase) on monoacylglycerol (43), this pathway has also been suggested to be active in cancer cells (44).

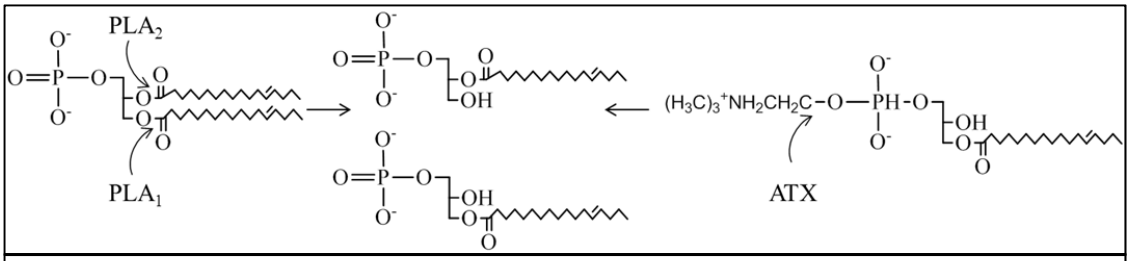


Figure 1.1 Two major routes for LPA production.

The effective concentration of LPA is regulated both by its production and by its degradation. The important players in the degradation processes are lipid phosphate phosphatases (LPPs). Three different isoforms of LPPs (LPP-1, LPP-2 and LPP-3) have been identified to date which are capable of dephosphorylating LPA, PA, sphingosine-1-phosphate (S1P), ceramide-1-phosphate and diacylglycerol pyrrophospate (DGPP) (45,46). Several publications have, shown LPP-1 to specifically inactivate LPA in vitro and in vivo (46-48) . These LPPs are membrane associated enzymes with their catalytic domain facing the extracellular environments and as their name suggests, they cleave off the phosphate group from LPA, generating monoacyl glycerol (MAG). Another mechanism for reducing LPA concentrations is by converting LPA to PA by the acylation reaction carried out by LPA acyl transferases (LPAAT) (49,50).

1.1.2 LPA RECEPTORS

LPA has numerous biological functions in physiological and pathophysiological conditions (reviewed in Table 1.3). These effects of LPA are mediated by signaling through its membrane-associated GPCRs. Seven LPA receptors have been identified to date. The expression patterns of these receptors vary in their relative amounts and from one tissue to another, thereby leading to a complex regulation of cellular processes by LPA in a tissue dependent manner. In cancer, LPA signaling is heightened due to increased levels of ligand (LPA) or receptor expression, influencing processes such as cancer cell proliferation, survival, migration, and invasion. LPA receptors fall into two

sub-groups, the endothelial differentiation gene (Edg) family of LPA receptors and non-Edg LPA receptors.

Table 1.3 Physiological and pathophysiological effects of LPA

Effect	Cell type/remarks
Cell proliferation and survival	Many cell types, normal and transformed (51)
Cell migration and invasion	Many cell types, normal and transformed (51)
Tumor progression	Mouse xenografts (overexpression or knockdown of LPA receptors) (51,52)
Wound healing *in vivo*	Skin (53); intestinal epithelium (54)
Cell contraction	Smooth muscle cells; myofibroblasts (55)
Platelet activation and aggregation	LPA in atherosclerotic plaques; synergy with ADP (56,57)
Cytokine production	Fibroblasts; astrocytes; leukocytes; epithelial and endothelial cells; carcinoma cells (51)
Stabilization of embryonic vessels	Mouse allantois explant culture (58)
Neurite retraction, collapse/turning of growth cones	Neuroblastoma cells (28); primary neurons (59)
Inhibition/reversal of differentiation	Neuroblastoma and glioma cells (60); astrocytes (61) vascular smooth muscle cells (62); pre-adipocytes (63)
Cerebral cortex growth and folding *ex vivo*	Action on neural progenitor cells; not observed in LPA_1/LPA_2-deficient mice (64)
Initiation of neuropathic pain and demyelination of the dorsal root *in vivo*	Reduced in LPA_1-deficient mice (65)
Demyelination of the dorsal root *ex vivo*	Direct action on myelinating Schwann cells (66)
Membrane depolarization (chloride efflux-mediated)	Neuronal cells (67); fibroblasts (68)
Blastocyst implantation (timing and spacing)	LPA_3-mediated (69)

The Edg LPA receptors (LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7) have been extensively studied. They are structurally similar with 46-50% amino acid homology between them (70). However the non-Edg subgroup of LPA receptors (LPA_4/GPR23/P2Y9, LPA_5/GPR92 and LPA_6/P2Y5) are phylogenetically distinct with less than 15% sequence homology among the family members (70) (Figure 1.2).

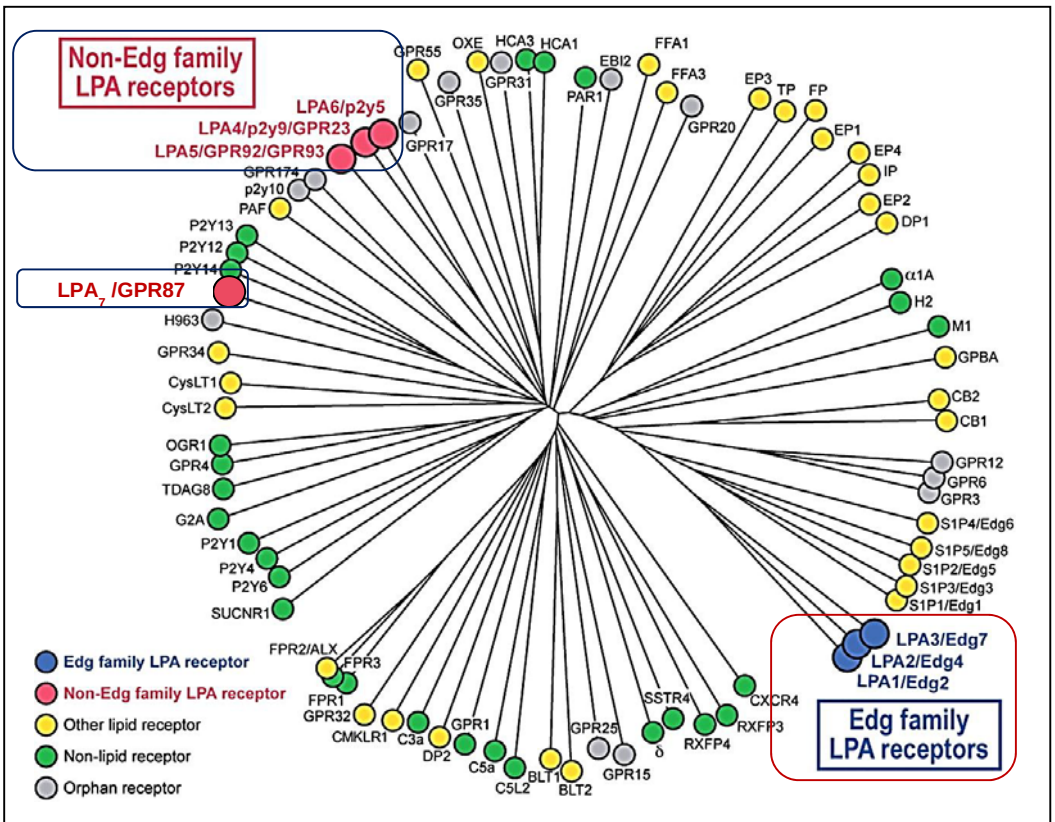


Figure 1.2 A phylogenetic tree of human GPCRs, depicting the phylogenetic difference between Edg and non-Edg GPCR receptors of LPA. (Adapted from Yanagida, K., and Ishii, S. (2011) Non-Edg family LPA receptors: the cutting edge of LPA research. *J Biochem* **150**, 223-232).

LPA_1/Edg2 was the first LPA receptor to be identified. In an effort to identify GPCRs regulating cortical neurogenesis, Chun and colleagues identified a GPCR expressed in the cortical neurogenic region and named it ventricular zone gene-1 (VZG-1). They overexpressed this gene in neuronal cells and observed cell rounding and adenyl cyclase inhibition specifically in response to LPA among other ligands including lipids (71), providing evidence for identification of the first LPA receptor. LPA_1 in humans is expressed in a variety of adult tissues including heart, small intestine, pancreas, kidney, prostate, ovary, and testis, but the highest expression of LPA_1 had been observed in the mouse brain (70,71). Hence the role of LPA_1 in the developing nervous system is a major focus of research. LPA_1 has been shown to be important for neurogenesis in the dentate gyrus, synapse formation in the hippocampus and for overall cortical development (72-74). The importance of LPA_1 was evident from studies using LPA_1 knockout mice which had a semi lethal phenotype (50% neonatal deaths). Among the mice that survived, some displayed craniofacial deformities, frontal hemorrhages and defects in suckling behavior (75). By subjecting the LPA_1 knockout mice to pathophysiological conditions, its roles in initiation of neuropathic pain (65), and in pulmonary and renal fibrosis have been established (76,77).

LPA_2/Edg4 was identified from GenBank searches of orphan GPCRs and its high amino acid sequence similarity to LPA_1 (55%) (78). In humans, *LPA_2* gene is located on chromosome 19 and encodes for a 39 kd protein (79). The expression of LPA_2 as compared to LPA_1 is relatively restricted. In adult mice, LPA_2 is expressed in lung, spleen, stomach, testis and kidney (80), and in humans, its expression has been detected

in pancreas, thymus, testis, spleen and leukocytes (81). LPA_2 knockout mice are viable, grossly normal and display no apparent breeding abnormality. Moreover the LPA_1 and LPA_2 double knockout mice did not show any phenotype additional to LPA_1 knockout mice, except for a minor increase in frontal hematomas (82). Thus it can be speculated that under physiological conditions, LPA_2 may not play a significant role and its functions might be redundant to LPA_1. However, LPA_2 has been found to be upregulated in various cancers and may contribute to pathogenesis of ovarian cancer, colorectal cancer and other malignancies (83,84).

LPA_3/Edg7 was initially identified as an orphan receptor using degenerate PCR-based cloning. Based on its responsiveness to LPA and its homology to LPA_{1-2} receptors (53.7 and 48.8% for LPA_1 and LPA_2 respectively), it was identified as the third Edg LPA receptor (85,86). The *LPA3* gene is located on chromosome 1 and encodes for a 40 kd protein. In human, LPA_3 expression is detectable in a number of tissues including heart, testis, prostate, pancreas, lung, ovary, and brain (85,86). In mice, LPA_3 is highly expressed in the uterus, and during post pregnancy almost exclusively in the luminal endometrial epithelium (69,70). Its expression has also been shown to be regulated by progesterone and estrogen (87). In accordance to its expression and potential hormone-mediated regulation, LPA_3 female knockout mice had a dramatic phenotype in the reproductive system. Loss of LPA_3 resulted in delayed implantation and an alteration in embryo spacing resulting in reduced litter size (69). It is, however, notable that LPA_3 knockout mice had no observable phenotype in the nervous system, in spite of its expression in brain.

LPA_4/GPR23/P2Y9 was the first non-Edg family LPA receptor to be identified. As part of their "de-orphaning" project, Noguchi et al. identified GPR23/P2Y9, a receptor of the purinergic sub-family of GPCRs as the fourth LPA receptor (88). Human LPA_4 is expressed in a variety of tissues, with highest expression observed in the ovary (88). It is located on the X chromosome and encodes a 42 kd protein. We were the first group to generate and characterize LPA_4 knockout mice. The LPA_4 null mice were viable and had no phenotypic abnormality. However, we demonstrated that the loss of LPA_4 sensitizes mouse embryonic fibroblasts (MEFs) to LPA-induced migration and tumor cell invasion (89), a process involving the inhibition of LPA1 receptor activity. This study was the first evidence for functional antagonism between LPA receptors. Following our study, Sumida et al also generated LPA_4 knockout mice and reported partial lethality and defects in blood vessel formation in their LPA_4 knockout mice (90). Although we did not observe any phenotypic defects in our LPA_4 null mice, the difference in phenotype could arise from difference in genetic backgrounds of mice used.

LPA_5/GPR97 is a recently identified LPA receptor that shares 35% homology with LPA_4 and is structurally distant from the Edg LPA receptors (91). The *LPA_5* gene is located on chromosome 12 and codes for a 41 kd protein. Using mouse and human tissue samples, LPA_5 has been shown to be expressed in small intestine, colon, stomach, spleen, heart and embryonic brain, with highest expression observed in the small intestine of mice and spleen of humans (91,92). Very few studies have been carried out on LPA_5, but it has been suggested to play a role in platelet activation (93) and cyclooxygenase (Cox-2) induction in some ovarian cancer cell lines (94).

The *LPA_6/P2Y5* gene is located on chromosome 13 at a locus (q14.11-13q21.33) and is linked to an autosomal recessive form of hypotrichosis, which lead to its discovery (95). Pasternack et al. has subsequently shown that LPA6 is the expressed in the hair follicle and is required to maintain hair growth (95).

LPA_7/GPR87 is the last known LPA receptor. The *LPA_7* gene is present on chromosome 3 in both mouse and humans. It has been shown that LPA_7 is expressed in placenta, ovary, testis, prostate, brain, and skeletal muscles in mice (96). Other GPCRs such as P2Y10 (97) and GPR35 (97) have been proposed as additional LPA receptors but they have not been validated by independent studies.

1.1.3 LPA RECEPTORS AND CANCER

LPA is known for its proliferative and migratory effects on a variety of cell types, and since LPA levels are elevated in cancer patients, LPA signaling is known to be heightened in cancer cells as well. LPA receptors are upregulated in a number of cancer types, thereby contributing to elevated LPA signaling in cancer.

Although LPA_1 is expressed in a wide range of human and mouse tissues, analysis of LPA_1 mRNA expression data from tumor samples failed to conclusively prove overexpression of this receptor in major cancers (98). Moreover several groups have suggested LPA_1 to be downregulated in cancers of the ovary, colon, vulva, thyroid, and testis compared with corresponding normal tissues (99-101). Irrespective of its expression changes in cancer, there is strong evidence that LPA_1 is involved in oncogenic processes, especially in promotion of tumor cell invasion and metastasis. Overexpression

of LPA_1 in cell lines where LPA receptors are either absent or had very low expression (B103, Rh7777 and SkBr3), resulted in increased proliferative and migratory responses to LPA and increased metastasis to the bone when injected into nude mice (56,102,103). The role of LPA_1 in enhancing migratory and metastatic potential of cancer cells was also supported by the observation that, Nm23-H1 (a metastatic suppressor) inhibited expression of LPA_1 (104,105).

Unlike LPA_1, expression of LPA_2 is known to be upregulated in a variety of cancers and is generally believed to be the major LPA receptor contributing to carcinogenesis. Our lab was the first to report overexpression of this reporter in ovarian cancer lines and primary ovarian cancer (106,107), following which several groups have identified overexpression of this receptor in other cancer types including breast (108), gastric (109), colorectal (110), and thyroid cancers (111). There are several lines of evidence that support a role of LPA_2 in driving tumorigenesis. Overexpression of LPA_2 in ovarian cancer cell lines increased production of oncogenic factors such as interleukin-6 (IL-6), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF), leading to an increased tumor burden in mice when these cells were injected subcutaneously (83). Breast tissue specific expression of LPA_2 (driven by the MMTV promoter) in mice was found to increase the incidence of mammary tumors, as compared to wild type litter mates. Moreover, LPA_2 knockout mice were found to be resistant to intestinal tumor formation in both the Adenomatous polyposis coli $(APC)^{+/-}$ mouse model (112) and dextran sulfate sodium model (113). It is thus evident that LPA_2 plays a

major role in tumor development. However, the exact mechanism by which LPA_2 regulates oncogenic processes remains elusive.

The role of LPA_3 in cancer is not fully understood. It has been found to be overexpressed in ovarian (107) and prostate (114) cancers but downregulated in some breast cancers (101). However in ovarian cancer, LPA_3 has been found to contribute to tumorigenicity (83). In other cancers such as colon cancers, there are conflicting reports. Although it is downregulated in colon cancer (99), there is experimental evidence that LPA_3 contributes to LPA-driven proliferation of these cells (115). In addition, LPA_3 negatively regulates LPA_1-driven migration of rat lung cancer cells (116). It is evident that further studies are required in order to clarify the role of LPA_3 in cancer.

As compared to the Edg LPA receptors, the contributions of non-Edg receptors in tumorigenesis have not been elaborately studied. LPA_4, the first non-Edg family receptor to be identified, is expressed at low levels as compared to other LPA receptors or is undetectable in a majority of cancer cell lines. Analysis of expression data from some cancer studies failed to provide any significant difference in LPA_4 levels between cancers and their corresponding non cancer tissues (Figure 1.3). Thus it is imperative to study LPA_4 expression in individual cancer types separately. We have recently shown that LPA_4 expression levels are reduced after Ras transformation, suggesting that reduced expression of this receptor is one of the molecular changes associated with oncogenic transformation (117).

Roles of LPA_5, LPA_6 and LPA_7 in cancer have not been adequately studied. However, expression analysis suggests their expression could be deregulated in cancer (Figure 1.3).

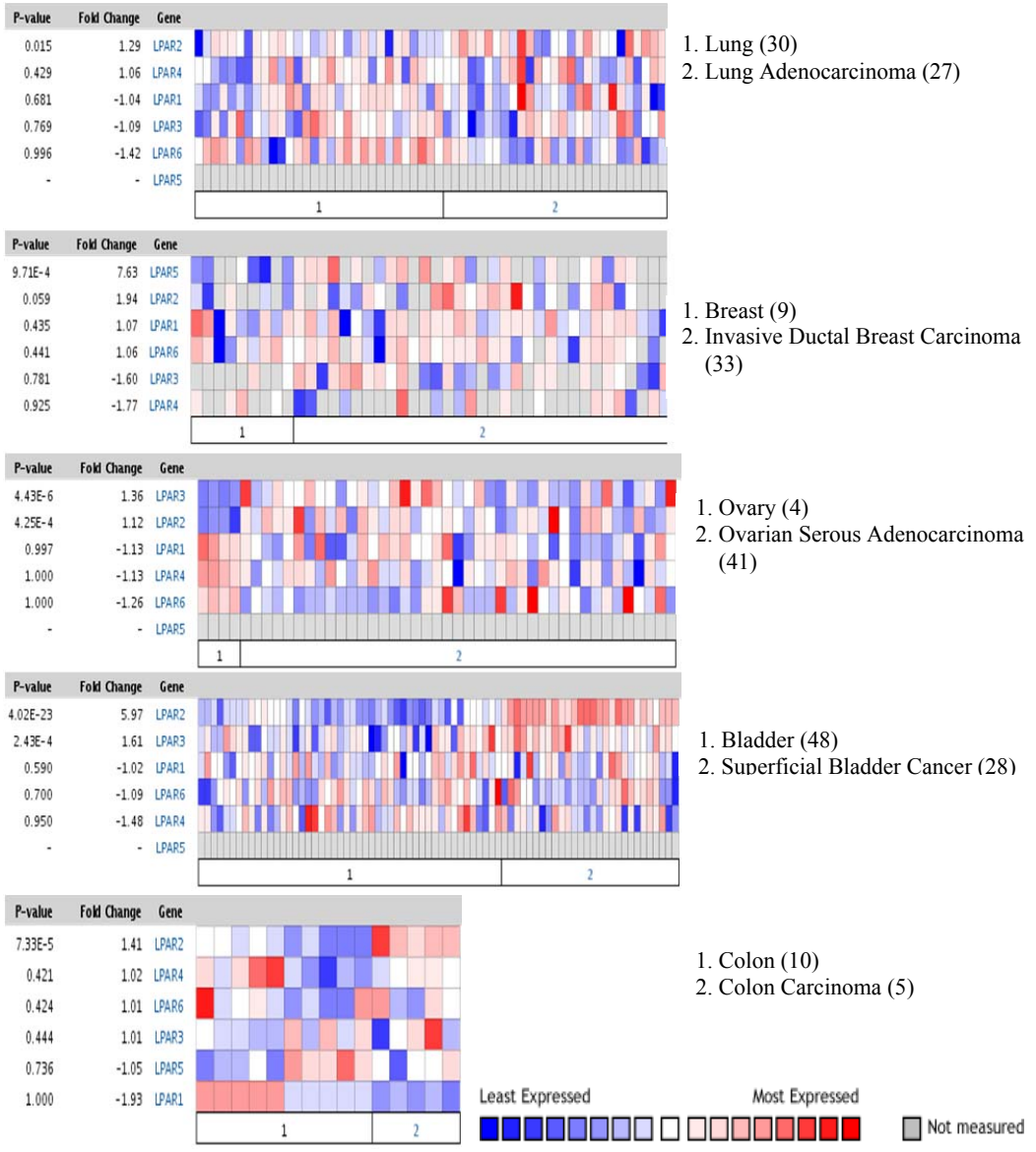


Figure 1.3 Expression of LPA receptors in cancer. Expression patterns of LPA receptors are depicted as normal vs. tumor of their log2 median-centered intensity. The Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization.

1.1.4 LPA RECETOR MEDIATED SIGNALING

Like other GPCRs, LPA receptors couple to multiple G-proteins. G-proteins are hetrotrimeric proteins (composed of α-, β- and γ- subunits) that transduce signals from the receptors to various effectors. In the inactive state, G-proteins remain attached to GDP (guanosine diphosphate); on activation, the GDP gets replaced by GTP (guanosine triphosphate) resulting in release of the α-subunit from βγ-subunits. These subunits then go on to activate various signaling pathways. As shown in Figure 1.4, LPA receptors are majorly known to couple to $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha 12/13}$ and in certain cases $G_{\alpha s}$. LPA_1 and LPA_2 receptors couple to $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$, whereas LPA_3 couples to $G_{\alpha i}$ and $G_{\alpha q}$. The non-Edg LPA receptors are not known to activate $G_{\alpha i}$ but are known to activate $G_{\alpha s}$ instead. This is a significant difference as coupling to $G_{\alpha s}$ leads to activation of adenly cyclase (AC) and hence leads to increase in cAMP (cyclic-adenosine monophosphate), whereas as $G_{\alpha i}$ mediates inhibition of AC. LPA via $G_{\alpha i}$ inhibits cAMP accumulation, activates Ras-MAPK (mitogen-activated protein kinase) pathways, Rac GTPases via TIAM1 (a GDP/GTP exchange factor) and the PI3K-AKT pathway (102,118-121). LPA mediated activation of $G_{\alpha q}$ is linked to activation of phospholipase C (PLC), which catalyzes hydrolysis of phosphoinositol biphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3) with subsequent release of intracellular calcium and activation of PKCs (protein kinase C) (118). LPA via $G_{\alpha 12/13}$ activates RhoA leading to cytoskeletal changes and cell rounding (122). Thus LPA via $G_{\alpha i}$ regulates cell proliferation and survival, via $G_{\alpha q}$ regulates the production of secondary messengers and by combined actions of $G_{\alpha i}$ and $G_{\alpha 12/13}$ regulates cell migration and invasion.

Apart from the G_{α}- subunit, $G_{\beta\gamma}$-subunits have also been shown to activate signaling pathways. Upon LPA stimulation, $G_{\beta\gamma}$-subunit has been shown to associate with Rab11a-containing early and late endosomes, leading to recruitment and activation of the PI3K-AKT pathway (123).

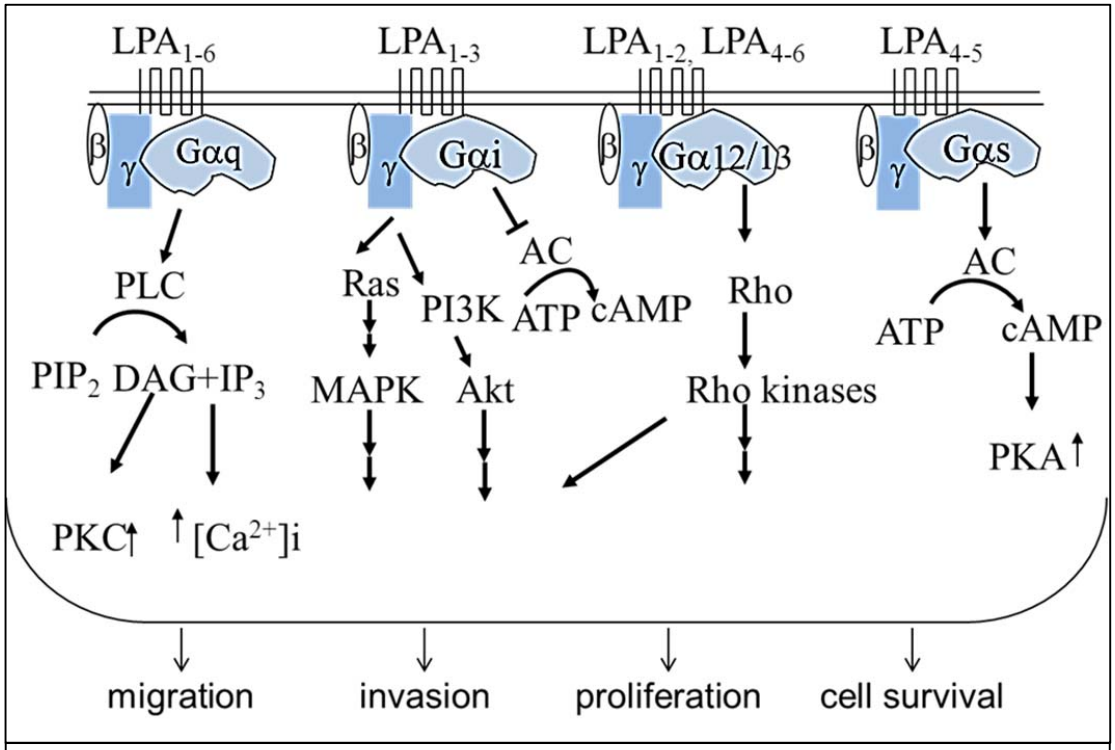


Figure 1.4 LPA receptor mediated signaling

1.1.5 LPA AND CELL PROLIFERATION

The mitogenic effect of LPA was first discovered in fibroblasts (118); subsequently, LPA has been found to increase cellular proliferation in cells of multiple lineages including transformed cells. The proliferative effect of LPA is generally

regarded to be driven by the pertussis toxin (PTX) sensitive Gαi pathway (118,120), however possible contribution by RhoA signaling has also been suggested (124).

Although LPA activates the $G_{\alpha q}$-PLC pathway, this pathway is not required for proliferation (118). Downstream of $G_{\alpha i}$, the Ras-MAPK and the PI3K-AKT pathways have been known to mediate LPA-induced cell proliferation (120,125). These pathways are also involved in promotion of cell survival (126,127).

All Edg receptors coupled to Gαi are capable of enhancing cell proliferation when analyzed individually in ovarian cancer cell lines (83). However, Edg LPA receptors are often found to be co-expressed, making it difficult to link a biological response to a specific receptor. The crosstalk among the Edg LPA receptors likely plays an important role in the proliferative response to LPA. Studies using mouse embryonic fibroblasts have showed that LPA_1 and LPA_2 have redundant functions in terms of cellular proliferation. Loss of both receptors caused a dramatic inhibition of LPA-dependent cell proliferation (82). In transformed cells, there is emerging evidence that implicates LPA_2 in driving cell proliferation and tumorigenesis. LPA_2 has been shown to activate a number of cell cycle regulators and oncogenic proteins including IL-6, VEGF, HIF1α, c-Myc, cyclin D1, kruppel-like factor 5, and Cox-2 (112,113,128-131). These protein factors could be important mediators of LPA_2's biological functions. LPA is known to transactivate epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and hepatocyte growth factor receptor (c-Met). A signal input from these receptor tyrosine kinases seems to be required for maximum induction of proliferation by LPA in various cellular contexts (132-134).

1.2 CANCER CELL METABOLISM

Cell metabolism refers to complex biochemical reactions within a cell that use nutrients as substrates to generate macromolecules (such as protein, DNA and lipids) and energy (Adenosine-5'-triphosphate, ATP). ATP inside a cell is generated by glycolysis, fatty acid β-oxidation and the tricarboxylic acid cycle (TCA). During glycolysis, glucose is converted into pyruvate with the net output of 2 ATP molecules, and in the absence of oxygen, lactate becomes the end product. However, if oxygen is in abundance, pyruvate enters the TCA cycle and subsequently a total of 36 molecules of ATP can be generated from complete catabolism of one glucose molecule. These processes are tightly coordinated and anabolic or catabolic processes are activated to meet the cellular requirements. Cancer cells have evolved an altered metabolic profile that is well suited for an increased rate of cellular proliferation.

The first evidence of an altered metabolic program in cancer cells was provided by Otto Warburg in 1920s. He showed that transformed cells continue to use glycolysis even in the presence of oxygen as the major ATP generation process, which later came to be known as the Warburg effect (135). This observation has been demonstrated in a variety of cancers and has been exploited in the detection of cancer by fluorodeoxyglucose positron emission tomography (FDG-PET). The Warburg effect initially provided a paradox as cancer cells by definition are rapidly proliferating cells and preferential use of glycolysis would generate less ATP per molecule of glucose. However, recent observations suggests that cancer cells utilize glycolysis to preferentially increase carbon flux inside the cells which is required to generate biomass needed to meet

the demand for rapid cellular proliferation. Thus cancer cells are characterized by an increased rate of glycolysis, highly active DNA and protein synthesis and hyperactive *de novo* lipogenesis.

1.2.1 *DE NOVO* LIPOGENEIS

Fatty acids are important constituents of cell membranes, signaling molecules, and secondary messengers. There are two sources of fatty acids for cellular metabolism, 1) external fatty acids obtained from diet and 2) endogenously synthesized fatty acids. The majority of cells in humans rely on dietary fats to meet their requirements and hence the inherent process of fatty acid synthesis (*de novo* lipogenesis) is generally inhibited. In contrast, cancer cells heavily depend on fatty acids from de novo synthesis. Using radio isotopes, it has been showed that more than 90% of fatty acids are generated by cancer cells themselves and only a minor fraction is contributed by cellular uptake of extracellular fatty acids (136). Fatty acid synthesis is carried out in the cytosol from acetyl CoA which acts as the carbon donor (Figure 1.5). The first step in fatty acid synthesis is the generation of malonyl CoA by carboxylation of acetyl CoA, which is carried out by acetyl CoA carboxylase (ACC). Sequential addition of 2 carbon unit leads to the generation of long chain fatty acids, catalyzed by a multi-functional protein known as fatty acid synthase (FAS).

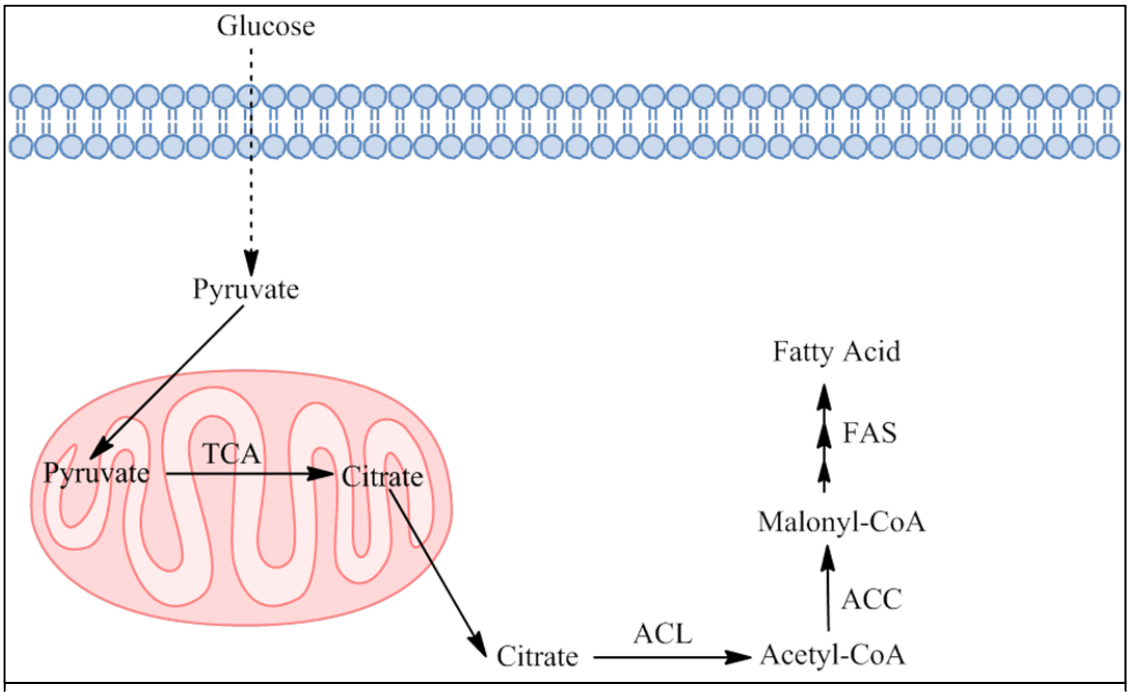


Figure 1.5 An illustration of fatty acid synthesis

1.2.2 KEY MEDIATORS OF LIPOGENESIS IN CANCER

Generation of fatty acids in the cytosol depends on the ability of cells to generate cytosolic acetyl CoA catalyzed by ATP citrate lyase (ACL), as acetyl CoA generated in the mitochondria cannot be directly transported to the cytoplasm. The acetyl CoA thus produced feeds into fatty acid synthesis, cholesterol synthesis and in acetylation reactions, suggesting the importance of this enzyme in multiple processes. In humans, ACL has been found to be abundantly expressed in liver and adipose tissues (137,138) and is often seen upregulated in lung, prostate, bladder, breast, liver, stomach, and colon tumors (139-145). The importance of ACL in *de novo* lipogenesis and proliferation of cancer cells is suggested by the observation that pharmacological and RNA interference (RNAi)

mediated inhibition of ACL leads to reduced cell growth, survival and tumorigenesis of cancer cells (146,147).

The second enzyme in fatty acid synthesis is ACC. It catalyzes the first committed step in fatty acid synthesis. Mammalian ACC consists of multi-functional domains (biotin carboxylase, biotin carboxyl carrier, and carboxyltransferase). ACC has two isoforms, ACC1 and ACC2; the former is the predominant one present in lipogenic tissues (liver, adipose tissue, lactating mammary glands) and cancer cells, and the latter is expressed in skeletal muscle, heart and liver (148). ACC1 has been shown to be upregulated in cancer of prostate, breast and liver (141,149,150). Interestingly, pre-neoplastic lesions with increased expression of ACC1 have been shown to have a higher chance of developing into breast cancers (150). Knockdown of ACC1 in cancer cells results in reduced proliferation and viability (151,152).

In 1994, Kuhajda and colleagues identified the oncogenic antigen-519 (OA-519) as FAS (153), and several subsequent studies showed OA-519 overexpression to correlate with poor patient outcomes (154). FAS has been shown to be upregulated in cancers of breast, colorectum, prostate, bladder, ovary, esophagus, stomach, lung, oral tongue, oral cavity, head and neck, thyroid and endometrium (154-156). There are two possible mechanisms by which FAS can be upregulated in cancer. The first involves the activity of growth factor receptors. In particular, the EGF family receptors ERBB1 and ERBB2 have been shown to regulate FAS transcriptionally (157-159), via PI3K or MAPK pathways (160-162). The second route is via a post-translational mechanism, supported by the observation that in prostate cancer cells FAS has been found to interact with

ubiquitin specific proteases USP2a. USP2a protected FAS from ubiquitin-mediated degradation, and knockdown of USP2a reduced FAS levels (163). Correlation between FAS and USP2a expression can also be seen in microarray analyses (164). It should be noted that to date such regulation of FAS has not been reported in any type of cancer cells.

1.2.3 REGULATION OF LIPOGENESIS IN CANCER

The lipogenic process in cancer cells shares certain similarities with non-transformed cells (liver and adipose tissue). In both cases, FAS expression is regulated by sterol regulatory element binding proteins (SREBPs). The SREBP family of transcription factors comprises three members SREBP-1a, SREBP-1c, and SREBP-2 (165,166). SREBP-1a and SREBP-1c are produced from one gene by the use of alternative promoters (166-168), and SREBP-2 is a product of another gene with no known other isoforms (169). Each SREBP has three domains: 1) a N-terminal transactivation domain, 2) a hydrophobic transmembrane region, and 3) a C-terminal regulatory domain (170). Although SREBPs have similar consensus DNA binding sites, there seems to be some specificity in transactivation of target genes. Studies using liver specific expression of SREBPs in mice have suggested that SREBP-1a and SREBP-1c regulate genes involved in fatty acid synthesis (171,172), while SREBP-2 regulates multiple genes in cholesterol synthesis (173).

SREBPs can be regulated by three possible mechanisms 1) transcriptional, 2) proteolytic cleavage of SREBP precursors, and (3) post-translational modification of

nuclear SREBPs. Studies involving fasting/refeeding regimes in rodents showed that changes in nutritional status regulated the expression of SREBP-1c in liver, white adipose tissue and skeletal muscles (174-177). The expression of SREBP-1c was found to be reduced during starvation and increased when fed carbohydrate rich diets. Consistent with these observations, insulin and glucagon were found to be the upstream modulators of the increase or decrease in SREBP-1c transcription respectively (172,178). SREBP-1c has also been shown to be regulated by activation of androgens (179), progesterone (180) and the nuclear hormone receptor LXRα (181). SREBP-1a and SREBP-2 also are regulated to a minor extent by transcriptional mechanisms, and under reduced sterol concentrations both proteins can be transcriptionally upregulated (170).

Proteolytic cleavage of SREBP is a highly complicated process as depicted in Figure 1.6. Following translation; SREBPs are localized to the endoplasmic reticulum (ER). When cholesterol and 25-hydroxycholesterol are present in adequate amounts, SREBPs forms a complex with sterol regulatory element binding protein cleavage activating protein (SCAP) and insulin-induced gene (INSIG) proteins (182,183). A fall in intracellular cholesterol levels leads to disruption of the complex, unmasking the sorting signal in SCAP. The SCAP-SREBP complex is then transported to the Golgi via COPII-mediated vesicular transport (182). In the Golgi, two proteases, site 1 protease (S1P) (184,185) and site 2 protease (S2P) (186), sequentially cleave the precursor forms of SREBPs thereby releasing the active N-termini, which translocates to the nucleus to bind and activate their target genes .

Inside the nucleus, the transcriptional activity of SREBPs is regulated by covalent modifications or by interactions with other proteins. Studies in cell lines suggest that insulin via the MAPK pathway leads to phosphorylation of SREBPs at several sites, which have been shown to increases transactivation capacities of SREBPs (187,188). In addition to MAPK, mammalian target of rapamycin (mTOR) has been recently found to influence the transcriptional activity of SREBPs (189). mTORC1 phosphorylates Lipin1 and prevents its nuclear entry. Since nuclear Lipin1 decreases transcriptional activities of both SREBP-1 and SREBP-2, activated mTOR enhances the transactivation potential of SREBP proteins. Reduced activities of SREBPs have also reported to be caused by sumoylation (190) and by degradation via an ubiquitin-proteasome pathway (191).

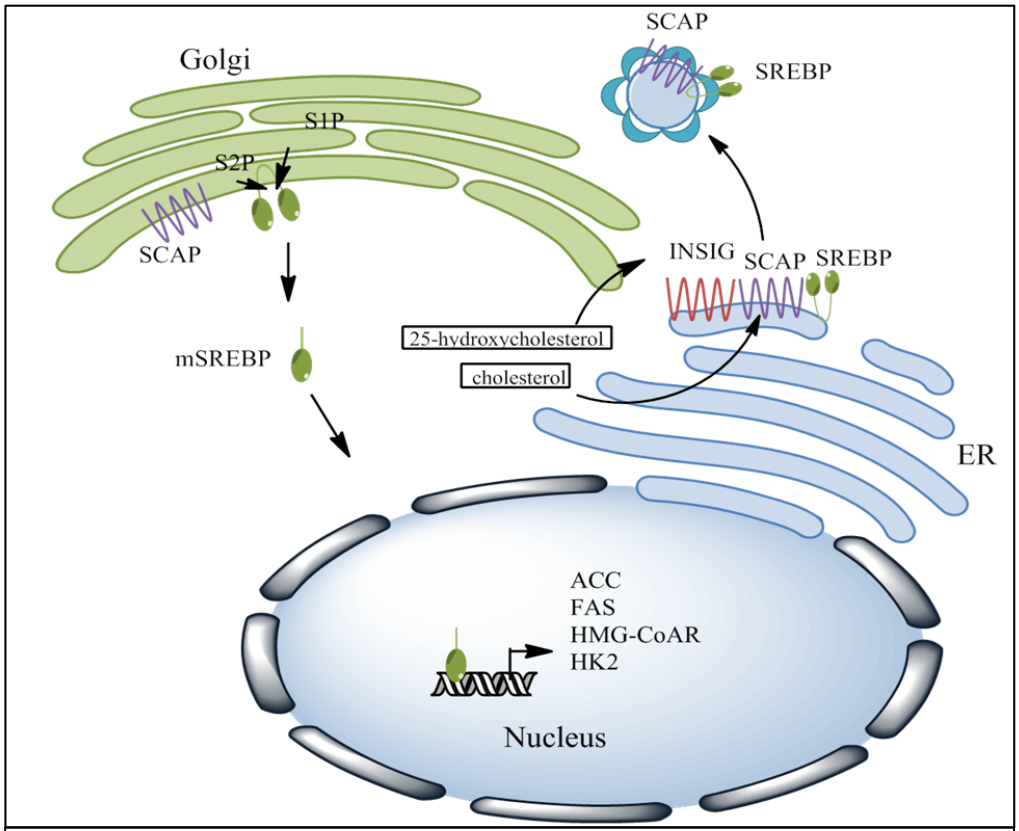


Figure 1.6 Proteolytic activation of SREBP.

1.2.4 AEROBIC GLYCOLYSIS

Glycolysis is a biochemical process that converts glucose to pyruvate, generating 2 molecules of ATP per molecule of glucose. The steps of the process and the enzymes involved are depicted in Figure 1.7. All living cells need energy (ATP) to maintain cellular homeostasis. Transformed cells, however, have increased requirements for energy and intracellular nutrients to carry on non-spontaneous anabolic reactions that support heightened cell growth. It is thus believed that to satisfy the above requirements, cancer cells have undergone a shift to aerobic glycolysis. Aerobic glycolysis generates less ATP but leads to increased carbon flux (as nutrients) in the cell, so to make up for the inefficiency in ATP generation, cancer cells have a heightened rate of glucose uptake. Hence by selection of glycolysis over oxidative phosphorylation, cancer cells have been able to maintain a balance between ATP generation and biomass production. It is interesting to note that some unicellular organisms also prefer glycolysis. For example, when fermentative yeast *Saccharomyces cerevisiae* was grown in media where glucose is not the primary source of carbon, oxidative phosphorylation was preferred, but when glucose was added, they rapidly shifted to the glycolytic pathway and this change resulted in a faster growth rate (192,193). However, the same is not true for aerobic yeast *Yarrowia lipolytica* or for aerobic bacteria Escherichia coli (194,195).

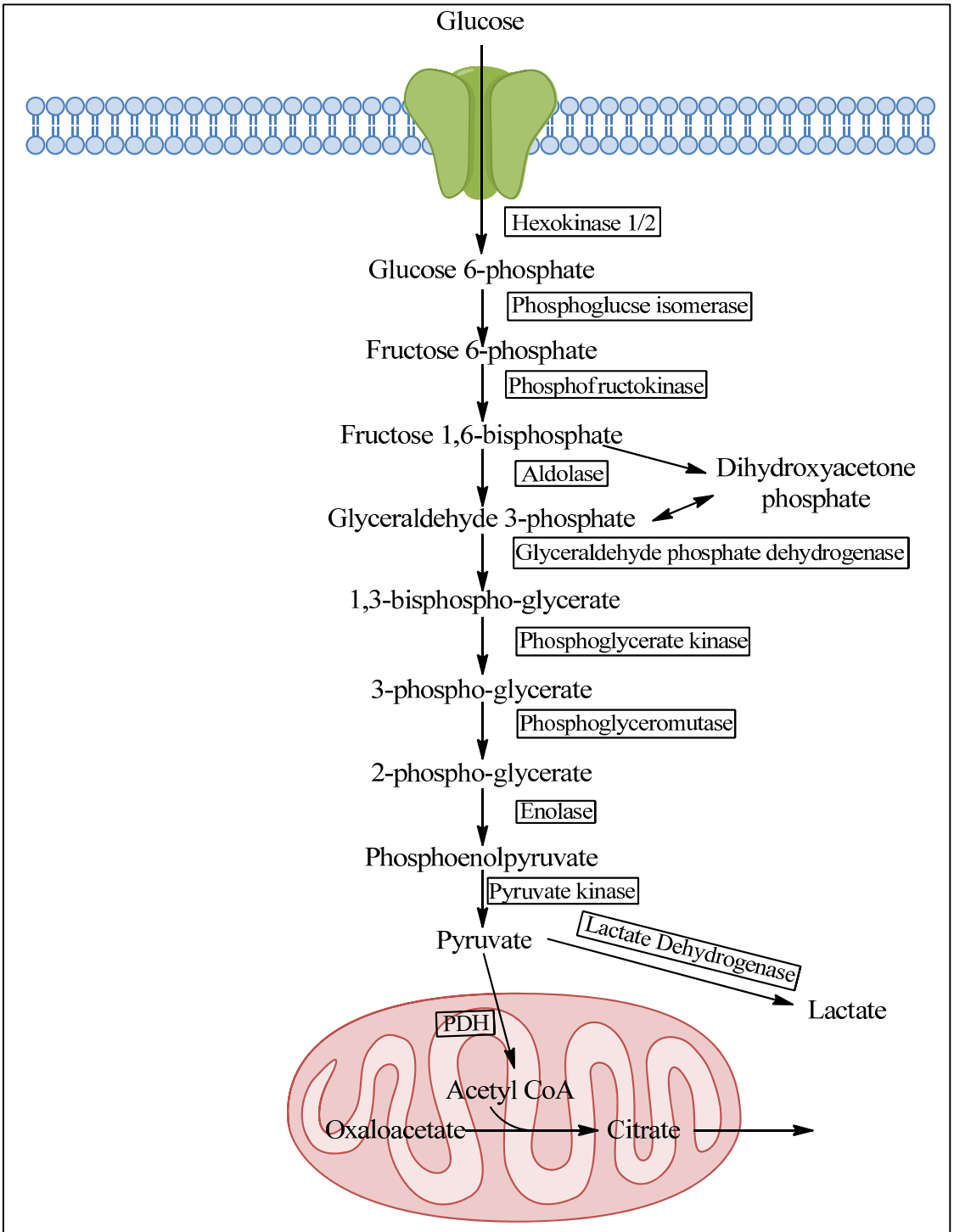


Figure 1.7 Enzymes involved in the glycolytic process.

Thus it can be said that increased glucose uptake for glycolytic ATP generation or anabolic reactions offers the following advantages to facilitate tumor cell growth:

1) Intermediates of the glycolytic pathway can be used for anabolic reactions thus branching into different pathways. For example glucose 6-phosphate can contribute to ribose 5-phosphate synthesis. Dihydroxyacetone phosphate can lead to triacylglyceride and phospholipid synthesis. 3-phosphoglycerate can contribute to cysteine, glycine, and serine synthesis and pyruvate can generate alanine and malate (193).
2) Lactate is the principle end product of glycolysis secreted outside the cell, which leads to acidification of the tumor microenvironments. This acidification aids in tumor cell invasion (196) and immune modulation (197) facilitating tumor growth.
3) A part of the glucose can be diverted to the pentose phosphate pathway (PPP), generating NADPH which is required for fatty acid synthesis.
4) Reliance on glycolysis could provide cancer cells with a survival advantage under reduced oxygen concentrations, which would be fatal for cells relying on oxidative phosphorylation (198).

1.2.5 KEY REGULATORS OF GLYCOLYSIS IN CANCERS

Several studies suggest that control over glycolytic flux primarily resides at the glucose transport and phosphorylation steps of glycolysis (199-201) and thus the key players regulating glycolysis in cancer are glucose transporter 1 (GLUT1), hexokinase 2 (HK-2) and pyruvate kinase muscle isozyme (PKM2).

GLUT1 is the most widely expressed, high affinity glucose transporter and has been reported to be upregulated in various malignancies (202-208). Abnormal expression of GLUT1 occurs early during tumorigenesis and RAS or SRC mediated cell transformation is associated with GLUT1 upregulation (209). In addition, GLUT1 overexpression is linked to poor prognosis of cancer (208,210).

HK-2 catalyzes the irreversible first step of glycolysis, converting glucose to glucose-6-phosphate. This is an important step as phosphorylation prevents exit of glucose from the cell. There are four different isoforms of hexokinases. HK-2 and to a lesser extent HK1 are the only enzymes that have been typically associated with cancer. The switch from HK4 expressed in liver to HK-2 in cancer cells is one of the earliest adaptations observed during tumorigenesis (211-213). Upregulation of HK-2 is seen in a variety of cancers and consequently inhibition of HK-2 is often regarded as a possible therapy against cancer (214,215). HK-2 is localized to the outer membrane of mitochondria and is thought to be attached to the voltage-dependent anion channel (VDAC) (216). This interaction between HK-2 and VDAC not only ensures an efficient supply of ATP to HK-2 but is also critical for prevention of apoptosis (217,218). Thus HK-2 regulates both cell proliferation and survival (219).

Pyruvate kinase (PK) regulates the conversion of phosphoenolpyruvate (PEP) to pyruvate. There are four types of PK. Type l is found in the liver and kidneys, type R in erythrocytes, type M1 in muscle and brain, and type M2 in self-renewing cells such as embryonic and adult stem cells and cancer cells (220,221). While PKM1 is known for its high activity and rapid generation of ATP, PKM2 in cancers has lower activity and

reduces the amount of ATP generated by glycolysis (222,223). A recent study by Christofk et al. showed that PKM2 provides an alternative path for the transfer of a phosphate group, transferring it to PGAM1 (Phosphoglyceric acid mutase 1) instead of ADP (Adenosine diphosphate**)** (224), providing explanation for the loss of ATP. This mechanism thus uncouples ATP generation from glycolysis, thereby allowing glycolytic intermediates to accumulate and enter other subsidiary pathways, including the hexosamine pathway, uridinediphosphate (UDP)–glucose synthesis, glycerol synthesis and the hexose monophosphate shunt.

1.2.6 REGULATION OF GLYCOLYSIS IN CANCER CELLS: CURRENT UNDERSTANDING

Cancers cells are known to change their surrounding environment to better suit their needs. As tumor mass increases, accessibility of tumor cells to oxygen reduces, leading to a hypoxic state which has a dramatic effect on metabolism. Hypoxia leads to stabilization of hypoxia inducible factor -1 (Hif-1) proteins, which regulate almost all genes of the glycolytic pathway (225). Hif-1 is heterodimeric transcription factor composed of α and β subunits; the β-subunit is constitutively expressed but the α-subunit is regulated by the levels of oxygen (226,227). Under normoxic conditions, Hif-1α gets hydroxylated by prolyl hydroxylase domain protein 2 (PHD2) on proline residue 402 and/or 564, and this modification leads to binding of the von Hippel–Lindau tumor suppressor protein (VHL). VHL recruits an E3 ubiquitin ligase that degrades Hif-1α. Under hypoxic conditions, the proline hydroxylation is inhibited, leading to accumulation

of Hif-1α protein, which then binds to Hif-1β and activates transcription of its target genes (225,228). In addition to stabilization under hypoxic conditions, under normoxic conditions Hif-1α may be stabilized by mutations in tumor suppressor proteins such as VHL (229,230), succinate dehydrogenase (SDH) (231) and fumarate hydratase (FH) (232). Hif-1α, once present in sufficient amounts, will transactivate glucose transporters (GLUT1, GLUT3) and enzymes contributing to increased glycolytic phenotype (HKI, HKII, PFK-L, ALD-A, ALD-C, PGK1, ENO-alpha, PYK-M2, LDH-A, PFKFB-3) (233).

In addition to the tumor microenvironment, oncogenes, tumor suppressor genes and their associated signaling pathways also play an important role in regulation of aerobic glycolysis. Activated PI3K and Ras pathways have been shown to activate glycolysis via regulating expression of various glycolytic genes (234). AKT, the downstream effector of PI3K, enhances the rate of glycolysis by multiple mechanisms including increased expression and membrane translocation of glucose transporters and by phosphorylating key glycolytic enzymes, such as hexokinase and phosphofructokinase-1(235,236).

Another oncogene with wide-ranging effects on glycolytic enzymes is c-Myc. It has been shown that c-Myc coordinately regulates genes such as HK-2 and pyruvate dehydrogenase kinase 1, along with Hif-1α (237). In addition to oncogenes, tumor suppressor genes such as TP53 have been shown to upregulate expression of TIGAR (TP53-induced glycolysis and apoptosis regulator), which as its name suggested, prevents glycolysis by decreasing levels of fructose-2, 6-bisphosphate, an allosteric regulator of phosphofructokinase-1. Thus, loss of TP53 in tumor cells leads to increased glycolysis.

CHAPTER 2

LYSOPHOSPHATIDIC ACID ACTIVATES LIPOGENIC PATHWAYS AND *DE NOVO* LIPID SYNTHESIS IN OVARIAN CANCER CELLS

Part of the work presented in this Chapter has been published in Journal of Biological Chemistry J Biol Chem. 2012 Jun 3. [Epub ahead of print]

2.0 ABSTRACT

One of the most common molecular changes in cancer is the increased endogenous lipid synthesis, mediated primarily by overexpression and/or hyperactivity of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). The changes in these key lipogenic enzymes are critical for the development and maintenance of the malignant phenotype. Previous efforts to control oncogenic lipogenesis have been focused on pharmacological inhibitors of FAS and ACC. Although they show anti-tumor effects in culture and in mouse models, these inhibitors are non-selective blockers of lipid synthesis in both normal and cancer cells. To target lipid anabolism in tumor cells specifically, it is important to identify the mechanism governing hyperactive lipogenesis in malignant cells. In the current study, we demonstrate that lysophosphatidic acid (LPA), a growth factor-

like mediator present at high levels in ascites of ovarian cancer patients, regulates the sterol regulatory element binding protein (SREBP)-FAS and AMP-activated protein kinase (AMPK)-ACC pathways in ovarian cancer cells but not in normal or immortalized ovarian epithelial cells. Activation of these lipogenic pathways is linked to increased *de novo* lipid synthesis. The pro-lipogenic action of LPA is mediated through LPA_2, a LPA receptor subtype overexpressed in ovarian cancer and other malignancies. Downstream of LPA_2, the $G_{\alpha 12/13}$ and $G_{\alpha q}$ signaling cascades mediate LPA-dependent SREBP activation and AMPK inhibition, respectively. Moreover, inhibition of *de novo* lipid synthesis dramatically attenuated LPA-induced cell proliferation. These results demonstrate that LPA signaling is causally linked to the hyperactive lipogenesis in ovarian cancer cells, which can be exploited for development of new anti-cancer therapies.

2.1 INTRODUCTION

One of the most common molecular changes in tumor cells is the heightened rate of *de novo* lipid synthesis compared to their normal counterparts. The aberrant lipogenesis in cancer cells is mediated by increased expression and activity of key lipogenic enzymes primarily fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). Interestingly, the alterations in these key lipogenic enzymes are critical for the development and maintenance of the malignant phenotype (238). It occurs at early stages of tumorigenesis and becomes more pronounced in advanced cancers (238,239). Overexpression of FAS correlates with poor prognosis in several types of human malignancies including ovarian cancer (240,241). Furthermore, tumor cells depend

heavily on or are "addicted to" *de novo* lipid synthesis to meet their energetic and biosynthetic needs, irrespective of the nutritional supplies in the circulation (238). Consistent with this, pharmaceutical inhibitors of FAS suppress tumor cell proliferation and survival, and enhance cytotoxic killing by therapeutic agents (158,242-246). However, one barrier to cancer patient application of these inhibitors is their non-selective suppression of fatty acid synthesis in both normal and malignant tissues, which could contribute to weight loss, anorexia, fatigue and other cancer-associated complications. To target lipid anabolism in tumors specifically, it is important to identify the mechanism for the hyperactive lipogenesis in cancer cells, which is, however, poorly understood.

Lysophosphatidic acid (LPA), the simplest phospholipid, has been long known as a mediator of oncogenesis (36). LPA is present at high levels in ascites of ovarian cancer patients and other malignant effusions (36,247,248). LPA is a ligand of at least six G protein-coupled receptors (GPCRs) (70). The LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 receptors are members of the endothelial differentiation gene (Edg) family, sharing 46 - 50% amino acid sequence identity (70). 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 distant from the LPA_{1-3} receptors (70,249). The Edg LPA receptors, in particular LPA_2, are overexpressed in many types of human malignancies including ovarian cancer (36,107). Strong evidence implicates LPA_2 in the pathogenesis of ovarian, breast and intestine tumors (83,107,113), although the exact oncogenic processes involved remain elusive.

In the present study, we observed that LPA stimulated proteolytic activation of two isoforms of the sterol regulatory element binding proteins (SREBPs), transcription factors involved in regulation of FAS and other lipogenic enzymes for biosynthesis of fatty acid and cholesterol. In addition, LPA induces dephosphorylation of AMPKα at Thr-172 and concomitant dephosphorylation of ACC at Ser-79. The dephosphorylation of ACC at Ser-79 is associated with activation of the enzyme (250). These LPA-induced changes in the lipogenic enzymes occurred hours after exposure to LPA and the effects were sustained for many hours. Consistent with LPA activating these lipogenic pathways, LPA increased *de novo* lipid synthesis. We identified LPA_2, the receptor subtype overexpressed in ovarian cancer and other human malignancies, as the key receptor responsible for delivery of the lipogenic effect of LPA. The intracellular $G_{\alpha 12/13}$-Rho signaling cascade is critical for LPA activation of the SREBP while $G_{\alpha q}$-PLC is involved in LPA-mediated dephosphorylation and inhibition of AMPK. These findings reveal a novel mode of the cancer cell-specific regulation of lipogenesis by an intercellular factor present in the circulation and tumor microenvironments.

2.2 EXPERIMENTAL PROCEDURES

Reagents – 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) purchased from Roche (Indianapolis, IN). Acetic acid (1-^{14}C) was obtained from Moravek Biochemicals (Brea, CA). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). The transfection reagent

Dharmafect 1 was obtained from Dharmacon, Inc. (Lafayette, CO) and TransIT-TKO was obtained from Mirus Bio (Madison, WI). Luciferase assay reagents were obtained from Promega (Madison, WI). Anti-SREBP-1 and anti-SREBP-2 antibodies were obtained from BD Biosciences (San Jose, CA). Anti-phospho-AMPKα (Thr-172), anti-AMPKα, anti-phospho-ACC (Ser-79), anti-ACC, and anti-FAS antibodies were obtained from Cell Signaling (Danvers, MA). Anti-Tubulin antibody was obtained from EMD4Biosciences (Gibbstown, NJ). BODIPY 493/503 and cell culture reagents were purchased from Invitrogen Inc. (Carlsbad, CA). The TaqMan Universal PCR Master Mix and qPCR probes for LPA_1, LPA_2, LPA_3, 3-Hydroxy-3-methylglutaryl-CoA (HGM-CoA) reductase and GAPDH were obtained from Applied Biosystems (Carlsbad, CA). Calpain I inhibitor, water soluble cholesterol, the FAS inhibitor C75, the ACC inhibitor TOFA and sodium palmitate were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture – The sources of ovarian cancer cell lines used in the study were described previously **(251)**. These cells were cultured in RPMI medium supplemented with 10% FBS, 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 **(252)**.

siRNA, plasmids and transfection – The siRNA oligos for LPA_1, LPA_2 LPA_3, and FAS were obtained from Applied Biosystems. These siRNAs were transfected into cells using Dharmafect 1 following the manufacturer's protocol. In brief, cells were plated in 6-well plates to reach 50-60% confluence before transfection. Cells were then transfected with

target specific siRNA or non-targeting control siRNA (150 picoM) with Dharmafect 1 (4 μL) for 12-16 hours. Approximately 48 hours post transfection, the cells were serum starved overnight before LPA treatment. Lentiviruses carrying short hairpin RNA (shRNA) for LPA_{1-3} receptors were kind gifts from Dr. S. Huang (Medical College of Georgia) (253). The expression vector pcDNA3 expressing dominant negative form of $G\alpha_i$ was provided by Dr. P. Hylemon (Virginia Commonwealth University) (254,255). The $G\alpha_q$ and $G\alpha_{12}$ cDNAs were provided by Dr. RD Ye (University of Illinois at Chicago). The dominant-negative mutants of $G\alpha_q$ (G208A) and $G\alpha_{12}$ (G228A) (256-258) in pcDNA3 were made using the QuikChange XL site directed mutagenesis kit (Stratagene, Santa Clara, CA). The plasmids and the vectors expressing N19Rho and Botulinum toxin C3 were described previously (259,260). These plasmids were transfected into ovarian cancer cell lines using Lipofectamine LTX plus (Invitrogen) following the manufacturer's instruction.

Luciferase assays – The SREBP responsive luciferase reporter vector (pGL2–3xSREBP-TK-Luc) was generated by cloning 3 repeats of the SREBP consensus sequence (AAAATCACC CCACTGCAAACTCCTCCCCCTGC) (261,262) into the *Nhe*I and *Hind*III sites in front of the herpes simplex virus thymidine kinase (TK) gene promoter (–35 to +50) in the pGL2-TK-Luc vector (128). Ovarian cancer cell lines were transfected with the luciferase vector using TransIT-TKO according to the manufacturer's protocol. About 48 hours after transfection, the cells were starved overnight and treated with LPA or vehicle (BSA) for 12 hours. Cell extracts were prepared and assayed for luciferase activity using the luciferase assay kits from Promega.

Western blotting – Cells were lysed as previously described (263). Total cellular proteins were resolved by SDS-PAGE, transferred to immunoblot membrane (polyvinylidene difluoride) (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).

Quantitative PCR (qPCR) – Total cellular RNA was isolated from cultured cells using Trizol (Invitrogen). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative levels of LPA_1, LPA_2, LPA_3, HMG-CoA reductase and GAPDH were determined by qPCR using gene specific probes, the TaqMan Universal PCR Master Mix, and the Applied Biosystems 7900HT Real-Time PCR System.

Measurement of *de novo* lipid synthesis – Cells were grown in 6-well plates and serum starved prior to treatment with LPA or vehicle for 24 hours. The cells were labeled with ^{14}C acetic acid (5 μCi/ml) for the last 6 hours of incubation. The cells were then washed twice with PBS and lysed with lysis buffer (25 mM HEPES, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.2 mM EDTA, 0.5% sodium deoxycholate, 20 mM glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Lipids were extracted using a chloroform: methanol solution (2:1). Phase separation was achieved by centrifugation at 3200 x g for 10 minutes. The organic phase was extracted and dried with speed vacuum. Lipids were dissolved in Ultima Gold Cocktail (Perkin Elmer, Waltham, MA) and counted using Beckman LS 6500 scintillation counter. Each

measurement was performed in triplicate and normalized to cell numbers.

Lipid staining – Cells were grown and serum starved prior to treatment with LPA or vehicle for 24 hours. Cells were then stained with BODIPY 493/503 at final concentration of 0.5 μg/ml in PBS at 37°C for 30 minutes, followed by counter staining with Hoechst (10 μg/ml) for 15 minutes. Cells were then fixed with 2% paraformaldehyde and visualized with fluorescence microscopy.

Quantification of triacylglycerols (TAG) and phospholipids – TAG and phospholipids were extracted and quantified with the EnzyChrom Triglyceride Assay kit and the EnzyChrom Phospholipid Assay kit (BioAssay Systems, Hayward, CA), respectively, according to the manufacturer.

HPLC analysis of ATP/AMP ratio – Cells were serum starved for 16-18 hours prior to LPA treatment. Nucleotides were extracted using 5% perchloric acid. Samples were then subjected to HPLC analysis using BioBasic AX column. The phases A and B were 5 mM KH_2PO_4 and 750 mM KH_2PO_4, respectively. The pH of both solutions (which both solutions?) was adjusted to 3.2 using phosphoric acid. Nucleotides were separated using a gradient of 0-100% of phase B in 30 min, at the flow rate of 1 ml/min and detected at 254 nm. The retention time for the AMP and ATP were obtained by running specific standards, based on which corresponding peaks of samples were identified. Data acquisition and analysis were carried out using the Shimadzu LC solution.

Anchorage-independent growth - Anchorage independent growth of cells was

determined by soft agar assays in 6-well plates. Briefly, bottom layer of 0.6% soft agar in complete medium was prepared. Following which and a top layer of 0.3% soft agar including 3000 cells were applied into each well. After incubating the plates for 14 days, colonies were stained with crystal violet solution and colonies were counted under microscope.

Statistics – All numerical data were presented as mean ± SD. The statistical significances of differences were analyzed using Student's *t* test where $p<0.05$ was considered statistically significant. In all figures, the statistical significances were indicated with * if $p < 0.05$ or ** if $p < 0.01$.

2.3 RESULTS

2.3.1 LPA INDUCES PROTEOLYTIC CLEAVAGE AND ACTIVATION OF SREBP IN A CHOLESTEROL-SENSITIVE MANNER

Hyperactive lipogenesis is a hallmark of tumor cells (154,238). To identify pathophysiological mechanisms driving the lipogenic program in cancer cells, we examined the potential role of LPA, an endogenous regulator of many cellular functions in ovarian cancer and other human malignancies. We first assessed whether LPA was capable of activating the SREBP transcription factors that play crucial roles in regulating expression of lipogenic enzymes. Treatment of Caov-3, OVCA-432 and other ovarian cancer cell lines including OVCAR-3 with LPA induced cleavage of the precursor forms of SREBP-1 and SREBP-2 in a time-dependent manner (Figure. 2.1). The cleaved, mature forms of SREBP-1 and SREBP-2 were detectable at 4 hours and peaked at 12

hours post LPA treatment. In contrast to the ovarian cancer cell lines, LPA failed to activate SREBP-1 or SREBP-2 in the immortalized ovarian surface epithelial cell line IOSE-29 (Fig. 2.1) or normal ovarian epithelial cells (data not shown), suggesting a cancer cell-specific mechanism for SREBP activation by LPA in ovarian cancer cells.

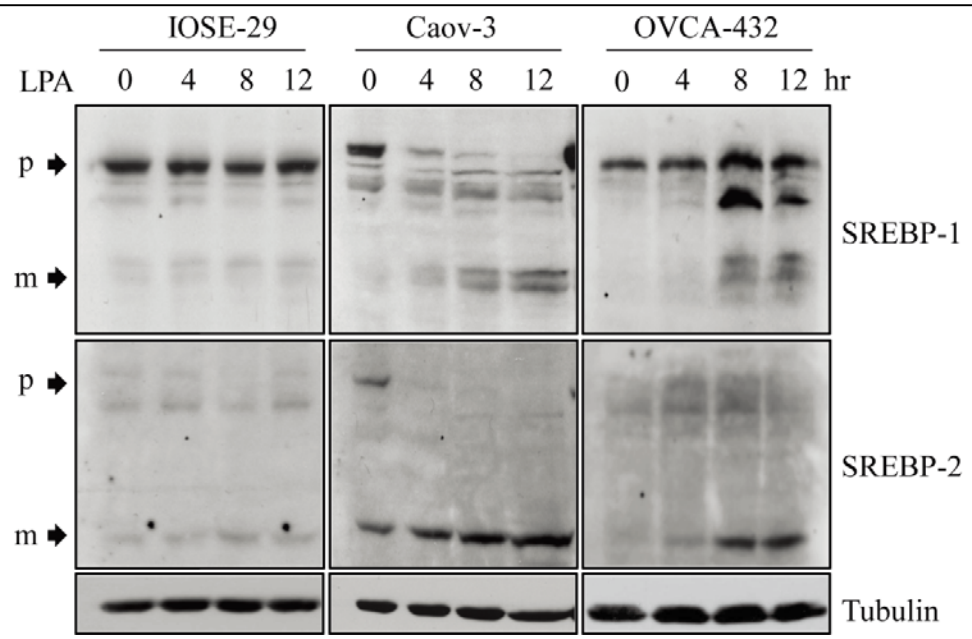


Figure 2.1 LPA activates SREBP in ovarian cancer cells. Ovarian cancer cell lines and IOSE-29 cells were treated with LPA (10 μM) for indicated periods of time. The calpain inhibitor I (25 μg/ml) was added to cells for the last 2 hours. Expression of SREBP-1 and SREBP-2 was analyzed by immunoblotting with antibodies that recognize both precursor (p) and active/mature (m) forms of SREBP-1 and SREBP-2.

Under physiological conditions, SREBP-1 and SREBP-2 are regulated by the intracellular sterol content. In their precursor forms, SREBPs are attached to the endoplasmic reticulum (ER). Specific signaling cues such as reduced cholesterol levels trigger SREBP cleavage-activating protein (SCAP)-mediated transport of SREBP from

the ER to the Golgi, where they are cleaved by proteases S1P and S2P to release the mature/active form (264). At high sterol concentrations, the SREBP/SCAP complex is retained in the ER due to increased binding to INSIG proteins (265). To determine whether LPA activation of SREBP could bypass cholesterol regulation, we preloaded Caov-3 and OVCA-432 cells with cholesterol (10 μg/ml) complexed with 0.1% fraction V fatty acid-free BSA in PBS, and then assessed activation of SREBP-1 in response to LPA. As shown in Fig. 2.2A, cholesterol treatment reduced both basal and LPA-induced active SREBP-1 levels, indicating that activation of SREBP by LPA remains sensitive to cholesterol availability.

To determine whether LPA-induced SREBP cleavage is sufficient to activate SREBP transcriptional activity, Caov-3 and OVCA-432 cells were transfected with the SREBP responsive reporter pGL2–3xSREBP-TK-Luc. As shown in Fig. 2.2B, treatment of transfected cells with LPA significantly enhanced luciferase activity in these cells. Similar to the SREBP cleavage, SREBP-dependent luciferase activity was also sensitive to cholesterol treatment (Fig. 2.2B).

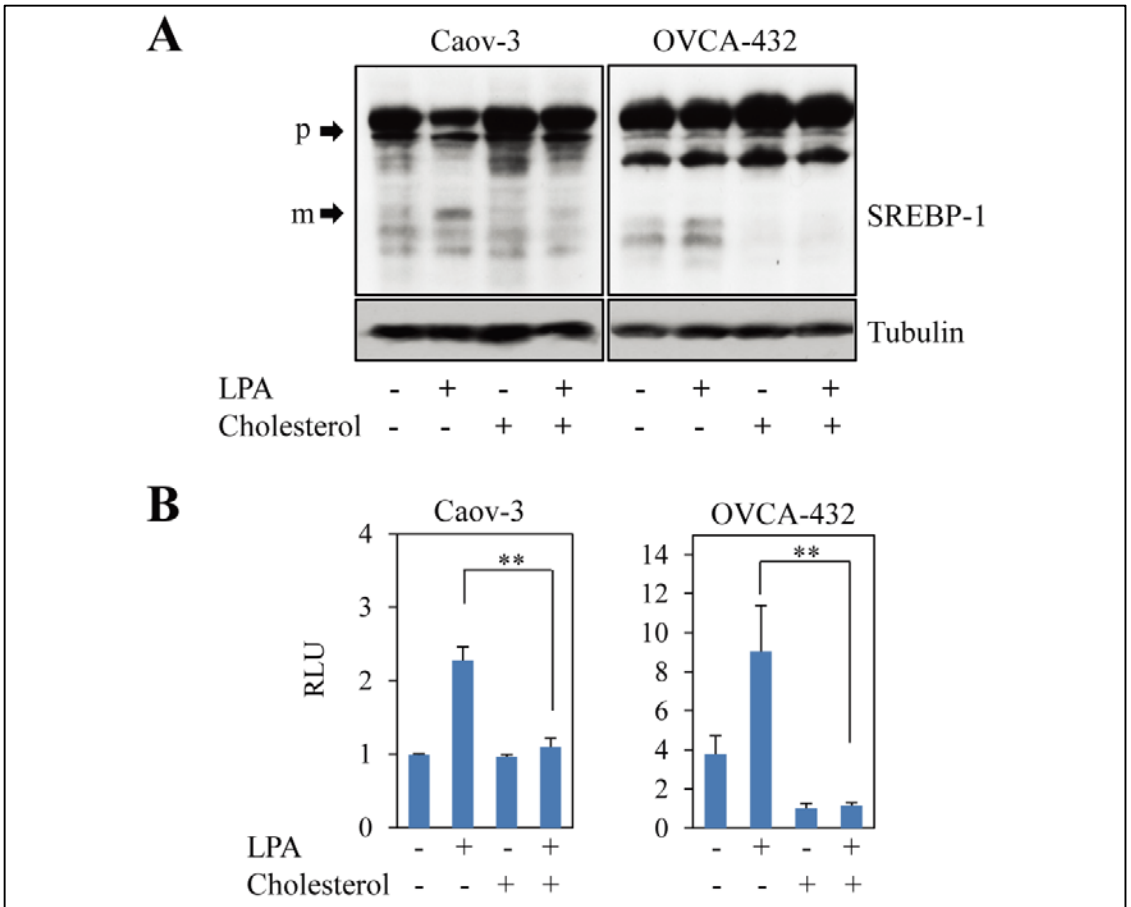


Figure 2.2 LPA activation of SREBP remains sensitive to cholesterol availability. In ***A***, Caov-3 and OVCA-432 cells were preloaded with or without cholesterol (10 μg/ml). The cells were treated with LPA and analyzed for expression of precursor and mature forms of SREBP as in ***Fig. 2.1.*** In ***B***, Caov-3 and OVCA-432 cells were transfected with pGL2–3xSREBP-TK-Luc and loaded with or without cholesterol before stimulation with LPA (10 μM) for 12 hours. The luciferase activity in cell extracts was determined as described in Experimental Procedures and the results presented as relative luciferase units (RLU).

2.3.2 LPA INDUCES EXPRESSION OF THE SREBP TARGET GENES FAS, ACC AND HMG-COA REDUCTASE

To substantiate the biological significance of SREBP activation by LPA, we monitored expression levels of FAS, ACC, and HMG-CoA reductase. These are well-known targets of SREBP-1 and SREBP-2 involved in biosynthesis of fatty acid and cholesterol. Treatment of Caov-3, OVCA-432 and OVCAR-3 cells with LPA increased expression levels of FAS and ACC proteins as shown in Fig. 2.3A. The mRNA levels of these key enzymes for fatty acid synthesis (data not shown) and the rate-limiting enzyme for cholesterol synthesis HMG-CoA reductase were also significantly increased by treatment of ovarian cancer cell lines with LPA (Fig. 2.3B), providing evidence that activation of SREBP-1 and SREBP-2 by LPA is sufficient to increase expression of key endogenous lipogenic enzymes in ovarian cancer cells.

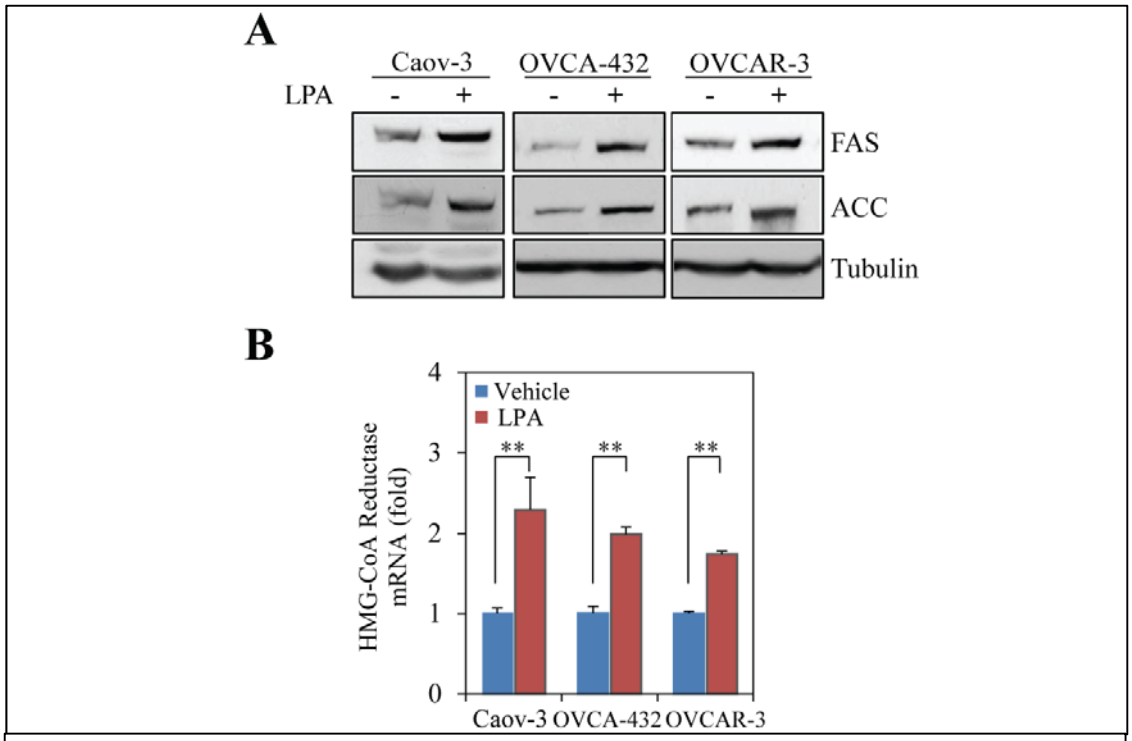


Figure 2.3 LPA induces expression of the SREBP target genes FAS, ACC and HMG-CoA reductase. Caov-3, OVCA-432 and OVCAR-3 cells were treated with or without LPA (10 μM) for 16 hours prior to immunoblotting analysis of FAS and ACC (***A***). Total cellular RNA was isolated from parallel samples and subjected to RT-qPCR analysis of expression of HMG-CoA reductase mRNA (***B***). The results were presented as fold increase relative to the value in the vehicle-treated cells (defined as 1).

2.3.3 LPA INDUCES DEPHOSPHORYLATION OF AMPK AND ACC

In addition to transcriptional upregulation, the activity of ACC is inhibited by AMPK mediated phosphorylation. AMPK, a highly conserved protein serine/threonine kinase, acts as an energy sensor and regulator of cellular metabolism, shutting down energy-consuming anabolic processes and activating energy-yielding catabolic processes (266). AMPK is activated through phosphorylation of Thr-172 within the activation

domain of the α-subunit (267). To determine the effect of LPA on AMPK and its downstream target ACC, we analyzed the phosphorylation status of AMPKα at this residue as a surrogate of activation of the enzyme. Treatment of Caov-3 and OVCA-432 cells with LPA induced a late onset and sustained dephosphorylation of AMPKα (Fig. 2.4A). The decrease in AMPKα phosphorylation was detectable at 8 hours and became prominent at 12 hours. Consistent with a predominant role of AMPKα in phosphorylation of ACC, AMPKα dephosphorylation in LPA-treated cells was accompanied by a decrease in ACC phosphorylation at Ser-79 (Fig. 2.4A). Dephosphorylation of this site is known to enhance ACC enzymatic activity. The effects of LPA on dephosphorylation of AMPKα and ACC were not detected in IOSE-29 cells (data not shown). These results establish that, in ovarian cancer cells, LPA signaling is coupled to activation of ACC via inhibition of AMPK. Moreover, we used HPLC to measure AMP/ATP ratios in Caov-3 cells treated with LPA for 12 hours. As seen in Fig 2.4B, LPA treatment led to a significant decrease in cellular AMP/ATP ratio. The decreased the AMP/ATP ratio could trigger the dephosphorylation/inactivation of AMPK seen in LPA-treated cells.

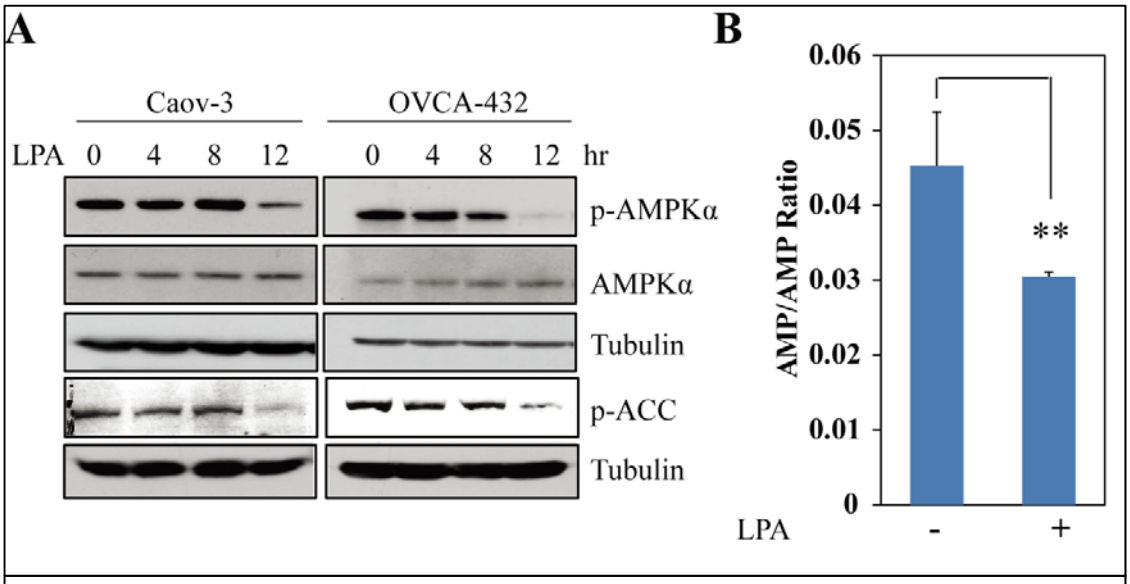


Figure 2.4 LPA induces dephosphorylation of AMPKα and ACC. ***A.*** Caov-3 and OVCA-432 cells were treated with or without LPA (10 μM) for the indicated periods of time. The cell lysates were analyzed with immunoblotting for phosphorylation status of AMPKα and ACC using their phospho-specific antibodies recognizing AMPKα phosphorylated at Thr-172 or ACC phosphorylated at Ser-79. ***B.*** Caov3 cells were serum starved overnight prior to LPA (10 μM) treatment for 12 hours. Nucleotides were extracted and analyzed with HPLC as described in Experimental Procedures.

2.3.4 LPA PROMOTES *DE NOVO* LIPID SYNTHESIS

Few studies have examined the role of exogenous factors in regulation of lipogenesis in cancer cells (157,158). We next examined whether LPA-induced activation of lipogenic enzymes is functionally sufficient to stimulate *de novo* lipid synthesis. The ovarian cancer cell lines Caov-3 and OVCA-432, and the immortalized IOSE-29 cells were treated with LPA or BSA as vehicle control and pulse labeled with ^{14}C acetic acid to monitor new lipid synthesis. As demonstrated in Fig. 2.5 (*left*), LPA treatment led to a significant increase in ^{14}C incorporation into the cellular lipid fractions, reflecting an

increase in newly synthesized lipids in response to LPA. The lipogenic effect of LPA was specifically detected in multiple ovarian cancer cell lines but not in the non-transformed IOSE-29 cells, wherein LPA failed to induce SREBP activation or AMPK dephosphorylation. Since these cells were treated with LPA in serum-free medium lacking extracellular fatty acids, we wanted to determine if the increase in lipogenesis in response to LPA was influenced by availability of extracellular lipids. As shown in Fig. 2.5 (*right*), exogenously supplemented palmitate slightly reduced LPA-driven lipogenesis. However, the reduction was statistically insignificant, indicating that the lipogenic role of LPA is largely independent of availability of extracellular fatty acids.

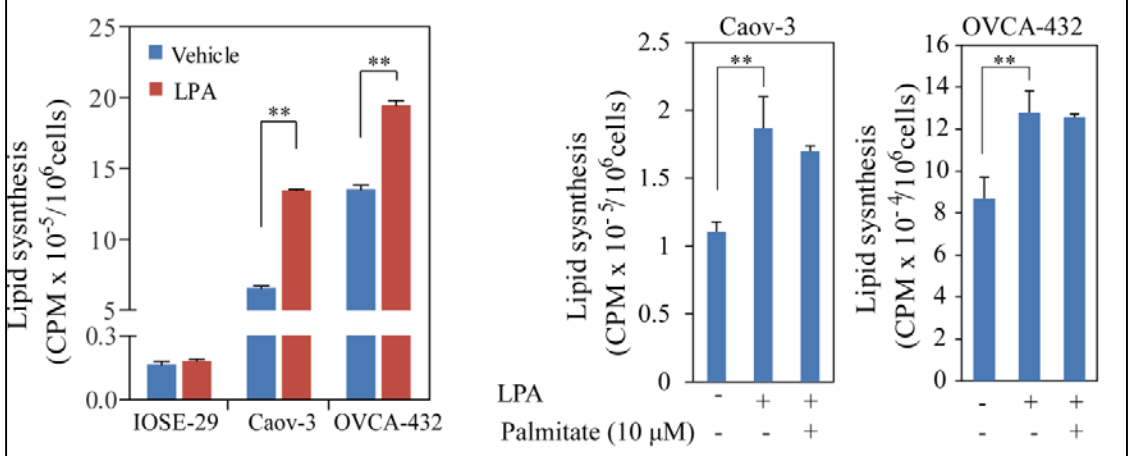


Figure 2.5 LPA stimulates *de novo* lipid synthesis independently of availability of extracellular fatty acids. Caov-3, OVCA-432 and IOSE-29 cells were treated with LPA (10 μM) or BSA (vehicle) for 24 hours. In the last 6 hours of incubation, the cells were pulse labeled with 5 μCi/ml of ^{14}C acetic acid before lipid extraction as described in Experimental Procedures. The incorporation of ^{14}C into lipid fractions was determined by scintillation counting. The results were presented as CPM per 1 x 10^6 cells (***left***). Caov-3 and OVCA-432 cells were treated with LPA in serum-free medium supplemented with palmitate (10 μM) and BSA (0.01 %). LPA-induced lipogenesis was measured as described above ***(right)***.

Consistent with the pro-lipogenic action of LPA, staining with a lipophilic dye BODIPY 493/503 revealed that LPA induced moderate increases in the intracellular contents of neutral lipids in Caov-3 and OVCA-432 cells but not in IOSE-29 cells (Fig. 2.6A). These results were further supported by the increases in both cellular TAG and phospholipids following LPA treatment (Fig. 2.6B & 2.6C).

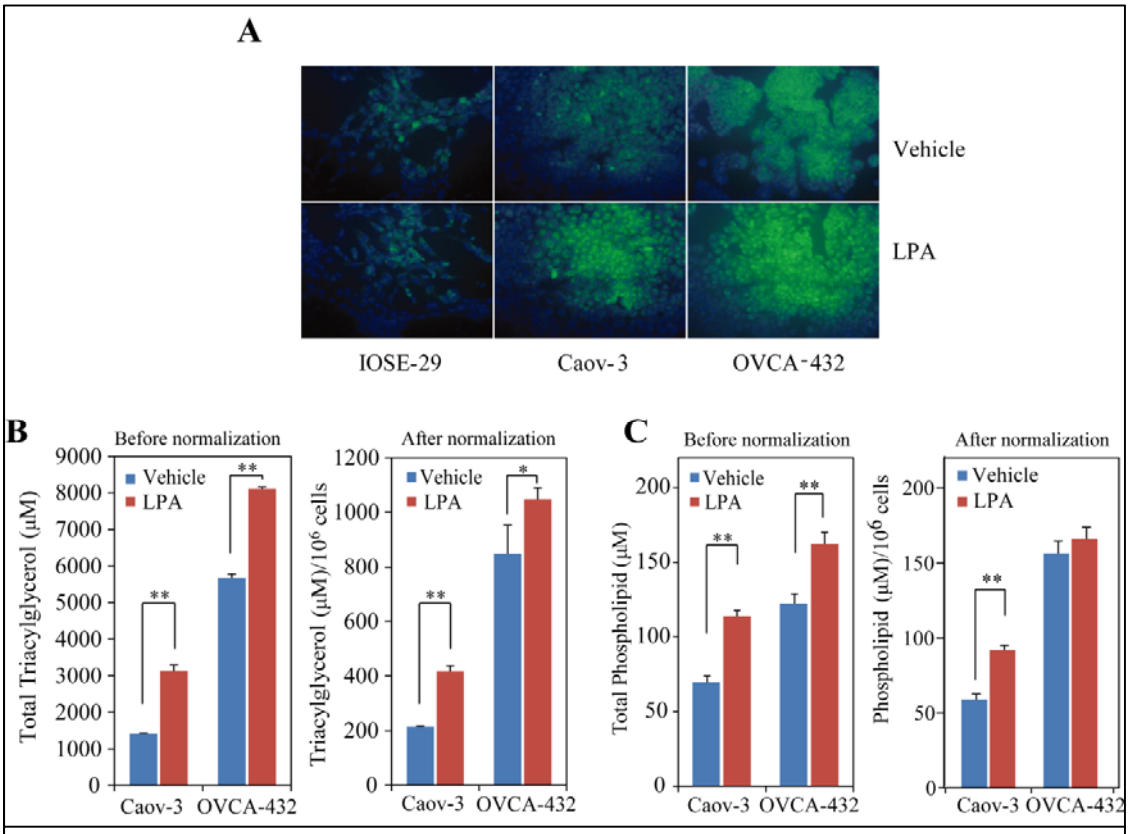


Figure 2.6 LPA increases neutral and phospholipid contents. ***A.*** Cells in 6-well plates were stained with BODIPY 493/503 fluorescent dye (0.5 μg/ml) for 30 minutes, followed by staining with Hoechst (10 μg/ml) for 15 minutes to monitor neutral lipid accumulation. Shown were fluorescence microscopic photographs of IOSE-29, Caov-3 and OVCA-432 cells treated with or without LPA (x 80 magnification). Total TAG (***B***) and phospholipids (***D***) in control and LPA-treated Caov-3 and OVCA-432 cells were determined as described in Experimental Procedures. The results were presented as amounts of lipids per well, or normalized on cell numbers to represent amounts of lipids per million cells.

2.3.5 LPA_2 IS THE MAJOR RECEPTOR SUBTYPE RESPONSIBLE FOR REGULATION OF SREBP AND AMPK

Caov-3, OVCA-432 and other ovarian cancer cell lines express the Edg LPA receptors LPA_1, LPA_2, and LPA_3 (Fig. 2.7A). The other non-Edg LPA receptors are either absent or expressed inconsistently in ovarian cancer cells (94,268). Thus, we focused on the potential role of LPA_{1-3} in the regulation of lipogenesis. We used siRNA to knockdown expression of LPA_1, LPA_2, and LPA_3 in Caov-3 cells and examined SREBP activation and AMPKα dephosphorylation in response to LPA treatment. Interestingly, only knockdown of LPA_2 significantly attenuated LPA-induced cleavage of SREBP-1, dephosphorylation of AMPKα at Thr-172 (Fig. 2.7B), and expression of FAS and ACC (Fig. 2.7C). There was minimal inhibitory effect on SREBP-1 activation, AMPKα dephosphorylation and expression of FAS and ACC in conjunction with LPA_1 or LPA_3 knockdown. We encountered a technical difficulty in achieving efficient knockdown of LPA receptors with transient siRNA in OVCA-432 cells. However, similar results were obtained from OVCA-432 cells when LPA receptors were stably knocked down by lentivirus-transduced shRNA (Fig. 2.7B & 2.7C). These results support a primary role of the LPA_2 receptor in LPA-dependent activation of SREBP-1 and inhibition of AMPKα. However, overexpression of LPA_2 in IOSE-29 cells was not sufficient to activate LPA-dependent induction of FAS and ACC (data not shown), suggesting that additional signaling player(s) present specifically in malignant cells is involved.

To verify this receptor subtype-specific regulation of lipogenesis, we examined the effect of LPA_2 knockdown on LPA-driven lipogenesis. The *de novo* lipid synthesis in LPA receptor knockdown and control cells was assessed as described earlier. The endogenous lipid synthesis induced by LPA was strongly attenuated by siRNA- or shRNA-mediated downregulation of LPA_2 (Fig 2.7D). In contrast, knockdown of LPA_3 (Fig. 2.7D) or LPA_1 (data not shown) did not inhibit LPA-induced lipid synthesis.

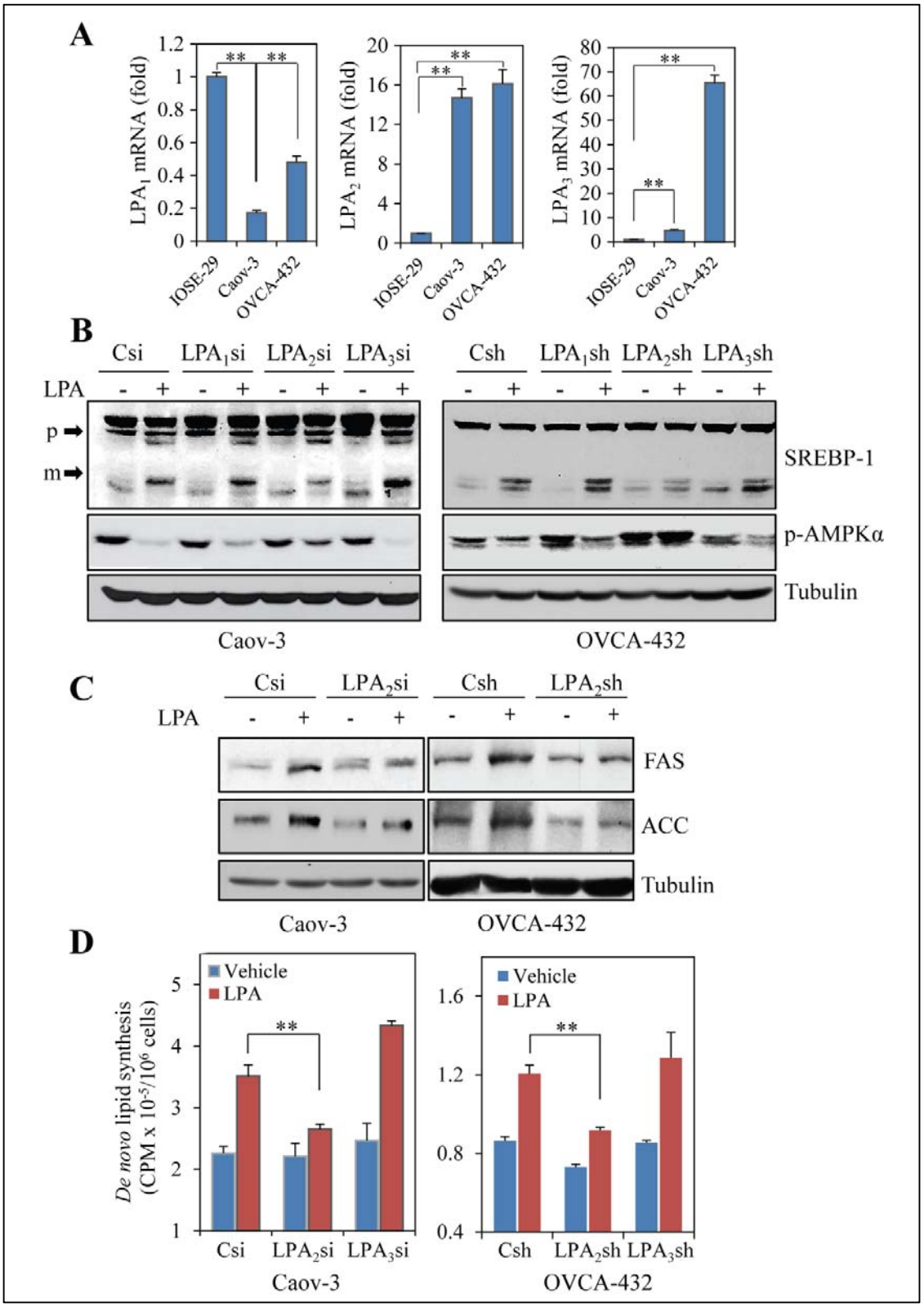

A
LPA1 mRNA (fold)
LPA2 mRNA (fold)
LPA3 mRNA (fold)
IOSE-29
Caov-3
OVCA-432
B
Csi
LPA1si
LPA2si
LPA3si
Csh
LPA1sh
LPA2sh
LPA3sh
LPA
p
m
SREBP-1
p-AMPKα
Tubulin
Caov-3
OVCA-432
C
FAS
ACC
Tubulin
D
Vehicle
LPA
De novo lipid synthesis (CPM x 10-5/106 cells)

Figure 2.7 LPA_2 mediates the lipogenic effect of LPA. ***A.*** Expression of mRNAs of LPA_{1-3} receptors in IOSE-29, Caov-3 and OVCA-432 cells was determined by qPCR analysis as detailed in Experimental Procedures. The results were presented as fold difference relative to the mRNA levels of LPA receptors in IOSE-29 cells (defined as 1). ***B.*** Caov-3 cells were transfected with siRNA for each LPA receptor (LPA_1si, LPA_2si and LPA_3si) or with non-targeting control siRNA (Csi). Expression of each LPA receptor in OVCA-432 cells was downregulated by lentivirus-transduced shRNA. The knockdown efficiencies for each LPA receptor in both cell lines range from 60 to 80% as determined by RT-qPCR analysis (data not shown). The cells were stimulated with LPA (10 μM) for 12 hours before immunoblotting analysis of SREBP-1 and phospho-AMPKα. In ***C***, the effects of LPA_2 knockdown on FAS and ACC induction in Caov-3 and OVCA-432 cells were examined by immunoblotting analysis. In ***D***, the effects on lipid synthesis of siRNA or shRNA knockdown of LPA_1, LPA_2 or LPA_3 receptor in Caov-3 and OVCA-432 cells were measured as described in Figure 2.5.

2.3.6 LPA_2 SIGNALING BIFURCATES TO REGULATE SREBP-1 AND AMPKα

We next examined the signaling effectors downstream of LPA_2 responsible for cleavage of SREBP-1 and dephosphorylation of AMPKα. The LPA_{1-3} receptors couple to $G_{\alpha i}$ and $G_{\alpha q}$, while only LPA_1 and LPA_2 couple to $G_{\alpha 12/13}$ (269). We transfected dominant negative forms of these G proteins into highly transfectable Caov-3 cells in an effort to screen for G proteins critical for LPA-dependent SREBP-1 cleavage and AMPKα dephosphorylation. As shown in Fig. 2.8A, expression of the dominant negative $G_{\alpha 12}$ attenuated LPA-induced SREBP-1 cleavage but not LPA-induced dephosphorylation of AMPKα. In contrast, expression of dominant negative $G_{\alpha q}$ inhibited AMPKα dephosphorylation but not SREBP-1 cleavage induced by LPA. Thus, different G protein cascades are implicated in the regulation of SREBP and AMPK by LPA. Since a

prominent effector of $G_{\alpha 12/13}$ is the Rho GTPase, we examined whether Rho is required for LPA activation of SREBP. As expected, expression of dominant negative Rho (N19Rho) or Botulinum toxin C3, a specific inhibitor of Rho GTPase, suppressed LPA-induced cleavage of SREBP-1 (Fig. 2.8B) as compared to vector-transfected cells. The results demonstrate that LPA_2 promotes SREBP activation in a Rho-dependent pathway. To determine the downstream effector of Rho that activates SREBP, we used inhibitors for various pathways to determine their effect on LPA-induced SREBP transcriptional activity. As shown in Figure 2.8C, Y-27632 (Rho-associated protein kinase, Rock inhibitor) abrogated LPA-driven SREBP activity.

To elucidate the regulatory network leading to AMPK dephosphorylation, we used pharmacological inhibitors of signaling molecules downstream of $G_{\alpha q}$. As shown in Fig. 2.8D, the PLC inhibitor U73122, but not it's inactive analog U73433, blocked AMPKα dephosphorylation induced by LPA. The data supports a $G_{\alpha q}$-PLC-dependent mechanism to control phosphorylation and activity of AMPKα in LPA-treated cells.

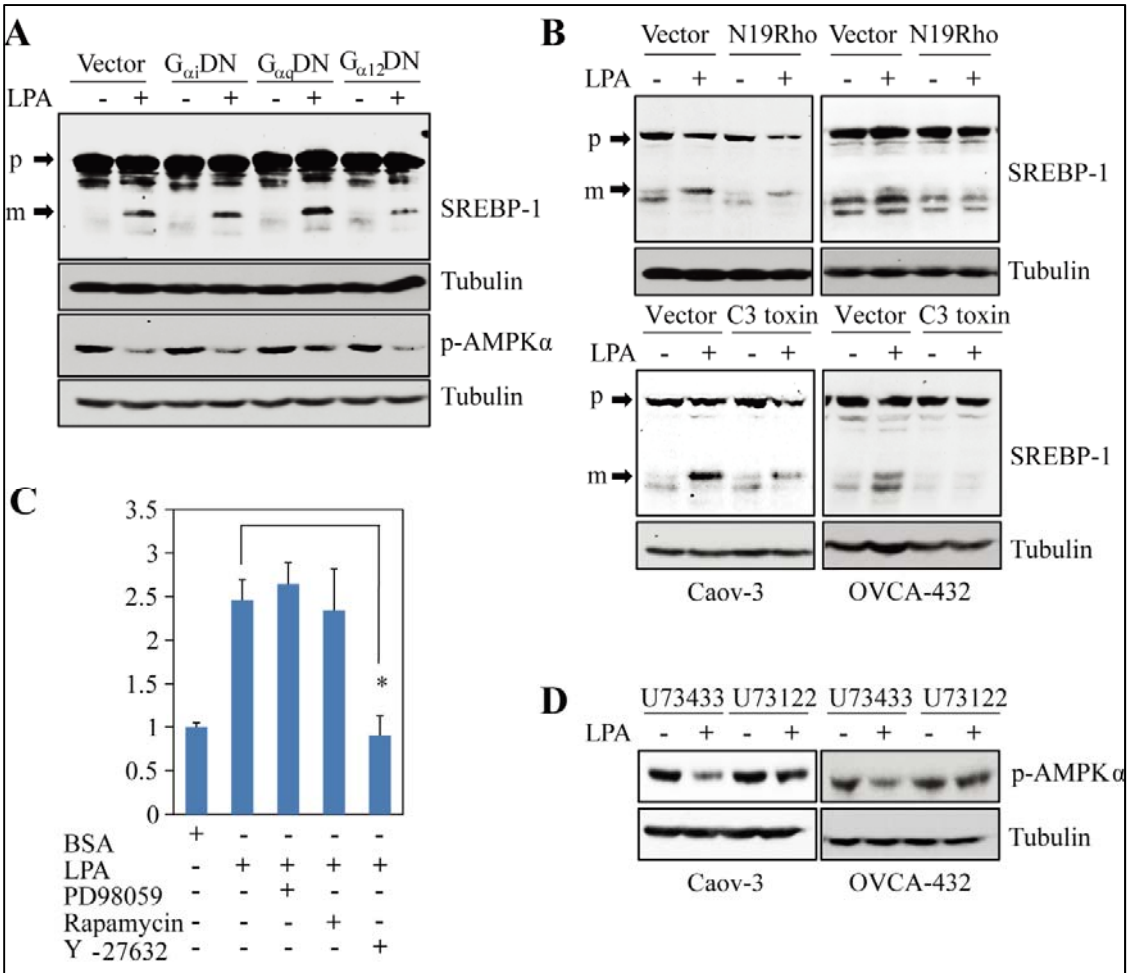


Figure 2.8 LPA regulates SREBP and AMPK through different G protein cascades. Caov-3 cells were transfected to express dominant negative forms of $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ or the control vector. The transfected cells were treated with LPA (10 μM) for 12 hours before immunoblotting analysis of SREBP-1 cleavage and AMPKα dephosphorylation (***A***). In ***B*** dominant negative Rho (N19Rho) or C3 toxin expression vector was transfected into Caov-3 and OVCA-432 cells. The effects of N19Rho and C3 toxin on LPA-induced SREBP-1 cleavage were analyzed by immunoblotting. In ***C,*** Caov3 cells were transfected with pGL2-3XSRE-TK-luc construct and treated with (10 μM) LPA alone or in the presence of the indicated inhibitors for 12 hours and subsequently assayed for luciferase activity. Concentrations of inhibitors used are as follows: PD98059 (10 μM), rapamycin (0.1 nM), Y-27632 (10 μM). In ***D***, Caov-3 and OVCA-432 cells were treated with LPA in the presence of the PLC inhibitor U73122 or its inactive analog U73433 (10 μM). LPA-induced AMPKα dephosphorylation was analyzed by immunoblotting.

2.3.7 LPA-DRIVEN CELL PROLIFERATION REQUIRES LPA_2 AND *DE NOVO* LIPID SYNTHESIS

LPA is a mitogen that stimulates proliferation of ovarian cancer cells (52,270-272). To understand the biological significance of LPA-induced lipogenesis, we examined whether the pro-lipogenic activity of LPA contributes to LPA-driven proliferation of ovarian cancer cells. C75 and TOFA are well characterized, specific inhibitors of FAS and ACC, respectively (273,274). The presence of C75 dramatically decreased cell numbers of Caov-3 and OVCA-432 in serum-free medium supplemented with LPA as a growth factor (Fig. 2.9A), suggesting that the blockade of *de novo* lipogenesis could attenuate LPA-induced cell proliferation. Similar effects were observed in the presence of the ACC inhibitor TOFA (data not shown). At the concentrations we used, C75 and TOFA did not induce significant increases in apoptosis or appreciable decreases in cell viability (data not shown), suggesting that these inhibitors mainly targeted cell proliferation rather than cell survival. We also tested if exogenously added palmitate could reverse the effect of C75 on LPA-induced cell proliferation. At 10 μM, palmitate partially prevented the effect of C75 (Fig. 2.9B). This ability of palmitate, however, was not seen at 20 μM, suggesting a possible cytotoxic effect of high concentrations of palmitate. To obtain molecular evidence for the involvement of FAS in LPA-induced cell proliferation, we used siRNA to knockdown FAS expression in Caov-3 and OVCA-432 cells. Downregulation of FAS expression indeed prevented proliferation of these cells induced by LPA (Fig. 2.9C).

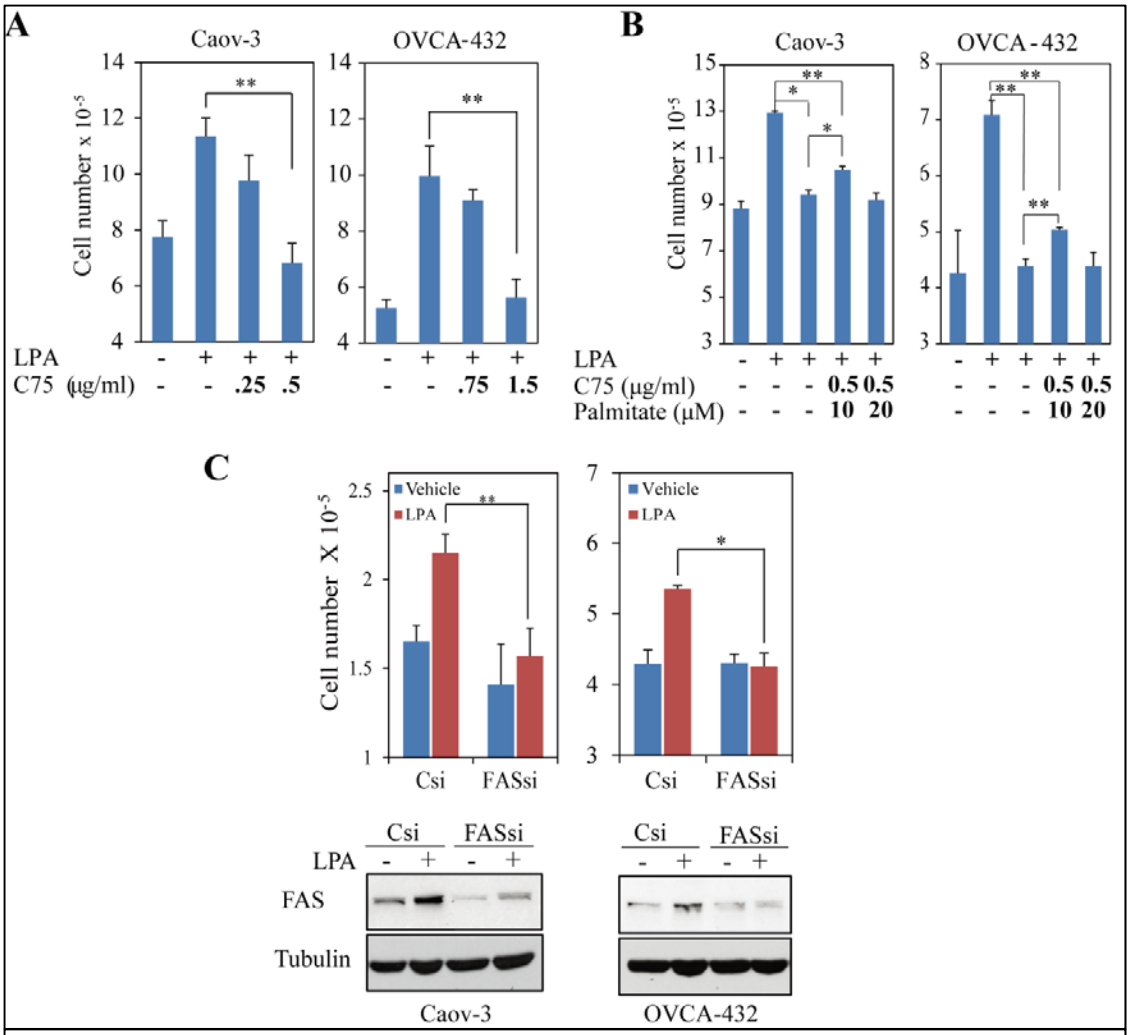


Figure 2.9 Inactivation of FAS attenuates LPA-induced cell proliferation. Caov-3 and OVCA-432 cells in 6-well plates were incubated for 48 hours in serum-free medium supplemented with 10 µM LPA in the presence of indicated concentrations of the FAS inhibitor C75 (***A).*** In ***B***, Caov-3 and OVCA-432 cells were incubated with LPA (10 µM) and C75 in the presence of the indicated concentrations of palmitate. BSA was kept at a final concentration of 0.01% for all treatments. In ***C,*** expression of FAS was downregulated by siRNA knockdown in Caov-3 and OVCA-432 cells to examine LPA-induced cell proliferation after 48 hours of incubation with 10 µM LPA.

Finally, since LPA_2 is the key receptor subtype required for LPA activation of lipogenesis, we knocked down its expression to determine whether LPA_2 is an integral component of LPA-induced cell proliferation. As shown in Fig. 2.10A, following downregulation of LPA_2, both cell lines exhibited a significant decrease in growth rate when the cells were incubated in serum-free medium containing LPA. Since LPA is a component of serum, we wondered if LPA signaling contributed to proliferation under a physiological setting. We observed that stable knockdown of LPA2 resulted in reduced growth of OVCA-432 cells grown in serum containing media (Fig. 2.10B). LPA_2 was also critical for anchorage-independent growth of ovarian cancer cells, as stable knockdown of LPA_2 in OVCA-432 cells inhibited the numbers and sizes of colonies grown in soft agar (Fig. 2.10C). Thus LPA_2 and its associated lipogenesis-promoting activity are critical for anchorage-dependent and independent growth of ovarian cancer cells.

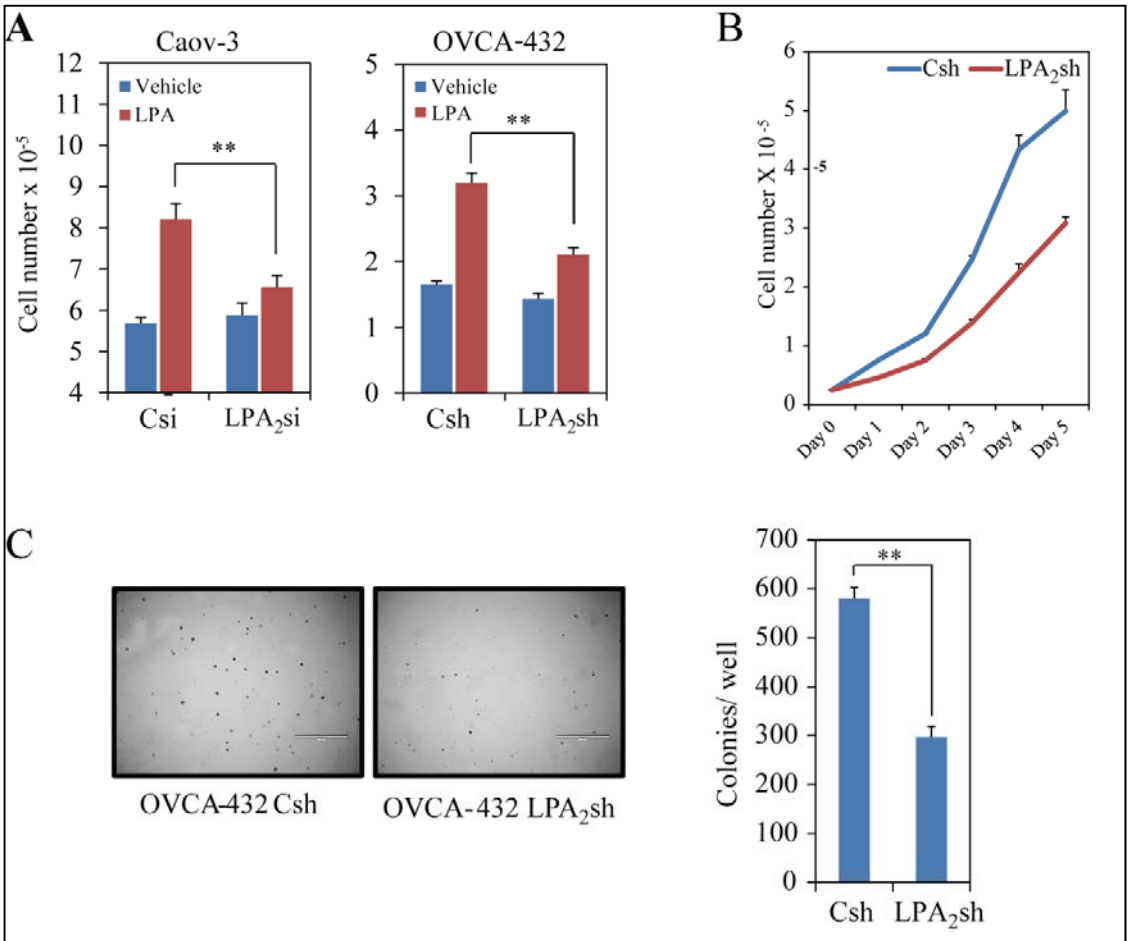


Figure 2.10 LPA_2 is required for cell proliferation and anchorage-independent growth. *A.* LPA_2 was downregulated by siRNA or shRNA in Caov-3 and OVCA-432 cells. The growth of these cells in serum-free medium supplemented with 10 μM LPA was examined after 48 hours of incubation. Cell numbers were quantitated with Coulter counter and presented as mean ± SD of triplicate assays, representative of three independent experiments. ***B.*** OVCA-432 cells were plated in 12 wells dishes in equal numbers and cell numbers were counted every 24 hours using coulter counter. ***C.*** OVCA-432 cells were plated in 6-well plates (3000 cells/well) coated with 0.6% soft agar and allowed to grow for two weeks. After which photographs were taken under microscope and colony numbers were quantified. Bar represents 2000 μM.

2.4 DISCUSSION

The majority of adult tissues depend on dietary fat to meet their nutritional needs. In contrast, cancer cells depend on *de novo* lipid synthesis for generation of fatty acids, irrespective of the available extracellular supplies. Malignant cells typically show a high rate of *de novo* fatty acid synthesis (136,275). Intracellular fatty acids in rapidly dividing cancer cells not only supply energy through beta oxidation but more importantly, serve as precursors for biosynthesis of membrane phospholipids, signaling lipids and secondary messengers (155). The lipogenic phenotype of cancer cells has been primarily attributed to increased expression or aberrant activity of the major lipogenic enzymes FAS and ACC. In particular, FAS, originally recognized as a tumor specific antigen present in serum of cancer patients (154), is overexpressed in a variety of human malignancies. However, the cellular mechanisms by which lipogenic enzymes are upregulated in cancer cells remain poorly understood, except for a few studies suggesting that steroid hormones and Her family ligands could increase FAS expression via the PI3K or MAPK pathways (160,161,276,277).

In the present study, we describe a novel LPA-mediated mechanism activating *de novo* lipogenesis in ovarian cancer cells. We demonstrated that treatment of ovarian cancer cell lines with LPA activates the SREBP-FAS and AMPK-ACC lipogenic cascades, culminating in increased *de novo* lipid synthesis. The lipogenic effect of LPA was specifically observed in cancer cells as LPA failed to induce *de novo* lipogenesis in non-transformed IOSE-29 cells. LPA has been long known as a mediator of ovarian

cancer. It is present at high concentrations in tumor microenvironments such as ascites of ovarian cancer patients and other malignant effusions (247,248). The present study highlights the possibility that LPA is an etiological factor in tumor microenvironments to promote lipogenesis in ovarian cancer cells, although the effect of LPA in other cancer cells remains to be determined.

A significant finding of the present work is the selective role of the LPA_2 receptor in LPA activation of the lipogenic pathways and LPA-driven lipogenesis. We and others have previously shown that LPA_2 and LPA_3 are overexpressed in significant fractions of ovarian cancers and in most ovarian cancer cell lines (107,272). LPA_1, which is expressed by both normal and malignant ovarian epithelial cells, is dispensable for the pro-lipogenic activity of LPA in ovarian cancer cells. It is somewhat surprising that in both Caov-3 and OVCA-432 cells, knockdown of LPA_3 slightly potentiated the lipogenic effect of LPA (Fig. 2.7D). The results imply that the crosstalk among co-expressed LPA receptors is important in the control of biological outcomes of LPA. The specific role of LPA_2 in the promotion of lipogenesis in tumor cells is consistent with the increased expression of this receptor in various malignancies (107,108,110,111). Although LPA_1 and LPA_3 have also been reported to be up or down-regulated in some cancers, overexpression of LPA_2 is most commonly seen in almost all cancer types examined (107,108,110,111). There is also strong evidence from xenograft mouse models and transgenic mice that LPA_2 is more oncogenic compared to LPA_1 and LPA_3 (83,84). The compelling evidence for the implication of LPA_2 as an oncogene stems from recent studies by Yun's group who showed the LPA_2-deficient mice were more resistant to

intestinal tumorigenesis induced by colitis or by ApcMin mutation (112,113). However, the molecular mechanisms for the oncogenic activity of LPA_2 are not well understood. Most previous studies have been focused on the ability of LPA_2 to stimulate expression of oncogenic protein factors including IL-6, VEGF, HIF1α, c-Myc, cyclin D1, kruppel-like factor 5, and Cox-2 (112,113,128,129,131,278). LPA_2 seems to be more potent than other LPA receptors in driving the transcriptional effects of LPA on these LPA target genes. The current study links LPA_2 to the lipogenic phenotype of ovarian tumor cells. The role of LPA_2 in lipid metabolism provides a new avenue to explore the oncogenic role of LPA.

Different G proteins downstream of LPA_2 are involved in regulation of the SREBP-FAS and AMPK-ACC pathways in LPA-treated cells. Our results showed that SREBP cleavage/activation lies downstream of the $G_{12/13}$-Rho pathway, while AMPK dephosphorylation/inhibition is mediated by the G_q-PLC cascade. LPA stimulated cleavage of the precursor SREBP into mature and active forms in a time-dependent manner, which was accompanied by increases in SREBP-dependent transcriptional activity and upregulation of endogenous SREBP target genes. In addition, the effect of LPA on SREBP cleavage and activation remains sensitive to cholesterol-mediated regulation, indicating that the sterol sensing machinery involved in SREBP cleavage is not disrupted by LPA. The proteolytic cleavage of SREBP is controlled by the combined action of SCAP and INSIG proteins (279). An increase in SCAP or decrease in INSIG proteins could lead to activation of SREBP. Since androgens and insulin have been shown to regulate expression or stability of SCAP or INSIG proteins (179,280), it will be

of interest to determine whether LPA modulates these proteins or their ratios to activate SREBP. This possibility is consistent with the observation that SREBP cleavage occurs hours after exposure of ovarian cancer cells to LPA.

It is yet to be determined how the G_q-PLC pathway is linked to dephosphorylation and inhibition of AMPKα. Obviously, our observation does not agree with Kim et al. who recently reported that LPA stimulated transient phosphorylation of AMPKα at Thr-172 within the first 10 minutes of LPA treatment in the SKOV-3 ovarian cancer cell line (281). In our experiments involving multiple ovarian cancer cell lines, there was little change in AMPKα phosphorylation status at the early time points. Instead, we observed a time-dependent decrease in phospho-AMPKα levels, which maximized after 12 hours of incubation with LPA. The serine-threonine kinase LKB1, encoded by the Peutz-Jeghers syndrome tumor suppressor gene, is believed to be the primary AMPK kinase as suggested by LKB1 knockout studies (282-284). LKB1 possesses a nuclear localization domain and is located predominantly in the nucleus. Upon phosphorylation, LKB1 translocates to the cytoplasm where it forms an active complex with Ste20-related adaptor (STRAD) and mouse protein 25 (MO25) (285). LPA may downregulate LKB1 activity via modulation of its phosphorylation, nuclear-cytoplasmic translocation or association with STRAD-MO25 in the cytosol. In addition, AMPK phosphorylation could be downregulated by inhibition of other candidate AMPK kinases such as calmodulin-dependent protein kinase kinase-beta (CAMKKβ) (285) or by activation of unknown AMPK phosphatase(s). A decrease in the AMP/ATP ratio in LPA-treated cells as shown in Fig. 2.4B could also change the conformation of AMPK to prevent the active

site (Thr-172) on the α-subunit from being exposed and phosphorylated by AMPK kinases.

CHAPTER 3

LYSOPHOSPHATIDIC ACID ACTIVATES HEXOKINASE-2 EXPRESSION AND GLYCOLYSIS IN CANCER CELLS

3.0 ABSTRACT

Most malignancies exhibit the "Warburg effect"- a phenomenon characterized by an enhanced glycolytic rate, thereby replacing oxidative phosphorylation as the major ATP generating process. Hyperactive glycolysis leads to increased carbon flux and abundant metabolic precursors which are required to maintain the high rate of biosynthesis of structural and signaling lipids and other cellular components required during rapid tumor cell division. Glycolytic enzymes are classically activated by hypoxia and its principal mediator hypoxia-inducible factor (Hif-1α). Here we describe regulation of this process under normoxic conditions by lysophosphatidic acid (LPA). We showed that LPA dose-dependently enhanced the glycolytic rate and subsequent lactate efflux in ovarian, breast and lung cancer cells, but failed to elicit these effects in non-transformed epithelial cells, suggesting a cancer cell-specific regulation of glucose metabolism by

LPA. We found that the LPA receptor 2, a receptor subtype overexpressed in various malignancies including ovarian and breast cancer, was the major LPA receptor underlying the pro-glycolytic action of LPA. RT-qPCR array analysis revealed a number of glycolytic genes up- or down- regulated in response to LPA. Among them, hexokinase 2 (HK-2) was the most dramatically induced by LPA and promoted the glycolytic activation in LPA-treated ovarian cancer cells. Mutation and deletion analysis of the human HK-2 gene promoter identified two sterol regulator elements (SREs) responsible for LPA activation of the promoter. Moreover, DNA pull down assays demonstrated that these SREs bound to sterol regulatory element binding protein-1 (SREBP-1) in LPA-treated cells where SREBPs were proteolytically activated by LPA, as we described recently. Binding of SREBP-1 to the native HK-2 promoter upon LPA stimulation was further confirmed by chromatin immunoprecipitation assays. In addition to activation of the SREBP-1-HK-2 cascade, LPA treatment also stabilized Hif-1α protein in cancer cell lines. However, LPA enhanced HK-2 expression and glycolysis largely independently of Hif-1α. These results established a novel role of LPA in regulation of glucose metabolism via LPA_2-SREBP-1-dependent activation of HK-2 expression in neoplastic cells. Combined with our recent discovery of LPA's lipogenic effect (CHAPTER 2), our results indicate that aberrant LPA signaling is causally linked to the lipogenic and glycolytic phenotypes of cancer cells.

3.1 INTRODUCTION

Hyperactive glycolysis is one of the fundamental changes observed in transformed cells. First identified by Otto Warburg in 1920s, this observation suggests that cancer cells preferentially utilize glycolysis to generate ATP, even in the presence of oxygen, resulting in enhanced lactate efflux (135). Recent studies, however, indicate that ATP production is probably secondary to the effect that glycolysis has on biomass generation (193) . Transformed cells have a high rate of proliferation and to sustain this effect, cells need to upregulate their synthetic machinery. Glycolysis serves as a primary route for carbon influx, which is required to generate complex macromolecules and organelles in the cell. The molecular mechanisms regulating aerobic glycolysis vary among cancers and a fundamental cause remains to be elucidated. However, upregulation and mutational activation of certain metabolic enzymes along with deregulated growth factor signaling have been found to affect cancer cell metabolism (286,287). Several glycolytic enzymes have been found to be upregulated in various cancers, and one of the most frequently upregulated enzymse is Hexokinase 2 (HK-2) (219,288). HK-2 catalyzes one of the rate limiting steps of glycolysis, converting glucose to glucose-6-phosphate at the expense of one ATP molecule. In mammals, there are four isozymes of hexokinase which vary in their affinity for glucose, tissue distribution and their physiological functions (289). HK-2 is localized to the mitochondrial outer membrane and has been reported to be associated with the voltage-dependent ion channel (VDAC) (216), thereby gaining access to ATP from the inner mitochondrial ATP synthase (290).

LPA is an oncogenic lysophospholipid mediator, elevated in the circulation and malignant effusions of cancer patients (30). LPA is known to regulate diverse biological processes including proliferation, migration, invasion, and cell survival (51). These effects of LPA are mediated via binding to its cognate G-protein coupled receptors (GPCRs). LPA_1, LPA_2 and LPA_3 are LPA receptors that belong to the endothelial gene (Edg) subfamily of GPCRs. The purinergic family receptor LPA_4 and related LPA_5, LPA_6 and LPA_7 receptors constitute the non-Edg subgroup of LPA receptors, which are structurally distant from the Edg LPA receptors (70). These LPA receptors are expressed differentially in adult tissues (70). Accumulating evidence suggests that LPA receptors are not functionally identical (70), hence the cellular effects of LPA depend on the combination of various LPA receptors present in a cell. Among LPA receptors, LPA_2 has been the most consistently shown to be upregulated in diverse human malignancies including cancers of ovary, breast (108), stomach (109), colorectal (110) and thyroid (111). LPA_2 mediated signaling has been shown to induce pro-oncogenic factors such as IL-6, IL-8, VEGF and to increase ovarian cancer cell proliferation and tumor burden in xenograft studies (83). Overexpression of LPA_2 has also been linked to proliferation of colon and breast cancer cells and mesothelioma cells (84,99,291). Although LPA_2 is known to activate various mitogenic and pro-survival pathways, the exact mechanism responsible for its oncogenic role is yet to be determined.

In this study, we provide evidence that LPA signaling contributes to the Warburg effect in various cancer cells. We show that LPA activates glycolysis and lactate efflux in cancer cells but not in non-transformed, immortalized epithelial cells. We identified HK-

2 as a major glycolytic enzyme upregulated by LPA to promote glycolysis. The detailed analysis of the HK-2 gene promoter led to identification of SREBP-1 as the key transcription factor to mediate LPA induction of HK-2. The effects of LPA on HK-2 and glycolysis were independent of Hif-1α, a major regulator of glycolytic enzymes under hypoxic conditions (233). Furthermore, we identified LPA_2 to be the primary LPA receptor subtype mediating the effects of LPA on HK-2 expression and glycolysis. These findings provide a novel route for upregulating aerobic glycolysis in cancer cells by a previously unrecognized pro-glycolytic factor LPA.

3.2 EXPERIMENTAL PROCEDURES

Reagents – 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) purchased from Roche (Indianapolis, IN). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). The transfection reagent Dharmafect 1 was obtained from Dharmacon, Inc. (Lafayette, CO) and TransIT-TKO was obtained from Mirus Bio (Madison, WI). Luciferase assay reagents were obtained from Promega (Madison, WI). Anti-SREBP-1, SREBP-2 and Hif-1α antibodies were obtained from BD Biosciences (San Jose, CA). Anti-HK-2 antibody was obtained from Cell Signaling (Danvers, MA). Anti-Tubulin antibody was obtained from EMD4Biosciences (Gibbstown, NJ). The TaqMan Universal PCR Master Mix and qPCR probes for HK-2, PGK1 and GAPDH were obtained from Applied Biosystems

(Carlsbad, CA). Calpain I inhibitor was purchased from Sigma-Aldrich (St. Louis, MO). D-[5-^{3}H(N)]-glucose was purchased from Perkin Elmer (Boston, MA).

Cell Culture – The sources of ovarian and breast cancer cell lines used in the study were described previously (251). Lung cancer cells H838, H2347 and NHBE cells were kindly provided by Dr. Charles Chalfant, VCU. These cancer cell lines were cultured in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The non-transformed NHBE cells were cultured in keratinocyte serum free medium (Invitrogen, Carlsbad, CA).

siRNA, plasmids and transfection – The siRNA oligos for LPA_1, LPA_2 LPA_3, Hif-1α, SREBP-1 and HK-2 were obtained from Applied Biosystems. These siRNAs were transfected into cells using Dharmafect 1 following the manufacturer's protocol. In brief, cells were plated in 6-well plates to reach 50-60% confluence before transfection. Cells were then transfected with target specific siRNA or non-targeting control siRNA (150 picoM) with Dharmafect 1 (4 μL) for 12-16 hours. Approximately 48 hours post transfection; the cells were serum starved overnight before LPA treatment.

Western blotting – Cells were lysed as previously described (263). Total cellular proteins were resolved by SDS-PAGE, transferred to immunoblot membrane (polyvinylidene difluoride) (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).

Quantitative PCR (qPCR) – Total cellular RNA was isolated from cultured cells using Trizol (Invitrogen). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative levels of LPA_1, LPA_2, LPA_3, HK-2 and GAPDH were determined by reverse transcription (RT) followed by qPCR using gene specific probes, the TaqMan Universal PCR Master Mix, and the Applied Biosystems 7900HT Real-Time PCR System.

Luciferase vectors and luciferase assays- The human HK-2 promoter sequence (-1476 to +73) was PCR amplified and cloned into the pGL2-Basic-Luc vector to construct the luciferase reporter vector pGL2-1476-HK-2-Luc. The PCR product was inserted into pGL2-Basic-Luc at *XhoI* and *HindIII* sites. The truncated forms (-478 to +73 and -273 to +73) were made by PCR amplification of the corresponding fragments from pGL2-1476-HK-2-Luc 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. Two potential SREBP consensus sites (CCAGTCGCCCACACC and CACGCTCCCCCCACCA) within pGL2-1476-HK-2-Luc were converted into inactive (CCAG*GTGTCTT*ACACC and CACGC*GTCTCTT*ACCA) sequences by site-directed mutagenesis using Lightning Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. Primers used for these mutant constructs were listed in Table 3.1. Caov3 cells were transfected with the luciferase vector using TransIT-TKO according to the manufacturer's protocol. About 48 hours after transfection, the cells were starved overnight and treated with LPA or vehicle (BSA) for 12 hours. Cell extracts

were prepared and assayed for luciferase activity using the luciferase assay kits from Promega.

PCR array - Human glucose metabolism, RT^2 profiler PCR Array were obtained from SABiosciences (Qiagen). Caov-3 cells were treated with LPA or vehicle control for 12 hours before RNA isolation using RNeasy mini kit (Qiagen). The Tissue Scan ^{TM} Cancer and Normal cDNA arrays for human lung cancer (HLRT102) were obtained from Origene and qPCR was performed using the Taqman mix and probes for LPA_2 and HK-2. The results were normalized to the levels of β-actin.

Measurement of glycolytic rate – Glycolysis was measured as describes (292) with a few modifications. Briefly, cells were plated in 12 well dishes, serum starved and treated with vehicle (BSA) or LPA for 16 hours. At the 12^{th} hour of LPA treatment, 5-^{3}H (N) glucose was added to the medium at a concentration of 1μCi/ml and incubated for the remaining 4 hours. Post treatment, hydrochloric acid was added to the medium at a final concentration of 0.2 N to terminate all biological reactions. The acidified medium was collected in a 15 ml tube. A 0.5 ml micro centrifuge tube containing 0.25 ml distilled water was uncapped and inserted into the 15 ml tube. Precautions were taken to make sure the two liquids remained separate. The 15 ml tubes were sealed to allow diffusion between two liquid phases for more than 24 hours. The glycolytic rate was calculated based on the ratio of the radioactivities present in water and in medium determined by liquid scintillation counting (293) .

Lactate measurement – Cells were treated with LPA or vehicle (BSA) for 16 hours before the culture supernatants were collected. The lactate contents were then determined using the lactate assay kit (Eton Bioscience, San Diego, CA) following the manufacturer's protocol.

Hexokinase activity assay –Cells were lysed with a lysis buffer containing 15 mM Tris pH 7.8, 0.25 mM sucrose, 0.5 mM dithiothreitol (DTT), 1 mM aminohexanoic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml leupeptin. The lysates were then sonicated (5 time for 30 seconds each) in a water bath, followed by centrifugation at 2000 g at 4°C for 5 min. The cell extracts (50 μl) were added to 950 μl of reaction buffer (100 mM Tris-HCl, pH 7.8, 5 mM ATP, 10 mM $MgCl_2$, 10 mM glucose, 0.4 mM NADP, and 0.15 U/ml of G6PD (Sigma-Aldrich) and incubated at 37°C. HK enzymatic activity was determined by following the G6P-dependent conversion of NADP to NADPH spectrophotometrically at 340 nm. One unit of activity was defined as micromoles of NADPH per milligram of protein per minute at 37°C.

DNA pull-down assay –Nuclear proteins was isolated from vehicle (BSA) or LPA treated cells as described previously (133). Equal amounts of nuclear proteins were incubated with 4 μg of biotinylated double-stranded oligonucleotides which contains wild type HK-2 promoter sequence or its mutated counterpart (Table 3.1) for 16 hours at 4 °C. The M-280 Streptavidin Dynabeads (Invitrogen) (30 μl) were then added to each sample and incubated for another hour at 4 °C. The Dynabeads were washed three times with PBS before western analysis of SREBP-1 and SREBP-2.

Chromatin immunoprecipitation (ChIP) assay - Vehicle or LPA treated cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The cells were then 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 pellet was recovered by centrifugation (5 minutes at 5000×g) and resuspended in a nuclear lysis buffer (50 mM HEPES, pH 8.0, 10 mM EDTA, 1% SDS, 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) and protein concentrations were determined by BCA protein estimation kit (Pierce). Equal amounts of proteins were incubated for 16 hours at 4 °C with 2 µg of either normal rabbit IgG (Santa Cruz) or rabbit anti-SREBP-1 antibody. Protein G Dynabeads was subsequently added and incubated for 2 hours. The DNA-protein-beads 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 with TE elution buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 1% SDS) at 65 °C for 15 minutes. The immunoprecipitated complexes and the inputs were the reverse cross linked by incubating samples overnight at 65 °C. The samples were then treated with RNase A and proteinase K and subsequently DNA was purified using the QIAquick Spin Columns and analyzed by PCR amplification of the

HK-2 promoter sequence using primers listed in Table 3.1.

Statistics - All numerical data were presented as mean ± SD of triplicate assays, representative of three independent experiments. The statistical significances were analyzed using Student's *t* test, unless otherwise stated, $p<0.05$ was considered statistically significant. In all figures, the statistical significances were indicated with * if $p<0.05$ or ** if $p<0.01$.

Table 3.1 Oligonucleotides used in study

Luciferase primers	
-1476 fwd	5'-GCACTCGAGGGATTATGATTTTTGTTTATTTTTCCT-3'
+73 rvs	5'-GCAAAGCTTCGGATTTTCTTAGCTGGGTG-3'
-478 fwd	5'-GCACTCGAGCCGGCCGTGCTACAATAG-3'
-273 fwd	5' -GCACTCGAGCTCATGCGCCTTTCCGTC-3'
SRE1 Mut fwd	5'-CAGAGGCCCGTTTTTCCAGGTGTCTTACACCCCGGGTCC GCGAT-3'
SRE1 Mut rvs	5'-ATCGCGGACCCGGGGTGTAAGACACCTGGA AAA ACG GGC CTC TG-3'
SRE2 Mut fwd	5'-GGGTCCGCGATCACGCGTCTCTTACCCATAGCCGAGCCTG-3'
SRE2 Mut rvs	5'-CAGGCTCGGCTATGGGTAAGAGACGCGTGATCGCGGACCCG-3'
DNA pull-down oligonucleotides	
HK-2 WT fwd	5'-CGTTTTTCCAGTCGCCCCACACCCCGGGTCCGCGATCACGCT CCCCCCACCCATAG CCGA-3'
HK-2 WT rvs	5'-TCGGCTATGGGTGGGGGGAGCGTGATCGCGGACCCGGGGTG TGGGGCGACTGGAAAAACG -3'
HK-2 Mut fwd	5'-CGTTTTTCCAGGTGTCTTACACCCCGGGTCCGCGATCACGCG TCTCTTACCCATAGCCG -3'

HK-2 Mut **rvs**	5'-TCGGCTATGGGTAAGAGACGCGTGATCGCGGACCCGGGGTGTAAGACACCTGGAAAAACG -3'
ChIP primers	
-478 SRE fwd	5'- AGAGGCCCGTTTTTCCAGTCG -3'
-478 SRE rvs	5'- GCTAAAGGCTGGGACGGAAAGG -3'
-1476 SRE fwd	5'- GGAAGTTTTGCTGAGAGGCT -3'
-1476 SRE rvs	5'- AAGATGAAGGTCTGCCATGTTC -3'

3.3 RESULTS

3.3.1 LPA ACTIVATES GLYCOLYSIS IN OVARIAN CANCER CELLS

LPA is found in abundance in ascites of ovarian cancer patients (248,294). In this study, we examined whether LPA signaling promotes aerobic glycolysis, a hallmark of ovarian and other cancer cells. During glycolysis, one molecule of water is released as 2-phosphoglycerate is converted to phosphoenolpyruvate. By labeling cells with ^{3}H-glucose we were able to quantitate the glycolytic rate by measuring generation of ^{3}H water in culture supernatants. We treated a panel of ovarian cancer cell lines and an immortalized ovarian surface epithelial cell line IOSE-80 with (10 μM) LPA and labeled the cells with 5-^{3}H-glucose. As shown in Figure 3.1A, LPA treatment led to a dramatic increase in glycolytic rate in ovarian cancer cell lines, but failed to elicit this effect in the non-transformed IOSE-80 cells. The LPA mediated increase in glycolysis was concurrent with a significant increase in lactate efflux from ovarian cancer cell lines. Consistent with the lack of stimulation of glycolysis by LPA in IOSE-80 cells, no net increase in lactate production was observed (Figure 3.1B). Moreover, the effect of LPA on glycolysis in

ovarian cancer cell lines was dose dependent (Fig. 3.1C), with significant stimulation observed at as low as 1 μM. The optimal activity was observed with 10 μM LPA.

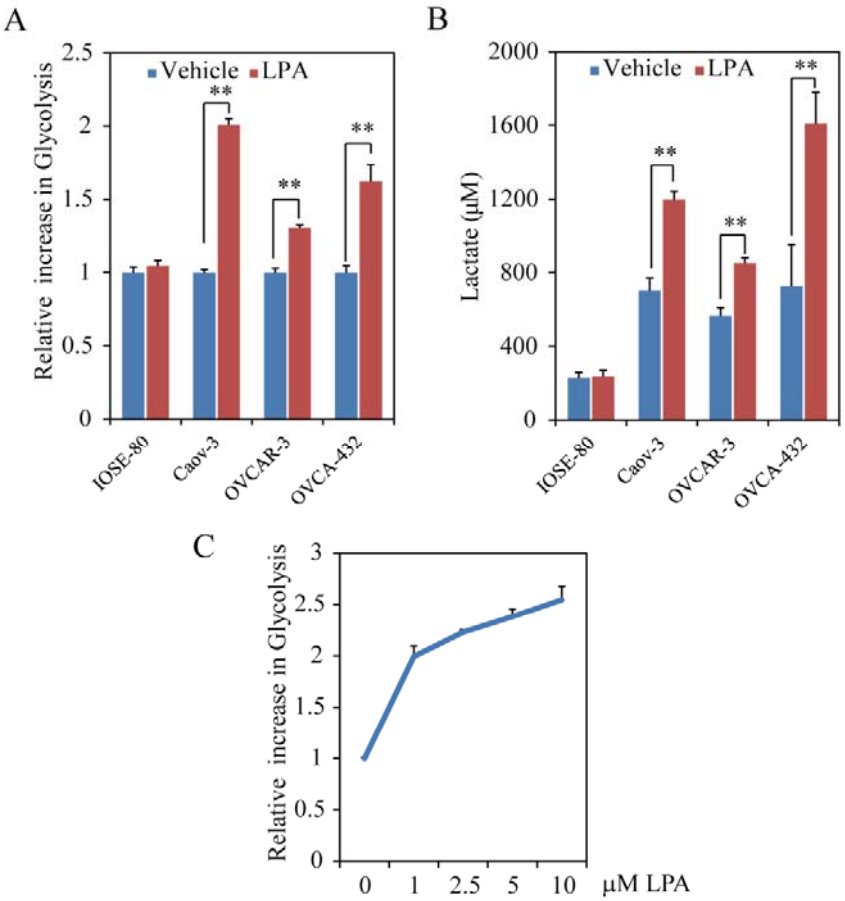
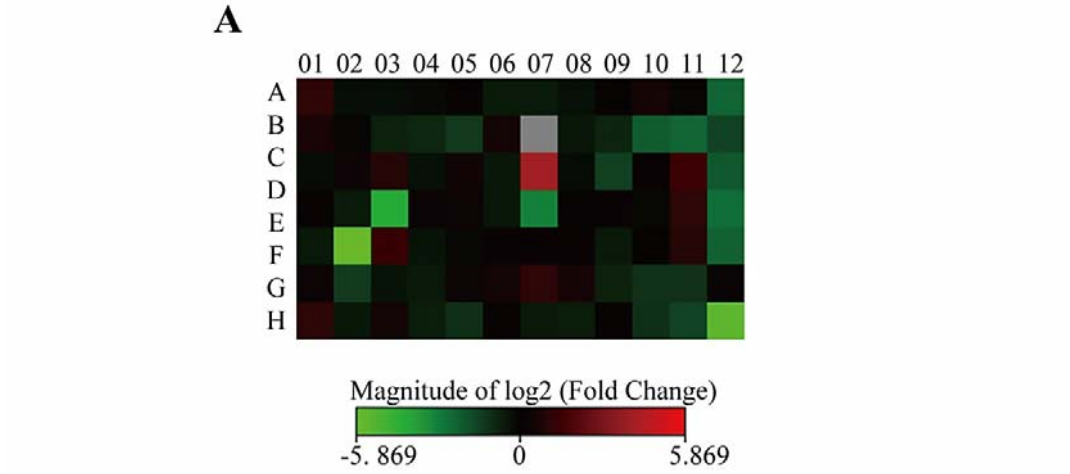


Figure 3.1 LPA activates glycolysis in ovarian cancer cells. *A.* IOSE-80 and ovarian cancer cell lines were treated with LPA (10 μM) or BSA (vehicle) for 16 hours. In the last 4 hours of incubation, cells were pulse labeled with 1μCi/ml of ^{3}H glucose before glycolytic rate was measured as described in Experimental Procedures. The results were presented as relative fold increase over vehicle treated control cells (defined as 1.0). ***B.*** Culture supernatants of the cell lines treated with LPA or BSA for 16 hours were collected and lactate concentrations were determined as described in Experimental Procedures. ***C.*** Caov-3 cells were treated with indicated concentrations of LPA and glycolytic rate was measured and presented as in ***A.***

3.3.2 LPA ACTIVATES TRANSCRIPTION OF GENES INVOLVED IN GLUCOSE METABOLISM

LPA is known to transactivate a variety of pro-oncogenic protein factors such as VEGF, COX-2, IL-6, IL-8, cyclin D1 and kruppel-like factor 5 (112,113,128,129,131). We wanted to determine if LPA transcriptionally activated genes involved in the glycolytic pathway, which could explain the enhanced glycolytic flux observed in ovarian cancer cell lines. We treated Caov-3 cells with LPA or its vehicle control for 12 hours and isolated RNA to determine the effect of LPA on glucose metabolism using an RT-qPCR array for genes involved in glucose metabolism (SABioscience). LPA treatment had dramatic effects on expression of multiple genes involved in glycolysis, the pentose phosphate pathway, the TCA cycle and gluconeogenesis, as shown in Figure 3.2A. However, HK-2 was the only glycolytic target gene that was strongly upregulated by LPA (Figure 3.2).



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACLY	ACO1	ACO2	AGL	ALDOA	ALDOB	ALDOC	BPGM	CS	DLAT	DLD	DLST
B	ENO1	ENO2	ENO3	FBP1	FBP2	FH	G6PC	G6PC3	G6PD	GALM	GBE1	GCK
C	GPI	GSK3A	GSK3B	GYS1	GYS2	H6PD	HK2	HK3	IDH1	IDH2	IDH3A	IDH3B
D	IDH3G	MDH1	MDH1B	MDH2	OGDH	PC	PCK1	PCK2	PDHA1	PDHB	PDK1	PDK2
E	PDK3	PDK4	PDP2	PDPR	PFKL	PGAM2	PGK1	PGK2	PGLS	PGM1	PGM2	PGM3
F	PHKA1	PHKB	PHKG1	PHKG2	PKLR	PRPS1	PRPS1L1	PRPS2	PYGL	PYGM	RBKS	RPE
G	RPIA	SDHA	SDHB	SDHC	SDHD	SUCLA2	SUCLG1	SUCLG2	TALDO1	TKT	TPI1	UGP2G

Figure 3.2 LPA regulates expression of genes involved in glucose metabolism. Caov-3 cells were treated with LPA (10 μM) or BSA (vehicle control) for 12 hours. cDNA was prepared from RNA followed by qPCR array as described in Experimental Procedures.

3.3.3 HK-2 IS A TARGET OF LPA SIGNALING AND REQUIRED FOR LPA DRIVEN GLYCOLYSIS

To confirm LPA upregulation of HK-2 expression, we treated Caov-3, OVCAR-3 and OVCA-432 cells with LPA and examined expression of HK-2 mRNA and protein.

Indeed, LPA upregulated HK-2 mRNA (Figure 3.3A) and protein levels (Figure 3.3B) in a time dependent manner. While the mRNA levels peaked around 8-12 hours, the protein levels reached a plateau between 12-16 hours post LPA treatment.

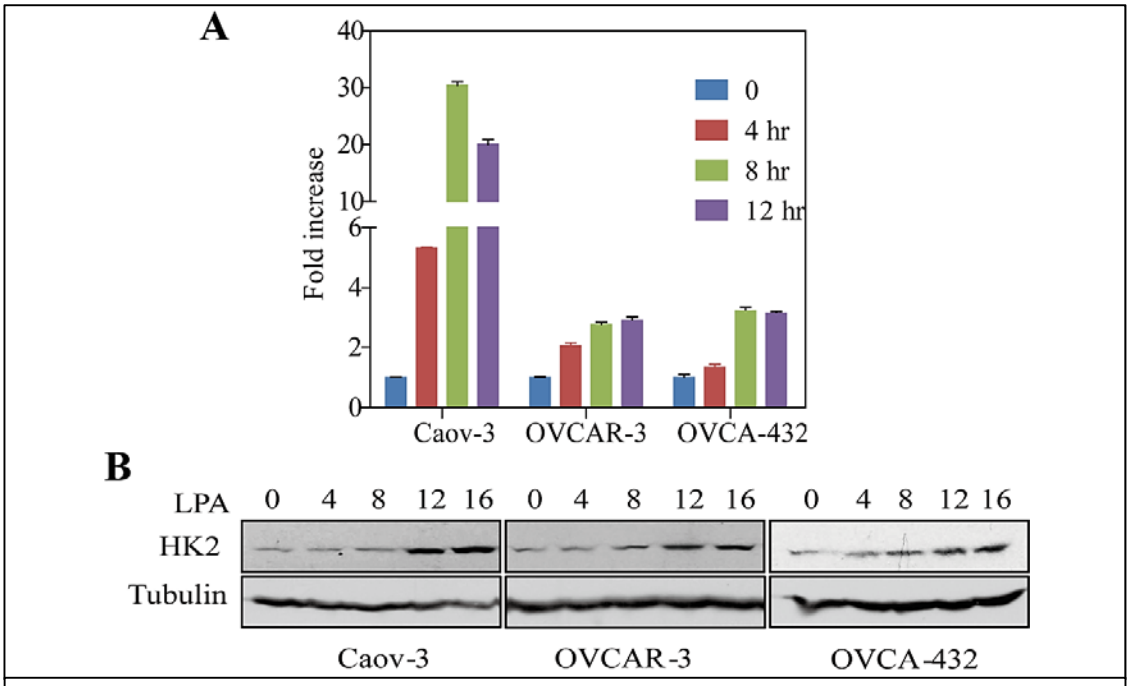


Figure 3.3 LPA upregulates HK-2 mRNA and protein expression in ovarian cancer cells. *A.* ovarian cancer cell lines were treated with LPA (10 μM) for indicated times (hours) and RT-qPCR analysis was carried out to determine HK-2 mRNA levels. The results were presented as fold increase relative to the mRNA level of untreated control cells. ***B.*** ovarian cancer cells lines were treated with LPA (10 μM) for indicated numbers of hours before immunoblotting analysis of HK-2 protein and tubulin (loading control).

The majority of HK-2 protein in a cell is attached to the mitochondria (216) and mitochondria-associated HK-2 is often regarded as the active form of the enzyme, contributing significantly to the glycolytic activity (219). LPA increased accumulation of

both cytosolic and mitochondrial HK-2 (Figure 3.4A). Consequently, LPA dramatically increased cellular hexokinase activity in ovarian cancer cell lines (Figure 3.4B).

To confirm the biological significance of HK-2 induction by LPA, we downregulated HK-2 expression induced by LPA in Caov-3 cells with HK-2 siRNA. As shown in Figure 3.5, we experienced technical difficulty in achieving high levels of HK-2 knockdown, probably due to the necessity of a basal level of HK-2 for cell proliferation or survival. However, even partial downregulation of HK-2 in Caov-3 cells was sufficient to significantly reduce LPA-driven glycolysis as shown in Figure 3.5.

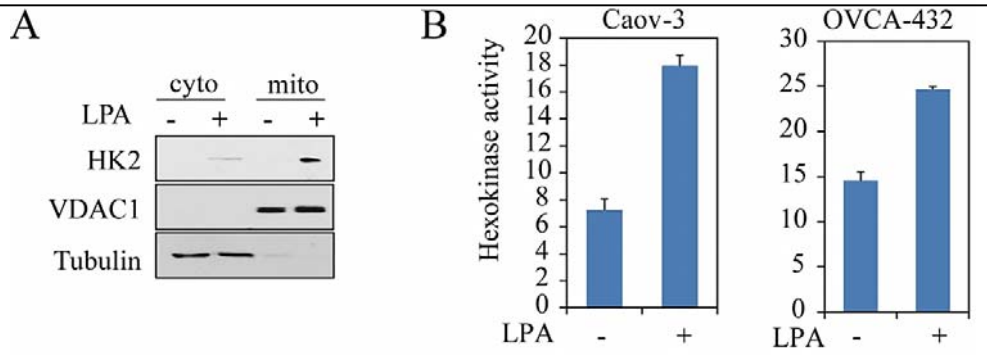


Figure 3.4 LPA induces HK-2 expression and cellular HK activity. ***A.*** Caov-3 cells were treated with LPA (10 μM) for 16 hours. Cytosolic and mitochondrial protein fractions were isolated and immunoblotted for HK-2, VDAC1 (mitochondrial marker) and tubulin (cytosolic protein). ***B.*** Ovarian cancer cell lines were treated with LPA (10 μM) for 16 hours before assaying for hexokinase activity as described in Experimental Procedures. Hexokinase activity is presented as NADPH (n moles)/mg ptn/min.

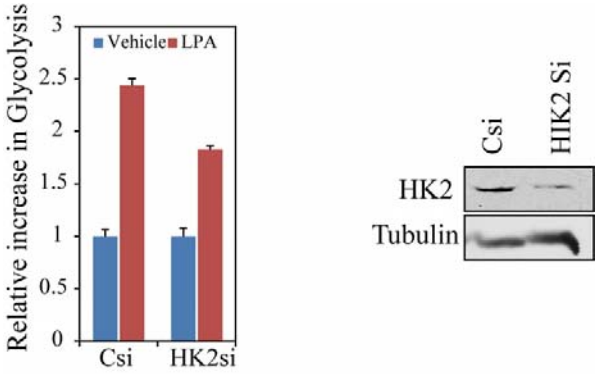


Figure 3.5 Downregulation of HK-2 attenuates LPA-induced glycolysis. Caov-3 cells were transfected with HK-2 siRNA or with non-targeting control siRNA. Cells were treated with LPA (10 μM) or vehicle and glycolytic rate was measured as described in Figure. 3.1. Cell lysates were analyzed with immunoblotting to determine efficiency of HK-2 knockdown.

3.3.4 LPA_2 IS THE MAJOR RECEPTOR THAT UPREGULATES HK-2 EXPRESSION AND GLYCOLYSIS

Caov-3, OVCA-432 and other ovarian cancer cell lines express the Edg LPA receptors LPA_1, LPA_2, and LPA_3 (295), while the non-Edg receptors are either absent or are expressed inconsistently in ovarian cancer cells (94). Thus to identify the LPA receptor responsible for the pro-glycolytic effect of LPA, we focused on the Edg LPA receptors. We used siRNA to knockdown expression of LPA_1, LPA_2, and LPA_3 in Caov-3 cells. Only knockdown of LPA_2 led to significant inhibition of LPA-induced HK-2 expression and glycolysis. (Figure 3.6A & Figure 3.6B). Similar observations were made in OVCA-432 cells where LPA receptors were stably knocked down using lentivirus-mediated shRNA (Figure 3.6A & Figure 3.6B). These results provided strong evidence

that LPA_2 is the major LPA receptor subtype accounting for LPA-driven HK-2 induction and glycolysis in ovarian cancer cells.

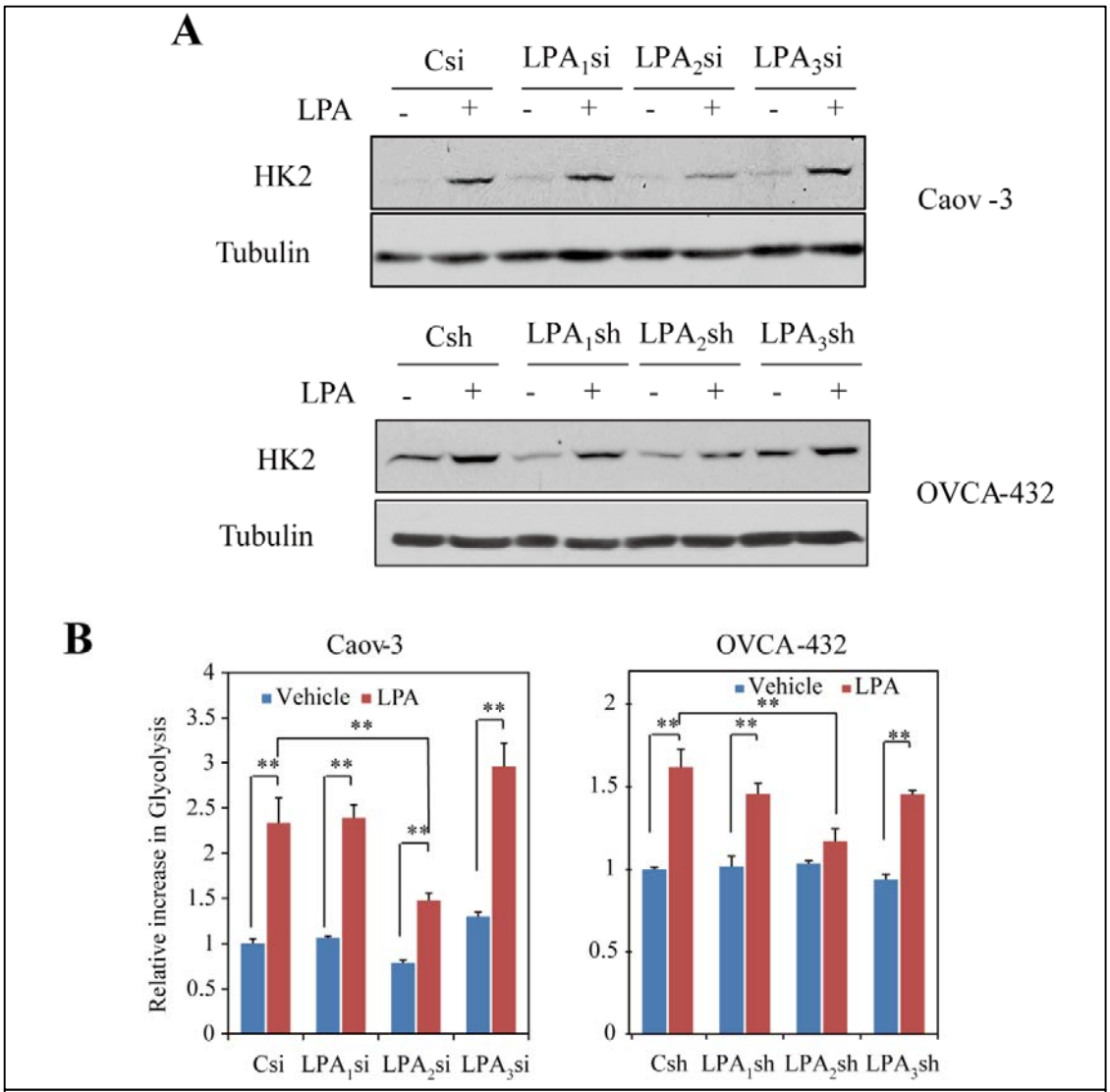


Figure 3.6 LPA_2 is the major LPA receptor subtype responsible for HK-2 induction (*A*) and glycolysis (*B*). Each of LPA_{1-3} receptors was knocked down by siRNA in Caov-3 cells or by lentivirus-transduced shRNA in OVCA-432 cells. The cells were treated with LPA (10 μM) or vehicle for 16 hours before immunoblotting analysis of HK-2 expression (***A***) and quantification of glycolysis (***B***).

3.3.5 LPA ENHANCES HK-2 EXPRESSION AND GLYCOLYSIS IN A HIF-1A INDEPENDENT MANNER

Hypoxia inducible factor (Hif) is the principle regulator of glycolysis under hypoxic conditions, upregulating expression of most glycolytic enzymes and their regulators, including HK-2 (233). We wondered if LPA mediated HK-2 induction and glycolysis are mediated by Hif-1α. We and others have shown that LPA increased Hif-1α protein levels (260). In addition to HK-2 induction and glycolysis, LPA treatment indeed increased Hif-1α levels in a time-dependent manner in all ovarian cancer cell lines examined (Figure 3.7A). However, when Hif-1α expression was downregulated by siRNA, LPA stimulation of HK-2 mRNA was unaffected in Caov-3 cells (Figure 3.7B). In contrast, LPA induction of another glycolytic gene, PGK1 (phosphoglycerate kinase-1), was dramatically reduced by Hif-1a knockdown in these cells (Figure 3.7C). Further, we examined whether HIF-1α knockdown compromised LPA-dependent glycolysis. As shown in Figure 3.7D, there was only slight inhibition of LPA-induced glycolysis, suggesting that LPA promotes glycolysis essentially via a HIF-1α-independent mechanism.

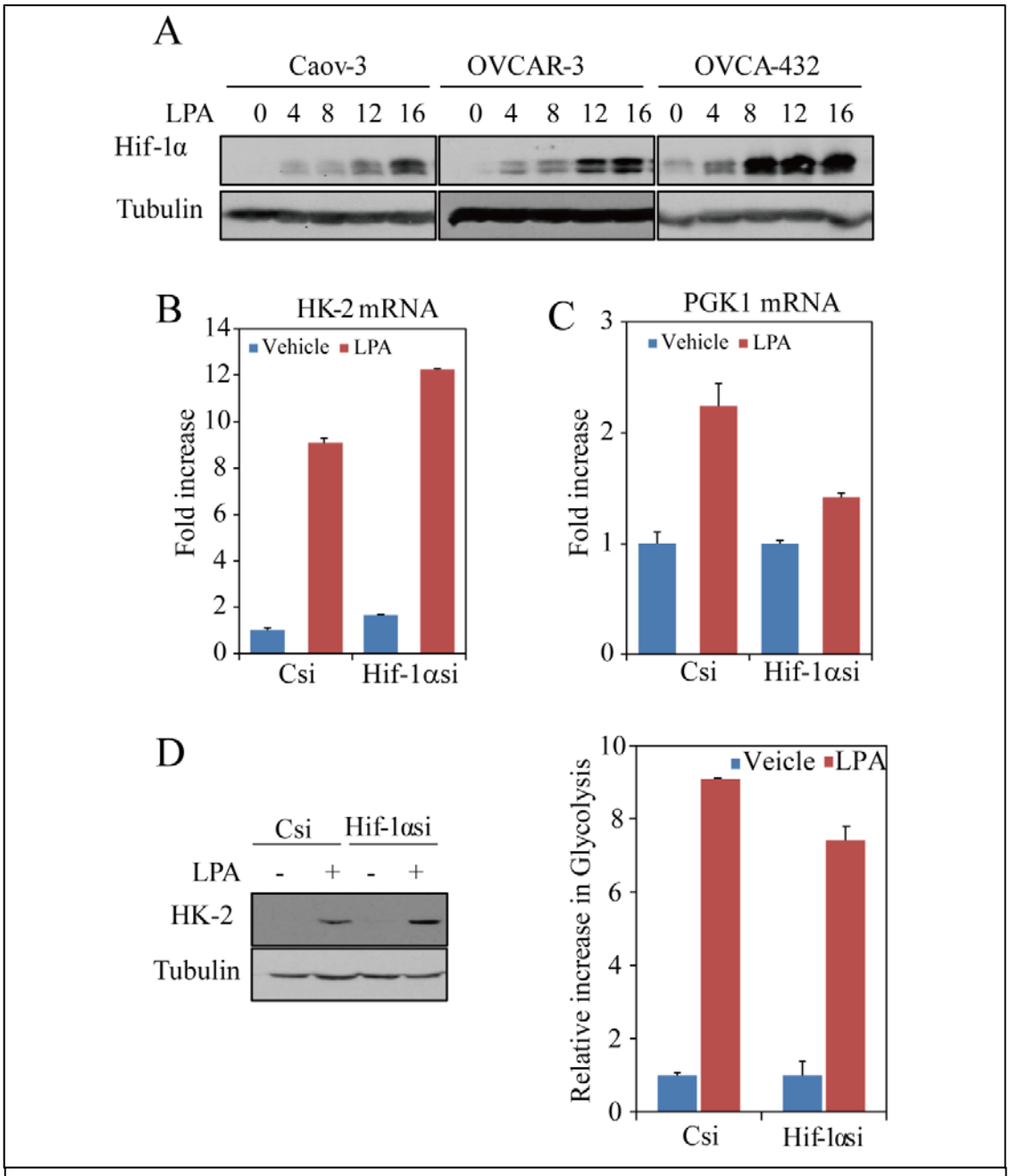


Figure 3.7 Hif-1α is not required for LPA induction of HK-2 and glycolysis. *A.* Ovarian cancer cell lines were treated with LPA (10 μM) for the indicated periods of time (hours) before immunoblotting analysis of Hif-1α protein. Hif-1α was knocked down by siRNA in Caov-3 cells. LPA-induced HK-2 mRNA expression (***B***), PGK1 mRNA expression (***C***) and glycolysis (***D***) were examined and compared between HK-2 knockdown cells and non-target control siRNA-transfected cells.

3.3.6 LPA STIMULATES HK-2 EXPRESSION THROUGH SREBP-1-MEDIATED TRANSCRIPTIONAL ACTIVATION

Since Hif-1α is not involved in LPA-mediated activation of HK-2 expression, we next investigated the underlying mechanism by analyzing the human HK-2 gene promoter. We cloned a fragment (-1476-+73) of the HK-2 promoter into the pGL2-Basic luciferase reporter vector. Further 5' deletion generated truncation mutants containing -478-+73 and -273-+73 fragments of the promoter. These luciferase reporter constructs were transfected into Caov-3 cells and LPA-induced luciferase activity was determined by luciferase assays. As illustrated in Figure 3.8, LPA treatment led to a robust increase in luciferase activity in Caov-3 cells transfected with the vector containing the full -1476-+73 fragment. The LPA-induced increase in luciferase activity remained intact when the HK-2 promoter sequence was shortened to -478-+73. However, further deletion to -273-+73 resulted in drastic loss of the response to LPA (Figure 3.8), suggesting that the major regulatory element(s) resided within the sequence between -478 to -273. Insilico analysis disclosed several potential transcription factor binding sites within this region, including cAMP-responsive element binding proteins (CREB), Hypoxia inducible factor (Hif-1α), Nuclear factor 1 (NF1), Kruppel-like factor 7 (KLF7), Specificity Protein 1 (SP1) and SREBP. The existence of two sterol regulator elements (SRE) within the responsive region and strong activation of SREBP by LPA prompted us to examine the potential role of SREBP in transcriptional activation of HK-2. As evident from Figure 3.8, point mutation of either SRE sites significantly reduced LPA-driven luciferase activity. Simultaneous mutation of both SRE sites led to a further reduction in the luciferase

activity but failed to eliminate the response to LPA completely. The remaining activity of the double mutant was similar to that of the -273 deletion mutant. These results indicated that the two SREs are necessary regulatory components of maximal activation of the HK-2 promoter by LPA.

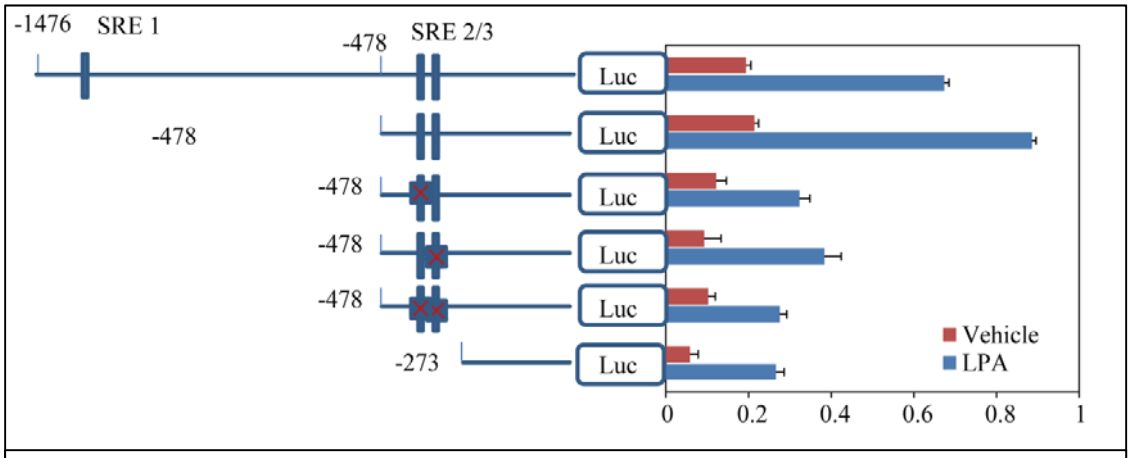


Figure 3.8 LPA activates the HK-2 gene promoter. Caov-3 cells were transfected with the indicated reporter constructs and luciferase activities were measured 12 hours after LPA (10 μM) treatment as described in Experimental Procedures.

3.3.7 LPA INDUCES BINDING OF SREBP-1 TO SRES OF THE HK-2 GENE PROMOTER

We have recently shown that LPA activates SREBP-1 and SREBP-2 transcription factors in ovarian cancer cells (295) & Chapter 2. To determine whether SREBP proteins are indeed capable of binding the SREs of the HK-2 promoter to activate transcription, we carried out a DNA pull down assay with a DNA sequence harboring the two wild type SREs (SRE2/3) or their mutated forms (see details of the sequences in Table 3.1). As

demonstrated in Figure 3.9A, increased binding of nuclear SREBP-1 to the wild type oligo was detected in LPA-treated Caov-3 cells compared to vehicle control cells. The LPA-stimulated binding of SREBP-1 was abrogated when the SREs of the oligo were mutated. In contrast, SREBP-2 was found to nonspecifically bind to both wild type and mutated oligos, which was not altered by LPA treatment of the cells (Figure 3.9A).

To confirm the binding of SREBP-1 to the native HK-2 gene promoter, we performed a chromatin immunoprecipitation (ChIP) assay. Following immunoprecipitation of SREBP-1 from LPA-treated Caov-3 cells, we were able to PCR amplify a 114 bp fragment corresponding to the region containing the SREs of the HK-2 promoter (Fig. 3.9B). Using the same precipitates, we were unable to amplify another region around -1478 bp (SRE1). In further support of an essential role of SREBP-1 in stimulation of HK-2 expression, siRNA knockdown of SREBP-1 in Caov-3 cells inhibited LPA-induced expression of HK-2 mRNA and protein (Fig. 3.10).

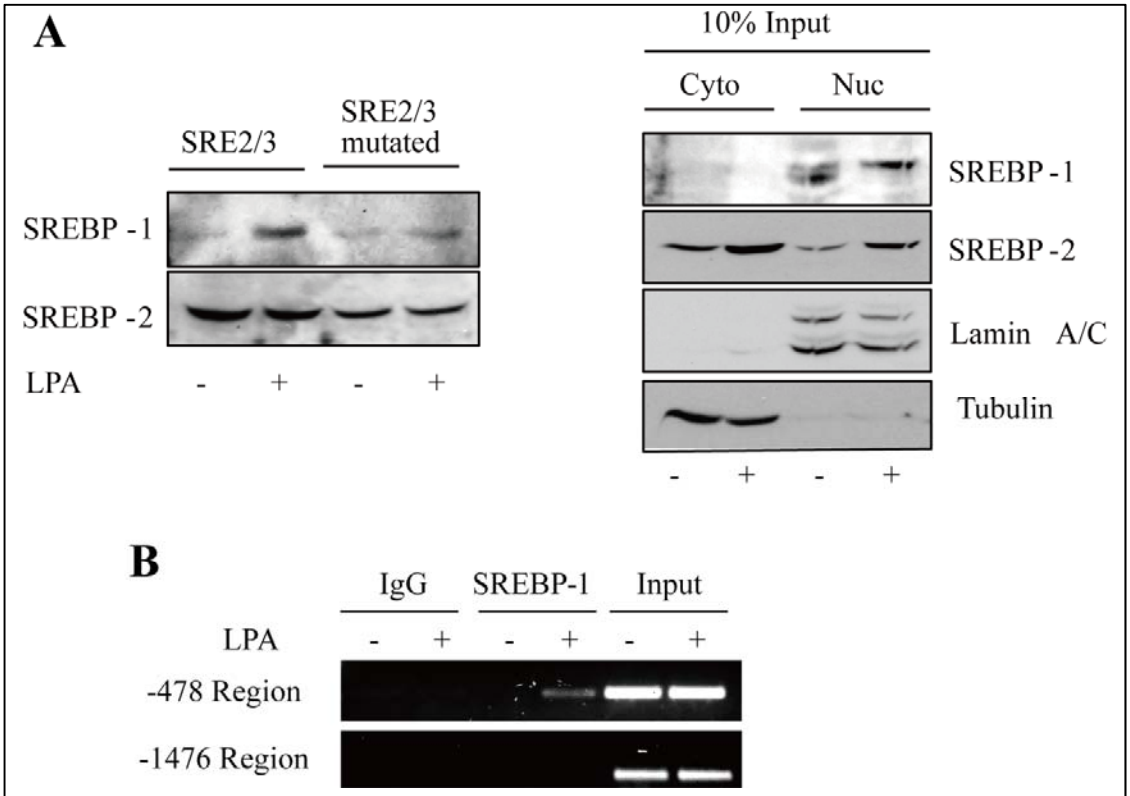


Figure 3.9 LPA induces SREBP-1 binding to the HK-2 promoter. Caov-3 cells were treated with LPA (10 μM) for 12 hours. DNA pull-down (***A***) was performed using nuclear extracts and biotin labeled oligonucleotides harboring the SREs from the HK-2 promoter (SRE2/3) or mutated form (SRE2/3 mutated). The SREBP-1 and SREPB-2 proteins bound to the oligos were examined by immunoblotting. LPA-induced binding of SREBP-1 to the native HK-2 promoter was analyzed with ChIP assays (***B***). Two regions containing SRE 2/3 SREs SRE 1, respectively, were PCR amplified.

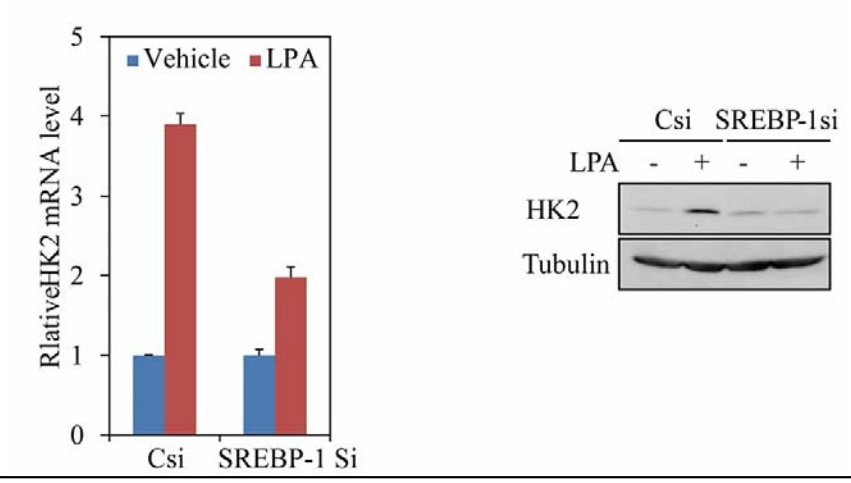


Figure 3.10 SREBP-1 is required for LPA-induced expression of HK-2 mRNA and protein. Caov-3 cells were transfected with SREBP-1 siRNA or non-targeting control siRNA. HK-2 mRNA (***A***) and protein (***B***) in the cells treated with LPA (10 μM) or vehicle for 16 hours were analyzed with RT-qPCR or immunoblotting.

3.3.8 LPA STIMULATES GLYCOLYSIS IN BREAST, COLON AND LUNG CANCER CELLS: A GENERAL PHENOMENON

Since LPA_2, the major receptor that regulates glycolysis in ovarian cancer cells, is also overexpressed in other types of cancers including breast (108) and colon cancers (99), we wanted to determine whether LPA could increase glycolysis in these cancer cells. As shown in Figure 3.14, LPA treatment promoted glycolysis in breast (MDA-MB-231 and MCF-7), colon (DLD-1) and lung cancer cells lines (H838).

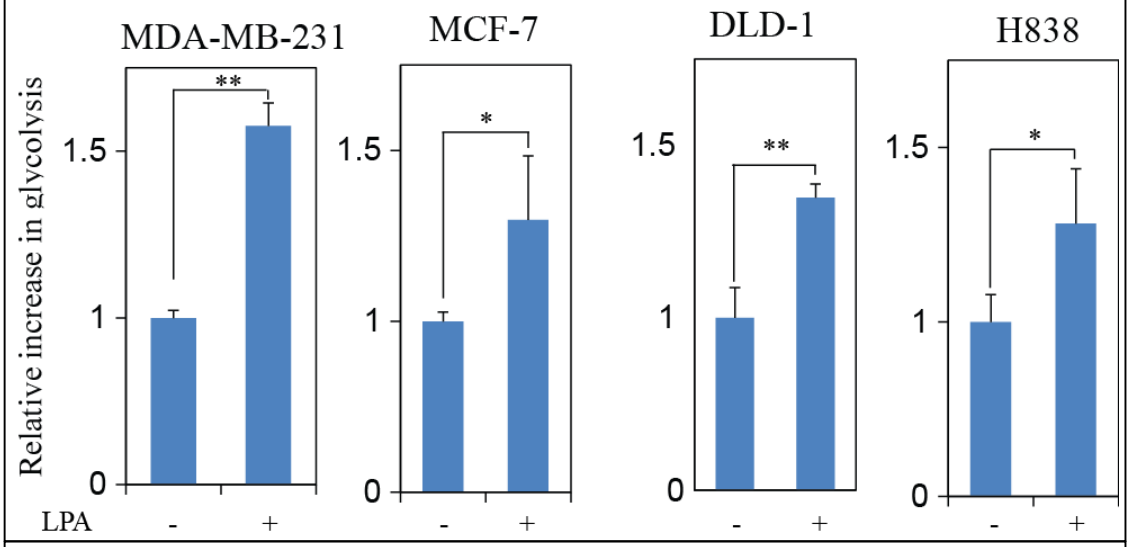


Figure 3.11 LPA activates glycolysis in multiple cancer cell lines. Cells were plated in 12-well plates and relative fold increase in glycolysis was determined as in experiments described earlier in ovarian cancer cell lines.

3.3.9 THE LPA_2 RECEPTOR AND HK-2 ARE ABERRANTLY OVEREXPRESSED IN LUNG

A significant finding of our studies described in Chapter 2 and herein (Chapter 3) is the important role of LPA_2 in lipid (Chapter 2) and glucose metabolism (Chapter 3) of cancer cells. This LPA receptor subtype has been reported to be overexpressed in ovarian, breast, colorectal and gastric cancers. In this last part of the Chapter, we examined expression and biological functions of LPA_2 in lung cancer, the most common human malignancy that causes more deaths than any other type of cancer. Non-small cell lung cancer (NSCLC) cell lines expressed LPA_2 mRNA at higher levels than non-transformed normal human bronchial-epithelial (NHBE) cells (Figure 3.12A). We further compared

LPA_2 expression in lung cancer and in normal lung tissues using the TissueScan™ Cancer and Normal Tissue cDNA Arrays (OriGene). As shown in Figure 3.12B, expression of LPA_2 mRNA was significantly increased in all stages of lung cancers including Stage I. Consistent with potential regulation of HK-2 via LPA_2 signaling, these lung cancer specimens also showed overexpression of HK-2 mRNA when compared with normal lung tissues. (Figure 3.12C).

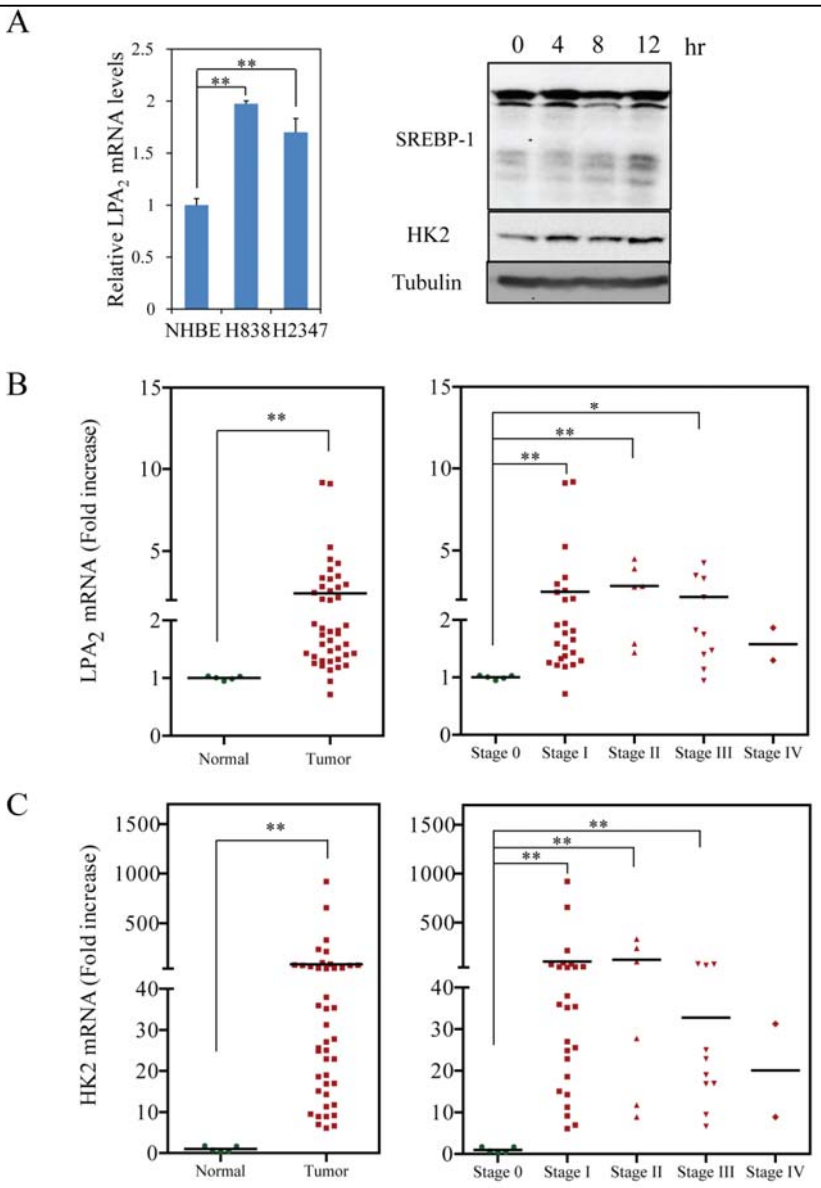


Figure 3.12 LPA_2 and HK-2 are abnormally overexpressed in lung cancer. *A.* RT-qPCR was carried out to determine the relative levels of LPA_2 in lung cancer cell lines and NHBE cells. Expression of LPA_2 (B) and HK-2 (C) in primary lung cancer and normal lung tissues was analyzed using the TissueScan™ Cancer and Normal Tissue cDNA Arrays (OriGene) as described in Experimental Procedures. For lung cancer TissueScan™ Cancer and Normal Tissue cDNA Arrays, the Mann Whitney test was performed to analyze significance between normal and tumor samples, and the Kruskal-Wallis ANOVA test was carried out to determine significance between samples of different stages.

3.4 DISCUSSION

Cancer cells exhibit an altered metabolic profile, exemplified by the Warburg Effect, which suggests that these cells utilize glycolysis, an inefficient pathway to generate ATP, instead of the more productive TCA cycle. This seemingly contradictory route of proliferative cells provides an elevated level of cellular nutrients and biosynthetic precursors to sustain a high cellular proliferation rate. Intracellular ATP concentration is often correlated with cell growth, particularly in bacteria, however, the correlation does not hold in mammalian cells especially tumor cells. Calculations based on cellular energy requirements clearly indicate that as opposed to unicellular organisms, a majority of cellular ATP is used to maintain cellular homeostasis in tumor cells (296). Moreover, cancer cells have been found to consume ATP to drive glycolytic processes and thus proliferation (297), which is consistent with the observation that high ATP is inhibitory for glycolytic processes. Thus, targeting this altered metabolic profile is often regarded as a potential therapeutic strategy for cancer treatment. Although multiple studies have been focused on understanding the regulation of the glycolytic process, to date no consensus mechanism has been identified to explain cancer-specific regulation of this process.

In this study, we provide a potential LPA-mediated mechanism for cancer specific regulation of the Warburg effect. We show that LPA, a bioactive lipid mediator, present at high levels in ascites of ovarian cancer and other malignant effusions (248,294) enhanced the glycolytic process in ovarian, breast, colon and lung cancers. This effect of LPA was cancer specific and undetectable in non-transformed ovarian IOSE-80 and

breast MCF10A (data not shown) epithelial cells, which lack the LPA_2 receptor that is critically involved in LPA-mediated activation of glycolysis.

This study thus provides evidence that LPA is one of the potential etiological factors in the tumor microenvironment that maintains hyperactive glycolysis in cancer cells. Many oncogenic factors and intracellular pathways, such as insulin and the PI3K-AKT or RAS-MAPK pathways, are known to enhance glucose uptake in cancer cells, thereby increasing glucose consumption (292,298,299). On the other hand, LPA does not increase glucose uptake (data not shown) but strongly enhances glycolysis via transcriptional activation of HK-2, the enzyme that catalyzes the first step of glycolysis. HK-2-mediated phosphorylation of glucose not only primes glucose for breakdown to generate ATP and metabolic intermediates, the step also prevents glucose from exiting the cell. Therefore, deregulated LPA signaling and other oncogenic pathways such as PI3K and RAS act in concert to promote distinct steps of glucose utilization in cancer cells.

Tumors at advanced stages often experience hypoxia, leading to stabilization of Hif-1α protein, a major regulator of almost all the glycolytic enzymes (233). However, hypoxia is not the causal factor underlying the glycolytic phenotype that occurs in both hypoxic and oxygenated regions of a tumor. Tumor cells in vitro also glycolyse when cultured in normoxic and neutral conditions. Ras, Akt, and c-Myc have been reported to upregulate expression of various glycolytic enzymes (300,301). In contrast, loss of the tumor suppressor TP53 inhibits the mitochondrial respiratory chain via suppression of

SCO2 (the synthesis of cytochrome c oxidase protein) and promotes glycolysis via TIGAR, a p53-inducible regulator of glycolysis and apoptosis (302). In addition, the mitochondrial respiratory function can be negatively affected by mutations in mitochondrial DNA. However, these defects are present only in some of human tumors and do not explain the generally altered glucose metabolism in a wide spectrum of cancers. Other unrecognized mechanisms are likely important in the development and maintenance of the glycolytic phenotype of malignant cells. Here we provided evidence for regulation of glycolysis in cancer cells by the LPA-LPA_2-SREBP-1-HK-2 pathway.

SREBP-1 is a master regulator of lipid metabolism regulating de novo lipogenesis in liver and in cancer cells (238). We have recently shown that LPA activates SREBP-1 in ovarian cancer cells, thereby leading to an increase in *de novo* lipogenesis in these cells (295). Taken together, these studies suggest that SREBP-1 serves as a convergence point of LPA signaling to regulate both lipid and glucose metabolism in cancer cells.

A major finding of this study is that LPA_2, a receptor subtype overexpressed in many malignancies including ovarian cancer (106,108-111), was the major receptor promoting glycolysis. LPA_1, which is expressed by both normal and malignant ovarian epithelial cells, was found to be dispensable for the effect of LPA on glycolysis in Caov-3 cells or to have only a minor contribution in OVCA-432 cells. In contrast, silencing of LPA_2 completely inhibited LPA-dependent glycolysis, suggesting a primary role of LPA_2 in the process. Given the importance of LPA_2 in cancer cell metabolism and the non-essential physiological role of this receptor in mice, inhibition of LPA_2 could thus be an ideal therapeutic strategy against cancer. This study thus provides a novel LPA signaling

mechanism linked to aerobic glycolysis in cancer cells, which can be exploited for cancer intervention.

CHAPTER 4

GENERAL DISCUSSION

In cancer cells, the control of proliferation is perturbed resulting in uncontrolled cell growth, one of the hallmarks of malignant cells (303). The classical notion of tumorigenesis is based on the premise that dysregulated oncogenes and tumor suppressor genes directly regulate cell cycle progression, maintain proliferative signals and help cells overcome growth suppression and cell death. However, recent advances in cancer cell metabolism suggest an alternative route for regulation of cell proliferation. Oncogenes and tumor suppressor genes could alter patterns of cellular metabolism and subsequently promote cell proliferation. There are several lines of evidences to support this proposition. A cell must pass though the interphase (G1, S and G2) before it enters the mitotic phase and cell division occurs. But before a cell divides, intracellular amounts of carbohydrates, lipids, nucleotides and amino acids must be sufficient for duplication of cellular contents, including DNA, cellular organelles and membranes. Since intracellular concentrations of these macromolecules could serve as limiting factors, it is not difficult to imagine the possibility of metabolic regulation of cell cycle. Yalcin et al. have recently shown that nuclear overexpression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzyme 3 (PFKFB3) favors transition from G1 to S phase and this subsequently upregulates cyclinD3, and M phase-promoting phosphatase Cdc25C, and reduces

expression of cell cycle inhibitor p27 (304). PFKFBs are enzymes that catalyze conversion of fructose-6-phosphate to fructose-2, 6-bisphosphate (Fru-2,6-BP), which is an allosteric activator of phosphofructokinase 1 (PFK1), thereby activating glycolysis. Consistent with the regulation of transition from G1 through M phase of the cell cycle by PFKFBs, activation of APC/C^{Cdh1} at the end of M phase has been found to degrade two critical enzymes involved in cellular metabolism, PFKFB3 and GLS1 (glutaminase 1) which regulates glycolysis and glutaminolysis, respectively (305). These studies highlight the roles of metabolic pathways in coordinated regulation of cell cycle.

An altered metabolic profile in cancer has been known for over nine decades since Otto Warburg's observation that cancer cells preferentially utilized glycolysis over oxidative phosphorylation to generate ATP (135). This observation was seen to be a paradox for quite some time, as glycolysis by itself is an inefficient process for generation of ATP. Recent observations, however, have demonstrated that the primary requirement of transformed cells is an abundance of precursors for biosynthetic processes, which are provided by a high glycolytic rate (193). It can thus be said that the requirement for ATP is secondary to that for intracellular biosynthetic precursors, providing an explanation for the paradoxical use of glycolysis by cancer cells. The heightened influx of carbon (as glucose), is utilized by transformed cells to generate fatty acids by *de novo* lipid synthesis. The fatty acids not only serve as precursors for protein lipidation reactions and as secondary messengers, they are also major constituents of cell membranes. Since a rapidly proliferating cell requires large amounts of membrane constituents for intracellular organelles and for plasma membranes, *de novo* lipogenesis is

often found to be a determinant in regulating cell proliferation and survival (147,151,153,306,307). Thus hyperactive glycolysis and enhanced de novo lipogenesis are two hallmarks of cancer cells.

Although growth factor mediated proliferation of cancer cells has been studied in detail, most of the focus has been on signaling from the receptor tyrosine kinases (such as EGFR, ERB2, PDGF, FGF and insulin receptor). Only recently, with the discovery of overexpressed GPCRs and their ligands in cancer, GPCR-mediated regulation of cell proliferation is being considered an important regulatory mechanism. One such class of GPCR ligands are bioactive phospholipids, such as LPA, and it related cousin sphingosin-1-phosphate (S1P). LPA is known for its role as an oncogenic lipid regulating various cellular processes including cell proliferation (36). Seven GPCRs for LPA have been identified to date, and both LPA and some of its receptors have been found to be upregulated in cancer. Our lab was the first to show overexpression of LPA_2 and LPA_3 in ovarian cancer (107,120), and subsequently several labs have provided evidence for overexpression of LPA_2 in various other malignancies. LPA_2 couples to $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$ G-proteins. Pertussis toxin sensitive $G_{\alpha i}$-driven signaling has been shown to regulate LPA-mediated cell proliferation (118,120). Downstream of $G_{\alpha i}$, the Ras-MAPK and the PI3K-AKT pathways have been shown to be involved in LPA induced cell proliferation (120,125). However, no conclusive mechanism has been elucidated that would explain how LPA regulates proliferation of cancer cells. In this study we wanted to determine if LPA had an effect on cancer cell metabolism and if this could explain the mitogenic role of LPA.

Since *de novo* lipogenesis has a direct impact on the cell cycle, we wondered if LPA could activate this process in cancer cells. As a model system, we chose ovarian cancer, as LPA mediated effects have been studied in great detail in this cancer type. We observed a dramatic increase in LPA mediated lipogenesis in ovarian cancer cells. However, LPA failed to activate this process in non-transformed cells, suggesting a cancer-specific action of LPA. This increase in lipid synthesis was not due to the increase in cell numbers as the results were normalized to the activity of a fixed numbers of cells. The lipid contents within cells were also increased dramatically after LPA treatment, as visualized by BODIPY 493/503 staining of neutral lipids and biochemical quantitation of different classes of intracellular lipids. It is important to note that the most significant increase in lipids was detected as TAG. There was also less dramatic increase in the content of phospholipids, which correlated with increases in cell number. Liver and adipogenic tissues are known to accumulate fat as lipid droplets which can then be broken down by β-oxidation to release energy. Such a mechanism can provide considerable advantages to cancer cells, such as reducing their dependence on growth factors and energy production, and promoting self-sufficiency as observed in co-culture experiment of adipocytes and cancer cells (308). Indeed, several studies have shown that there is increased lipid accumulation in breast (309), brain (310), lung metastasis (311) and in adenomas of the adrenal gland (312). Moreover, accumulation of lipids in cells in proximity to cancer cells has an indirect effect on carcinogenesis; for examples, lipid accumulation in dendritic cells has been shown to promote cancer metastasis (313).

Similar to lipogenic tissues (liver or adipose tissue), LPA mediated lipogenesis in cancer cells was found to be regulated by activation of SREBP proteins. In most cancer cell lines we analyzed, LPA was found to activate SREBP proteins by facilitating proteolytic cleavage of their precursor forms. Since both SREBP-1 and SREBP-2 are activated in a similar manner, LPA treatment led to the accumulation of mature forms of both proteins in nuclei. LPA-induced activation of SREBP proteins is sufficient to increase expression levels of critical lipogenic targets of SREBP proteins - SREBP-1 mediated FAS and ACC and SREBP-2 mediated HMGCoA reductase. Unlike activation of SREBP proteins by receptor tyrosine kinases (RTK), LPA induced activation of SREBP and lipogenesis was found to be dependent on the Rho-Rock pathway. This is the first report that implicates the Rho-Rock pathway in the activation of SREBP. This observation is significant as it indicates that multiple SREBP-activating pathways need to be inhibited to block SREBP-dependent lipogenesis in cancer cells.

The exact mechanism by which LPA activates SREBP proteins remains to be fully elucidated. We hypothesize that LPA facilitates transport of SREBP proteins from the ER to the Golgi, where constitutively active proteases S1P and S2P process SREBP to release its active form. This effect of LPA could be achieved by increasing the ratio of SCAP to INSIG proteins in cells. It will be interesting to test this hypothesis when appropriate antibodies against SCAP and INSIG become commercially available.

Lipogenesis is regulated at multiple levels. One such critical regulator is the serine threonine kinase AMPK, which is known to be activated by an increase in the

AMP: ATP ratio, indicative of reduced ATP levels in cells. AMPK is a master regulator that shuts down anabolic processes to activate energy yielding catabolic processes. It is known to inhibit lipogenesis by targeting various components of the pathway with the most classical target being ACC. Active AMPK phosphorylates ACC at Ser-79 to inhibit its activity, thereby attenuating lipogenic processes. Consistent with the activation of lipogenesis, LPA treatment was found to inhibit AMPK phosphorylation in a Gq-PLC dependent manner. LPA was also found to modestly reduce AMP: ATP levels, a possible mechanism leading to inactivation of AMPK in LPA-treated cells.

A significant finding of this study was the identification of LPA_2 as the major receptor regulating these processes. LPA_2 is the Edg LPA receptor known to be most often overexpressed in various cancer types including, ovarian cancer. The cell lines used in the study express several fold higher level of LPA_2 than non-transformed IOSE-29 cells. This could explain the LPA_2-specific activation of SREBP proteins and lipogenesis in ovarian cancer cells. It was interesting that LPA_2-mediated these processes selectively in the presence of other co-expressed receptors. In particular, although highly expressed in OVCA-432 cells, LPA_3 was not involved in LPA-mediated lipogenesis. On the contrary, downregulation of LPA_3 was consistently associated with slight potentiation of LPA-induced lipogenesis, indicating possible crosstalk between LPA_2 and potentially negative LPA_3 in modulation of the lipogenic response to LPA.

Another question we asked is whether LPA mediated cell proliferation depended on *de novo* lipogenesis. We inhibited lipogenesis in ovarian cancer cell lines by targeting

critical lipogenic enzymes FAS and ACC that were upregulated by LPA treatment. Chemical inhibitors and molecular approaches against these two proteins led to complete attenuation of LPA-induced cell growth. Also, since LPA_2 was the receptor responsible for the LPA-driven lipogenesis, inhibition of LPA_2 also caused a dramatic reduction of cell proliferation. These results indicate a causal role for de novo lipid synthesis in LPA-driven cell proliferation. Hence LPA signaling, especially LPA_2 receptor linked to activation of lipogenic enzymes, can be targeted as possible therapeutic approaches against cancer.

Since *de novo* lipogenesis is an important determinant in LPA driven proliferation, we extended the study to understand the lipogenic phenotype of cancer cells. The first step of fatty acid synthesis involves carboxylation of acetyl-CoA to malonyl-CoA, which is carried out by ACC. FAS then carries out the next steps of synthesis, generating long chain fatty acids by the subsequent addition of 2 carbon units. Acetyl-CoA thus acts as the limiting factor in this process, and so cells need to increase acetyl-CoA levels in the cytosol for lipogenesis to proceed. One of the primary routes for generation of acetyl-CoA is by glucose metabolism. Pyruvate generated via glycolysis is converted into acetyl-CoA by the pyruvate dehydrogenase complex in mitochondria. Since acetyl-CoA cannot exit the mitochondria, it is used to generate citrate, which can exit the mitochondria and is then converted to acetyl-CoA in the cytosol by ATP citrate lyase. Hence, the rate of glycolysis can control lipogenesis in cells, and not surprisingly cancer cells are known to have hyperactive glycolysis. We thus asked if LPA can activate glycolysis in cancer cells to ultimately lead to an increase in lipogenesis.

LPA treatment was found to dramatically activate glycolytic processes in cancer cells; this effect was not seen in non-transformed cells (IOSE-80 and MCF-10A), an observation similar to LPA mediated regulation of lipogenesis. This indicated that LPA regulated both lipogenesis and closely associated glycolytic metabolism. As a consequence of increased glycolysis, treatments with LPA lead to concomitant lactate efflux from cells, which were again observed only in cancer cells. Lactate efflux leads to acidification of the tumor microenvironment, favoring tumor cell invasion (196) and immune modulation (197) which facilitates tumor growth. Hyperactive glycolysis is often associated with enhanced glucose uptake mediated by increased expression of glucose transporters (such as Glut1). Glucose uptake has been shown to be upregulated by Hif-1α, c-Myc, and ATK (298,314,315). Although growth factors including LPA have been shown to regulate all of these mediators, the LPA-mediated increase in glycolysis could not be explained by changes in Glut1 expression. In fact, LPA did not increase glucose uptake in ovarian cancer cell lines (data not shown). Instead, our results demonstrate that LPA enhances glycolysis through transcriptional activation of HK-2, one of the glycolytic genes widely upregulated in cancers (219). This increase in HK-2 levels was functionally sufficient to promote glycolysis in cancer cell lines.

Using an RT-PCR array, we were able to profile transcriptional changes in glycolytic genes induced by LPA. The effect of LPA was not limited to glycolysis as LPA was found to alter mRNA levels of genes involved in various pathways of glucose metabolism including gluconeogenesis, the TCA cycle, the pentose phosphate pathway and glycogen metabolism. In was interesting to observe that there was a concomitant

reduction in transcripts of several genes involved in the TCA cycle such as malate dehydrogenase 1B (MDH1B), pyruvate dehydrogenase kinase 2 (PDK2) and pyruvate dehydrogenase kinase 4 (PDK4). It remains, however, to determine if LPA treatment inhibits the TCA cycle in cancer cells. Consistent with the role of LPA in activation of biosynthetic processes, genes involved in the reductive pentose phosphate pathway (PPP), such as phosphoribosyl pyrophosphate synthetase 1-like 1 (PRPS1L1) and Ribose-5-phosphate isomerase (RPIA), were also upregulated based on the data of RT-PCR array, which could lead to regeneration of glycolytic intermediates. However, the most significant effect of LPA on glucose metabolic genes was found to be on HK-2.

HK-2 is a well-established target of Hif-1α and c-Myc transcription factors (237,316). In this study, LPA increased HK-2 expression in cancer cells via SREBP-1 transcription factors, which are master regulators of fatty acid synthesis. Regulation of HK-2 by SREBP-1 is not an unknown phenomenon. There have been a few studies that connect SREBP activation to HK-2 expression. SREBP-1 has been reported to bind to the HK-2 promoter and activate its expression in human myocytes (317,318) and in rat liver, adipose tissue, and skeletal muscle. (319). However, SREBP-1 regulation of the HK-2 promoter activity and expression has not been observed in cancer cells. Here we show that LPA induced SREBP-1 binding to the -340 to -296 region of the HK-2 promoter, leading to its transactivation. It was interesting that this region had two potential SREs (SRE 2/3), located very close to each other. Mutation of either of the two sites impaired LPA-induced HK-2 promoter activity, indicating that both SREBP-1 sites contribute to optimal activation of the HK-2 promoter in LPA-treated cells. It is also possible that the

two closely linked SREs form a complex with more than one molecule of SREBP-1. Although SREBP-1 functions as the major transcription factor driving HK-2 expression in response to LPA, our mutational analysis of the HK-2 promoter suggest possible involvement of other transcriptional factors that could contribute to LPA- induced HK-2 expression, as the double SRE mutant remained partially responsive to LPA treatment.

In our study, LPA-mediated glycolysis was found to be independent of Hif-1α. Hif-1α is the principal regulator of hypoxia-mediated gene regulation. Hif-1α has been shown to play a major role in regulation of glycolysis in hypoxia. However, hyperactive glycolysis occurs in tumor cells in both hypoxic and normoxic conditions. Although oncogenic pathways such as PI3K (320,321), mutations in VHL (230), succinate dehydrogenase (SDH) (231) and fumarate hydratase (FH) (232) have been shown to stabilize Hif-1α under normoxic conditions, the amount of Hif-1α generated by these effects may not be enough to elicit a transcriptional response, and the majority of tumor samples possess modest amounts of Hif-1α (322). Thus, alternative pathways might exist that could activate glycolysis under normoxic conditions. LPA, by an unidentified mechanism, does lead to stabilization of Hif-1α proteins, but its effect on glycolysis was found to be independent of Hif-1α. These observations indicate that LPA could be one of the causative factors underlying the glycolytic phenotype of cancer cells under normoxic conditions.

Another significant finding from this study was the regulation of LPA-induced HK-2 expression and enhanced glycolysis by LPA_2, providing yet another piece of evidence for coupling of glycolytic and lipogenic processes via a co-regulator.

Interestingly, LPA_2 and to some extent LPA_1, contributed to stabilization of Hif-1α proteins (data not shown), yet the mechanism involved in activation of glycolysis was found to be independent of Hif-1α protein. Our lab has previously demonstrated that LPA-mediated upregulation of VEGF an established Hif-1α target, in ovarian cancer cells is independent of Hif-1α (260). These studies point to alternative Hif-1α-independent pathways mediated by LPA receptors as critical mediators of carcinogenesis. Moreover we provided evidence that the glycolytic effect of LPA was not limited to ovarian cancer. The effect was also observed in cancers of breast, colon and lung. The general effect of LPA in these cancers is consistent with overexpression of the LPA_2 receptor in these cancers. LPA_2 expression in lung cancer has not been studied previously. We here showed for the first time that LPA_2 is overexpressed in lung cancer cell lines as well as in primary lung carcinomas. Further, LPA induced expression of HK-2 and glycolysis in lung cancer cell lines.

Taken together, the results presented in this thesis provide compelling evidence that LPA induces both *de novo* lipid synthesis and glycolysis in diverse types of cancer cells. These effects of LPA are mediated by LPA_2, an LPA receptor subtype overexpressed in many types of human cancers. We have also presented evidence that LPA induction of lipogenesis and glycolysis in cancer cells is an integral component of the cellular proliferative program. Thus our studies have elucidated a novel role of LPA and its receptor LPA_2 in regulation of cancer cell metabolism and cell proliferation. These studies therefore open a new avenue for research in LPA and cancer cell biology.

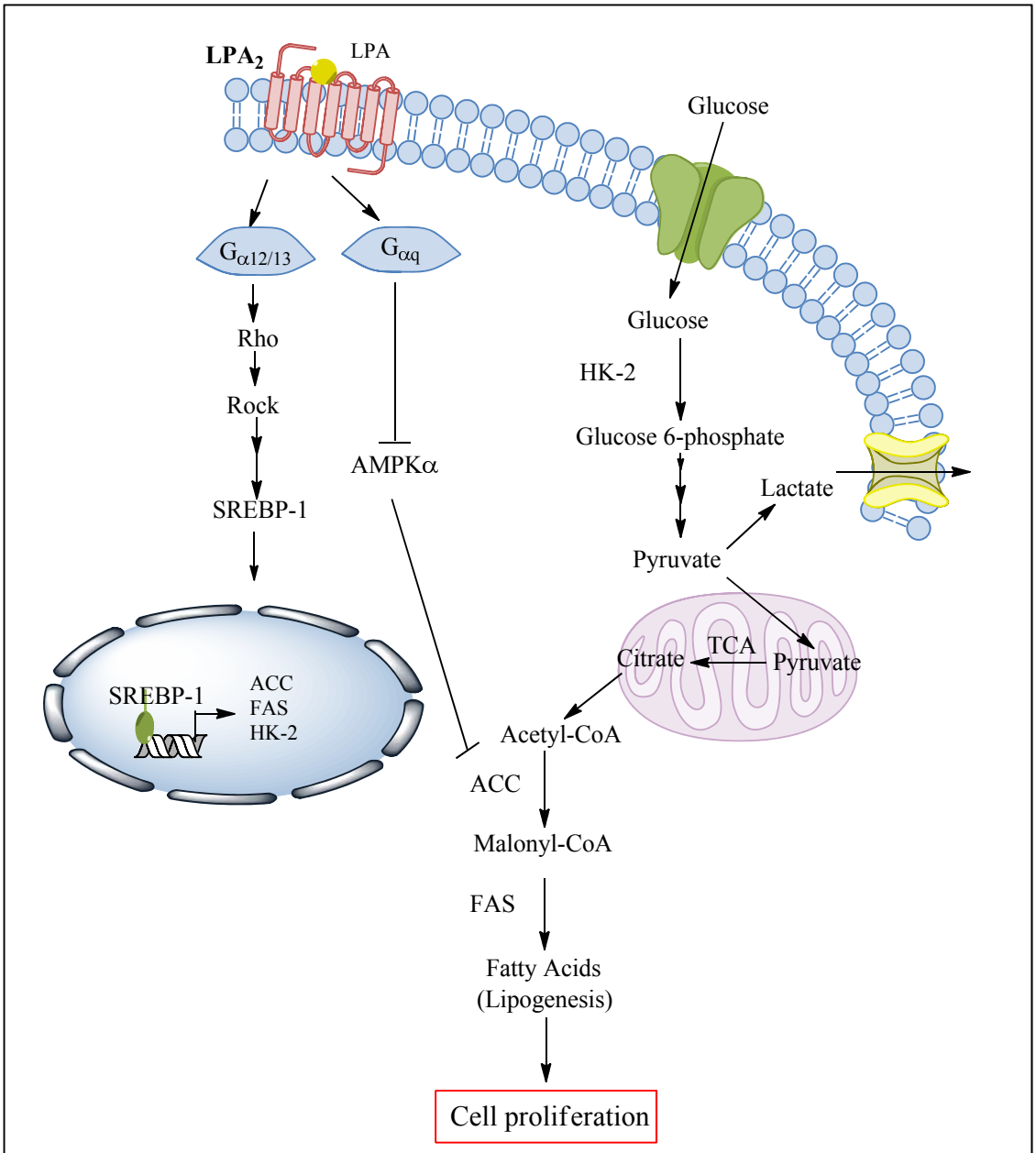


Figure 4.1 General Model of LPA mediated regulation of cancer cell metabolism.

LIST OF REFERENCES

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1. American Cancer, S. (2012) *Cancer Facts and Figures 2012*, American Cancer, Society

2. Cho, K. R., and Shih Ie, M. (2009) Ovarian cancer. *Annu Rev Pathol* **4**, 287-313

3. Risch, H. A., McLaughlin, J. R., Cole, D. E., Rosen, B., Bradley, L., Fan, I., Tang, J., Li, S., Zhang, S., Shaw, P. A., and Narod, S. A. (2006) Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada. *J Natl Cancer Inst* **98**, 1694-1706

4. Jacobs, I. J., Kohler, M. F., Wiseman, R. W., Marks, J. R., Whitaker, R., Kerns, B. A., Humphrey, P., Berchuck, A., Ponder, B. A., and Bast, R. C., Jr. (1992) Clonal origin of epithelial ovarian carcinoma: analysis by loss of heterozygosity, p53 mutation, and X-chromosome inactivation. *J Natl Cancer Inst* **84**, 1793-1798

5. Bast, R. C., Jr., Hennessy, B., and Mills, G. B. (2009) The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer* **9**, 415-428

6. Havrilesky, L., Darcy, M., Hamdan, H., Priore, R. L., Leon, J., Bell, J., and Berchuck, A. (2003) Prognostic significance of p53 mutation and p53 overexpression in advanced epithelial ovarian cancer: a Gynecologic Oncology Group Study. *J Clin Oncol* **21**, 3814-3825

7. Gorno-Tempini, M. L., Dronkers, N. F., Rankin, K. P., Ogar, J. M., Phengrasamy, L., Rosen, H. J., Johnson, J. K., Weiner, M. W., and Miller, B. L. (2004) Cognition and anatomy in three variants of primary progressive aphasia. *Ann Neurol* **55**, 335-346

8. Feng, W., Marquez, R. T., Lu, Z., Liu, J., Lu, K. H., Issa, J. P., Fishman, D. M., Yu, Y., and Bast, R. C., Jr. (2008) Imprinted tumor suppressor genes ARHI and PEG3 are the most frequently down-regulated in human ovarian cancers by loss of heterozygosity and promoter methylation. *Cancer* **112**, 1489-1502

9. Ibanez de Caceres, I., Battagli, C., Esteller, M., Herman, J. G., Dulaimi, E., Edelson, M. I., Bergman, C., Ehya, H., Eisenberg, B. L., and Cairns, P. (2004) Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. *Cancer Res* **64**, 6476-6481

10. Bondurant, A. E., Huang, Z., Whitaker, R. S., Simel, L. R., Berchuck, A., and Murphy, S. K. (2011) Quantitative detection of RASSF1A DNA promoter methylation in tumors and serum of patients with serous epithelial ovarian cancer. *Gynecol Oncol* **123**, 581-587

11. Ryu, S. Y., Kim, K., Lee, W. S., Kwon, H. C., Lee, K. H., Kim, C. M., and Kang, S. B. (2009) Synergistic growth inhibition by combination of adenovirus mediated p53 transfer and cisplatin in ovarian cancer cell lines. *J Gynecol Oncol* **20**, 48-54

12. Cheng, K. W., Lahad, J. P., Kuo, W. L., Lapuk, A., Yamada, K., Auersperg, N., Liu, J., Smith-McCune, K., Lu, K. H., Fishman, D., Gray, J. W., and Mills, G. B. (2004) The RAB25 small GTPase determines aggressiveness of ovarian and breast cancers. *Nat Med* **10**, 1251-1256

13. Cheng, K. W., Lahad, J. P., Gray, J. W., and Mills, G. B. (2005) Emerging role of RAB GTPases in cancer and human disease. *Cancer Res* **65**, 2516-2519

14. Baker, V. V., Borst, M. P., Dixon, D., Hatch, K. D., Shingleton, H. M., and Miller, D. (1990) c-myc amplification in ovarian cancer. *Gynecol Oncol* **38**, 340-342

15. Darcy, K. M., Brady, W. E., Blancato, J. K., Dickson, R. B., Hoskins, W. J., McGuire, W. P., and Birrer, M. J. (2009) Prognostic relevance of c-MYC gene amplification and polysomy for chromosome 8 in suboptimally-resected, advanced stage epithelial ovarian cancers: a Gynecologic Oncology Group study. *Gynecol Oncol* **114**, 472-479

16. Bookman, M. A., Darcy, K. M., Clarke-Pearson, D., Boothby, R. A., and Horowitz, I. R. (2003) Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the Gynecologic Oncology Group. *J Clin Oncol* **21**, 283-290

17. Nakayama, K., Nakayama, N., Jinawath, N., Salani, R., Kurman, R. J., Shih Ie, M., and Wang, T. L. (2007) Amplicon profiles in ovarian serous carcinomas. *Int J Cancer* **120**, 2613-2617

18. Enomoto, T., Inoue, M., Perantoni, A. O., Terakawa, N., Tanizawa, O., and Rice, J. M. (1990) K-ras activation in neoplasms of the human female reproductive tract. *Cancer Res* **50**, 6139-6145

19. Bast Jr, R. C., and Mills, G. B. (2008) Chapter 34 - Molecular Pathogenesis of Epithelial Ovarian Cancer. in *The Molecular Basis of Cancer (Third Edition)* (John, M., Md, Peter, M. H., Mark, A. I., Joe, W. G., PhD, and Craig B. Thompson, M. D. eds.), W.B. Saunders, Philadelphia. pp 441-454

20. Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E., and Meyerson, M. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497-1500

21. Nielsen, J. S., Jakobsen, E., Holund, B., Bertelsen, K., and Jakobsen, A. (2004) Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. *Int J Gynecol Cancer* **14**, 1086-1096

22. Stadlmann, S., Gueth, U., Reiser, U., Diener, P. A., Zeimet, A. G., Wight, E., Mirlacher, M., Sauter, G., Mihatsch, M. J., and Singer, G. (2006) Epithelial growth factor receptor status in primary and recurrent ovarian cancer. *Mod Pathol* **19**, 607-610

23. Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zoltick, P. W., Biegel, J. A., Hayes, R. L., and Wong, A. J. (1995) Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* **55**, 5536-5539

24. Hogdall, E. V., Christensen, L., Kjaer, S. K., Blaakaer, J., Bock, J. E., Glud, E., Norgaard-Pedersen, B., and Hogdall, C. K. (2003) Distribution of HER-2 overexpression in ovarian carcinoma tissue and its prognostic value in patients with ovarian carcinoma: from the Danish MALOVA Ovarian Cancer Study. *Cancer* **98**, 66-73

25. Ciardiello, F., and Tortora, G. (2008) EGFR antagonists in cancer treatment. *N Engl J Med* **358**, 1160-1174

26. Eichholtz, T., Jalink, K., Fahrenfort, I., and Moolenaar, W. H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* **291 (Pt 3)**, 677-680

27. Thumser, A. E., Voysey, J. E., and Wilton, D. C. (1994) The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. *Biochem J* **301 (Pt 3)**, 801-806

28. Tigyi, G., and Miledi, R. (1992) Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. *J Biol Chem* **267**, 21360-21367

29. Ridley, A. J., and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399

30. Aoki, J., Inoue, A., and Okudaira, S. (2008) Two pathways for lysophosphatidic acid production. *Biochim Biophys Acta* **1781**, 513-518

31. Pages, C., Simon, M. F., Valet, P., and Saulnier-Blache, J. S. (2001) Lysophosphatidic acid synthesis and release. *Prostaglandins Other Lipid Mediat* **64**, 1-10

32. Tokumura, A., Harada, K., Fukuzawa, K., and Tsukatani, H. (1986) Involvement of lysophospholipase D in the production of lysophosphatidic acid in rat plasma. *Biochim Biophys Acta* **875**, 31-38

33. Tokumura, A., Miyake, M., Nishioka, Y., Yamano, S., Aono, T., and Fukuzawa, K. (1999) Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of In vitro fertilization patients. *Biol Reprod* **61**, 195-199

34. van Dijk, M. C., Postma, F., Hilkmann, H., Jalink, K., van Blitterswijk, W. J., and Moolenaar, W. H. (1998) Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca2+ signaling pathways. *Curr Biol* **8**, 386-392

35. Umezu-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G. B., Inoue, K., Aoki, J., and Arai, H. (2002) Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J Cell Biol* **158**, 227-233

36. Mills, G. B., and Moolenaar, W. H. (2003) The emerging role of lysophosphatidic acid in cancer. *Nat Rev Cancer* **3**, 582-591

37. Pamuklar, Z., Federico, L., Liu, S., Umezu-Goto, M., Dong, A., Panchatcharam, M., Fulkerson, Z., Berdyshev, E., Natarajan, V., Fang, X., van Meeteren, L. A., Moolenaar, W. H., Mills, G. B., Morris, A. J., and Smyth, S. S. (2009) Autotaxin/lysopholipase D and lysophosphatidic acid regulate murine hemostasis and thrombosis. *J Biol Chem* **284**, 7385-7394

38. Nam, S. W., Clair, T., Campo, C. K., Lee, H. Y., Liotta, L. A., and Stracke, M. L. (2000) Autotaxin (ATX), a potent tumor motogen, augments invasive and metastatic potential of ras-transformed cells. *Oncogene* **19**, 241-247

39. Ptaszynska, M. M., Pendrak, M. L., Bandle, R. W., Stracke, M. L., and Roberts, D. D. (2008) Positive feedback between vascular endothelial growth factor-A and autotaxin in ovarian cancer cells. *Mol Cancer Res* **6**, 352-363

40. Yang, S. Y., Lee, J., Park, C. G., Kim, S., Hong, S., Chung, H. C., Min, S. K., Han, J. W., Lee, H. W., and Lee, H. Y. (2002) Expression of autotaxin (NPP-2) is closely linked to invasiveness of breast cancer cells. *Clin Exp Metastasis* **19**, 603-608

41. Hausmann, J., Kamtekar, S., Christodoulou, E., Day, J. E., Wu, T., Fulkerson, Z., Albers, H. M., van Meeteren, L. A., Houben, A. J., van Zeijl, L., Jansen, S., Andries, M., Hall, T., Pegg, L. E., Benson, T. E., Kasiem, M., Harlos, K., Kooi, C. W., Smyth, S. S., Ovaa, H., Bollen, M., Morris, A. J., Moolenaar, W. H., and Perrakis, A. Structural basis of substrate discrimination and integrin binding by autotaxin. *Nat Struct Mol Biol* **18**, 198-204

42. Nishimasu, H., Okudaira, S., Hama, K., Mihara, E., Dohmae, N., Inoue, A., Ishitani, R., Takagi, J., Aoki, J., and Nureki, O. (2011) Crystal structure of autotaxin and insight into GPCR activation by lipid mediators. *Nat Struct Mol Biol* **18**, 205-212

43. Simpson, C. M., Itabe, H., Reynolds, C. N., King, W. C., and Glomset, J. A. (1991) Swiss 3T3 cells preferentially incorporate sn-2-arachidonoyl monoacylglycerol into sn-1-stearoyl-2-arachidonoyl phosphatidylinositol. *J Biol Chem* **266**, 15902-15909

44. Bektas, M., Payne, S. G., Liu, H., Goparaju, S., Milstien, S., and Spiegel, S. (2005) A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells. *J Cell Biol* **169**, 801-811

45. Waggoner, D. W., Gomez-Munoz, A., Dewald, J., and Brindley, D. N. (1996) Phosphatidate phosphohydrolase catalyzes the hydrolysis of ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate. *J Biol Chem* **271**, 16506-16509

46. Dillon, D. A., Chen, X., Zeimetz, G. M., Wu, W. I., Waggoner, D. W., Dewald, J., Brindley, D. N., and Carman, G. M. (1997) Mammalian Mg2+-independent phosphatidate phosphatase (PAP2) displays diacylglycerol pyrophosphate phosphatase activity. *J Biol Chem* **272**, 10361-10366

47. Tomsig, J. L., Snyder, A. H., Berdyshev, E. V., Skobeleva, A., Mataya, C., Natarajan, V., Brindley, D. N., and Lynch, K. R. (2009) Lipid phosphate phosphohydrolase type 1 (LPP1) degrades extracellular lysophosphatidic acid in vivo. *Biochem J* **419**, 611-618

48. Xu, J., Zhang, Q. X., Pilquil, C., Berthiaume, L. G., Waggoner, D. W., and Brindley, D. N. (2000) Lipid phosphate phosphatase-1 in the regulation of lysophosphatidate signaling. *Ann N Y Acad Sci* **905**, 81-90

49. Eberhardt, C., Gray, P. W., and Tjoelker, L. W. (1997) Human lysophosphatidic acid acyltransferase. cDNA cloning, expression, and localization to chromosome 9q34.3. *J Biol Chem* **272**, 20299-20305

50. Leung, D. W. (2001) The structure and functions of human lysophosphatidic acid acyltransferases. *Front Biosci* **6**, D944-953

51. Moolenaar, W. H., van Meeteren, L. A., and Giepmans, B. N. (2004) The ins and outs of lysophosphatidic acid signaling. *Bioessays* **26**, 870-881

52. Pua, T. L., Wang, F. Q., and Fishman, D. A. (2009) Roles of LPA in ovarian cancer development and progression. *Future Oncol* **5**, 1659-1673

53. Balazs, L., Okolicany, J., Ferrebee, M., Tolley, B., and Tigyi, G. (2001) Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo. *Am J Physiol Regul Integr Comp Physiol* **280**, R466-472

54. Sturm, A., and Dignass, A. U. (2002) Modulation of gastrointestinal wound repair and inflammation by phospholipids. *Biochim Biophys Acta* **1582**, 282-288

55. van Nieuw Amerongen, G. P., Vermeer, M. A., and van Hinsbergh, V. W. (2000) Role of RhoA and Rho kinase in lysophosphatidic acid-induced endothelial barrier dysfunction. *Arterioscler Thromb Vasc Biol* **20**, E127-133

56. Boucharaba, A., Serre, C. M., Guglielmi, J., Bordet, J. C., Clezardin, P., and Peyruchaud, O. (2006) The type 1 lysophosphatidic acid receptor is a target for therapy in bone metastases. *Proc Natl Acad Sci U S A* **103**, 9643-9648

57. Haseruck, N., Erl, W., Pandey, D., Tigyi, G., Ohlmann, P., Ravanat, C., Gachet, C., and Siess, W. (2004) The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: involvement of P2Y1 and P2Y12 receptors. *Blood* **103**, 2585-2592

58. Tanaka, M., Okudaira, S., Kishi, Y., Ohkawa, R., Iseki, S., Ota, M., Noji, S., Yatomi, Y., Aoki, J., and Arai, H. (2006) Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J Biol Chem* **281**, 25822-25830

59. Yuan, X. B., Jin, M., Xu, X., Song, Y. Q., Wu, C. P., Poo, M. M., and Duan, S. (2003) Signalling and crosstalk of Rho GTPases in mediating axon guidance. *Nat Cell Biol* **5**, 38-45

60. Fukushima, N., Shano, S., Moriyama, R., and Chun, J. (2007) Lysophosphatidic acid stimulates neuronal differentiation of cortical neuroblasts through the LPA1-G(i/o) pathway. *Neurochem Int* **50**, 302-307

61. Ramakers, G. J., and Moolenaar, W. H. (1998) Regulation of astrocyte morphology by RhoA and lysophosphatidic acid. *Exp Cell Res* **245**, 252-262

62. Hayashi, K., Takahashi, M., Nishida, W., Yoshida, K., Ohkawa, Y., Kitabatake, A., Aoki, J., Arai, H., and Sobue, K. (2001) Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids. *Circ Res* **89**, 251-258

63. Ferry, G., Tellier, E., Try, A., Gres, S., Naime, I., Simon, M. F., Rodriguez, M., Boucher, J., Tack, I., Gesta, S., Chomarat, P., Dieu, M., Raes, M., Galizzi, J. P., Valet, P., Boutin, J. A., and Saulnier-Blache, J. S. (2003) Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity. *J Biol Chem* **278**, 18162-18169

64. Kingsbury, M. A., Rehen, S. K., Contos, J. J., Higgins, C. M., and Chun, J. (2003) Non-proliferative effects of lysophosphatidic acid enhance cortical growth and folding. *Nat Neurosci* **6**, 1292-1299

65. Inoue, M., Rashid, M. H., Fujita, R., Contos, J. J., Chun, J., and Ueda, H. (2004) Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. *Nat Med* **10**, 712-718

66. Fujita, R., Kiguchi, N., and Ueda, H. (2007) LPA-mediated demyelination in ex vivo culture of dorsal root. *Neurochem Int* **50**, 351-355

67. Dubin, A. E., Bahnson, T., Weiner, J. A., Fukushima, N., and Chun, J. (1999) Lysophosphatidic acid stimulates neurotransmitter-like conductance changes that precede GABA and L-glutamate in early, presumptive cortical neuroblasts. *J Neurosci* **19**, 1371-1381

68. Postma, F. R., Jalink, K., Hengeveld, T., Bot, A. G., Alblas, J., de Jonge, H. R., and Moolenaar, W. H. (1996) Serum-induced membrane depolarization in quiescent fibroblasts: activation of a chloride conductance through the G protein-coupled LPA receptor. *Embo J* **15**, 63-72

69. Ye, X., Hama, K., Contos, J. J., Anliker, B., Inoue, A., Skinner, M. K., Suzuki, H., Amano, T., Kennedy, G., Arai, H., Aoki, J., and Chun, J. (2005) LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* **435**, 104-108

70. Choi, J. W., Herr, D. R., Noguchi, K., Yung, Y. C., Lee, C. W., Mutoh, T., Lin, M. E., Teo, S. T., Park, K. E., Mosley, A. N., and Chun, J. (2010) LPA receptors: subtypes and biological actions. *Annu Rev Pharmacol Toxicol* **50**, 157-186

71. Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. (1996) Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J Cell Biol* **135**, 1071-1083

72. Matas-Rico, E., Garcia-Diaz, B., Llebrez-Zayas, P., Lopez-Barroso, D., Santin, L., Pedraza, C., Smith-Fernandez, A., Fernandez-Llebrez, P., Tellez, T., Redondo, M., Chun, J., De Fonseca, F. R., and Estivill-Torrus, G. (2008) Deletion of lysophosphatidic acid receptor LPA1 reduces neurogenesis in the mouse dentate gyrus. *Mol Cell Neurosci* **39**, 342-355

73. Estivill-Torrus, G., Llebrez-Zayas, P., Matas-Rico, E., Santin, L., Pedraza, C., De Diego, I., Del Arco, I., Fernandez-Llebrez, P., Chun, J., and De Fonseca, F. R. (2008) Absence of LPA1 signaling results in defective cortical development. *Cereb Cortex* **18**, 938-950

74. Musazzi, L., Di Daniel, E., Maycox, P., Racagni, G., and Popoli, M. Abnormalities in alpha/beta-CaMKII and related mechanisms suggest synaptic dysfunction in hippocampus of LPA1 receptor knockout mice. *Int J Neuropsychopharmacol*, 1-13

75. Contos, J. J., Fukushima, N., Weiner, J. A., Kaushal, D., and Chun, J. (2000) Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc Natl Acad Sci U S A* **97**, 13384-13389

76. Tager, A. M., LaCamera, P., Shea, B. S., Campanella, G. S., Selman, M., Zhao, Z., Polosukhin, V., Wain, J., Karimi-Shah, B. A., Kim, N. D., Hart, W. K., Pardo, A., Blackwell, T. S., Xu, Y., Chun, J., and Luster, A. D. (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* **14**, 45-54

77. Pradere, J. P., Klein, J., Gres, S., Guigne, C., Neau, E., Valet, P., Calise, D., Chun, J., Bascands, J. L., Saulnier-Blache, J. S., and Schanstra, J. P. (2007) LPA1 receptor activation promotes renal interstitial fibrosis. *J Am Soc Nephrol* **18**, 3110-3118

78. Contos, J. J., and Chun, J. (1998) Complete cDNA sequence, genomic structure, and chromosomal localization of the LPA receptor gene, lpA1/vzg-1/Gpcr26. *Genomics* **51**, 364-378

79. Contos, J. J., and Chun, J. (2000) Genomic characterization of the lysophosphatidic acid receptor gene, lp(A2)/Edg4, and identification of a frameshift mutation in a previously characterized cDNA. *Genomics* **64**, 155-169

80. Contos, J. J., Ishii, I., and Chun, J. (2000) Lysophosphatidic acid receptors. *Mol Pharmacol* **58**, 1188-1196

81. An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1998) Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* **273**, 7906-7910

82. Contos, J. J., Ishii, I., Fukushima, N., Kingsbury, M. A., Ye, X., Kawamura, S., Brown, J. H., and Chun, J. (2002) Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Biol* **22**, 6921-6929

83. Yu, S., Murph, M. M., Lu, Y., Liu, S., Hall, H. S., Liu, J., Stephens, C., Fang, X., and Mills, G. B. (2008) Lysophosphatidic acid receptors determine tumorigenicity and aggressiveness of ovarian cancer cells. *J Natl Cancer Inst* **100**, 1630-1642

84. Liu, S., Umezu-Goto, M., Murph, M., Lu, Y., Liu, W., Zhang, F., Yu, S., Stephens, L. C., Cui, X., Murrow, G., Coombes, K., Muller, W., Hung, M. C., Perou, C. M., Lee, A. V., Fang, X., and Mills, G. B. (2009) Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases. *Cancer Cell* **15**, 539-550

85. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999) Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* **274**, 27776-27785

86. Im, D. S., Heise, C. E., Harding, M. A., George, S. R., O'Dowd, B. F., Theodorescu, D., and Lynch, K. R. (2000) Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol Pharmacol* **57**, 753-759

87. Hama, K., Aoki, J., Bandoh, K., Inoue, A., Endo, T., Amano, T., Suzuki, H., and Arai, H. (2006) Lysophosphatidic receptor, LPA3, is positively and negatively regulated by progesterone and estrogen in the mouse uterus. *Life Sci* **79**, 1736-1740

88. Noguchi, K., Ishii, S., and Shimizu, T. (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278**, 25600-25606

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

90. Sumida, H., Noguchi, K., Kihara, Y., Abe, M., Yanagida, K., Hamano, F., Sato, S., Tamaki, K., Morishita, Y., Kano, M. R., Iwata, C., Miyazono, K., Sakimura, K., Shimizu, T., and Ishii, S. (2010) LPA4 regulates blood and lymphatic vessel formation during mouse embryogenesis. *Blood* **116**, 5060-5070

91. Lee, C. W., Rivera, R., Gardell, S., Dubin, A. E., and Chun, J. (2006) GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* **281**, 23589-23597

92. Kotarsky, K., Boketoft, A., Bristulf, J., Nilsson, N. E., Norberg, A., Hansson, S., Owman, C., Sillard, R., Leeb-Lundberg, L. M., and Olde, B. (2006) Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. *J Pharmacol Exp Ther* **318**, 619-628

93. Williams, J. R., Khandoga, A. L., Goyal, P., Fells, J. I., Perygin, D. H., Siess, W., Parrill, A. L., Tigyi, G., and Fujiwara, Y. (2009) Unique ligand selectivity of the GPR92/LPA5 lysophosphatidate receptor indicates role in human platelet activation. *J Biol Chem* **284**, 17304-17319

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

95. Pasternack, S. M., von Kugelgen, I., Aboud, K. A., Lee, Y. A., Ruschendorf, F., Voss, K., Hillmer, A. M., Molderings, G. J., Franz, T., Ramirez, A., Nurnberg, P., Nothen, M. M., and Betz, R. C. (2008) G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. *Nat Genet* **40**, 329-334

96. Tabata, K., Baba, K., Shiraishi, A., Ito, M., and Fujita, N. (2007) The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **363**, 861-866

97. Murakami, M., Shiraishi, A., Tabata, K., and Fujita, N. (2008) Identification of the orphan GPCR, P2Y(10) receptor as the sphingosine-1-phosphate and lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **371**, 707-712

98. Murph, M. M., Nguyen, G. H., Radhakrishna, H., and Mills, G. B. (2008) Sharpening the edges of understanding the structure/function of the LPA1 receptor: expression in cancer and mechanisms of regulation. *Biochim Biophys Acta* **1781**, 547-557

99. Yun, C. C., Sun, H., Wang, D., Rusovici, R., Castleberry, A., Hall, R. A., and Shim, H. (2005) LPA2 receptor mediates mitogenic signals in human colon cancer cells. *Am J Physiol Cell Physiol* **289**, C2-11

100. Hendrix, N. D., Wu, R., Kuick, R., Schwartz, D. R., Fearon, E. R., and Cho, K. R. (2006) Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas. *Cancer Res* **66**, 1354-1362

101. Radvanyi, L., Singh-Sandhu, D., Gallichan, S., Lovitt, C., Pedyczak, A., Mallo, G., Gish, K., Kwok, K., Hanna, W., Zubovits, J., Armes, J., Venter, D., Hakimi, J., Shortreed, J., Donovan, M., Parrington, M., Dunn, P., Oomen, R., Tartaglia, J., and Berinstein, N. L. (2005) The gene associated with trichorhinophalangeal syndrome in humans is overexpressed in breast cancer. *Proc Natl Acad Sci U S A* **102**, 11005-11010

102. Van Leeuwen, F. N., Olivo, C., Grivell, S., Giepmans, B. N., Collard, J. G., and Moolenaar, W. H. (2003) Rac activation by lysophosphatidic acid LPA1 receptors through the guanine nucleotide exchange factor Tiam1. *J Biol Chem* **278**, 400-406

103. Boucharaba, A., Serre, C. M., Gres, S., Saulnier-Blache, J. S., Bordet, J. C., Guglielmi, J., Clezardin, P., and Peyruchaud, O. (2004) Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer. *J Clin Invest* **114**, 1714-1725

104. Horak, C. E., Lee, J. H., Elkahloun, A. G., Boissan, M., Dumont, S., Maga, T. K., Arnaud-Dabernat, S., Palmieri, D., Stetler-Stevenson, W. G., Lacombe, M. L., Meltzer, P. S., and Steeg, P. S. (2007) Nm23-H1 suppresses tumor cell motility by down-regulating the lysophosphatidic acid receptor EDG2. *Cancer Res* **67**, 7238-7246

105. Horak, C. E., Mendoza, A., Vega-Valle, E., Albaugh, M., Graff-Cherry, C., McDermott, W. G., Hua, E., Merino, M. J., Steinberg, S. M., Khanna, C., and Steeg, P. S. (2007) Nm23-H1 suppresses metastasis by inhibiting expression of the lysophosphatidic acid receptor EDG2. *Cancer Res* **67**, 11751-11759

106. Fang, X., Gaudette, D., Furui, T., Mao, M., Estrella, V., Eder, A., Pustilnik, T., Sasagawa, T., Lapushin, R., Yu, S., Jaffe, R. B., Wiener, J. R., Erickson, J. R., and Mills, G. B. (2000) Lysophospholipid growth factors in the initiation, progression, metastases, and management of ovarian cancer. *Ann N Y Acad Sci* **905**, 188-208

107. Fang, X., Schummer, M., Mao, M., Yu, S., Tabassam, F. H., Swaby, R., Hasegawa, Y., Tanyi, J. L., LaPushin, R., Eder, A., Jaffe, R., Erickson, J., and Mills, G. B. (2002) Lysophosphatidic acid is a bioactive mediator in ovarian cancer. *Biochim Biophys Acta* **1582**, 257-264

108. Kitayama, J., Shida, D., Sako, A., Ishikawa, M., Hama, K., Aoki, J., Arai, H., and Nagawa, H. (2004) Over-expression of lysophosphatidic acid receptor-2 in human invasive ductal carcinoma. *Breast Cancer Res* **6**, R640-646

109. Yamashita, H., Kitayama, J., Shida, D., Ishikawa, M., Hama, K., Aoki, J., Arai, H., and Nagawa, H. (2006) Differential expression of lysophosphatidic acid receptor-2 in intestinal and diffuse type gastric cancer. *J Surg Oncol* **93**, 30-35

110. Shida, D., Watanabe, T., Aoki, J., Hama, K., Kitayama, J., Sonoda, H., Kishi, Y., Yamaguchi, H., Sasaki, S., Sako, A., Konishi, T., Arai, H., and Nagawa, H. (2004) Aberrant expression of lysophosphatidic acid (LPA) receptors in human colorectal cancer. *Lab Invest* **84**, 1352-1362

111. Schulte, K. M., Beyer, A., Kohrer, K., Oberhauser, S., and Roher, H. D. (2001) Lysophosphatidic acid, a novel lipid growth factor for human thyroid cells: over-

expression of the high-affinity receptor edg4 in differentiated thyroid cancer. *Int J Cancer* **92**, 249-256

112. Lin, S., Lee, S. J., Shim, H., Chun, J., and Yun, C. C. (2010) The absence of LPA receptor 2 reduces the tumorigenesis by ApcMin mutation in the intestine. *Am J Physiol Gastrointest Liver Physiol* **299**, G1128-1138

113. Lin, S., Wang, D., Iyer, S., Ghaleb, A. M., Shim, H., Yang, V. W., Chun, J., and Yun, C. C. (2009) The absence of LPA2 attenuates tumor formation in an experimental model of colitis-associated cancer. *Gastroenterology* **136**, 1711-1720

114. Zeng, Y., Kakehi, Y., Nouh, M. A., Tsunemori, H., Sugimoto, M., and Wu, X. X. (2009) Gene expression profiles of lysophosphatidic acid-related molecules in the prostate: relevance to prostate cancer and benign hyperplasia. *Prostate* **69**, 283-292

115. Yang, M., Zhong, W. W., Srivastava, N., Slavin, A., Yang, J., Hoey, T., and An, S. (2005) G protein-coupled lysophosphatidic acid receptors stimulate proliferation of colon cancer cells through the {beta}-catenin pathway. *Proc Natl Acad Sci U S A* **102**, 6027-6032

116. Hayashi, M., Okabe, K., Yamawaki, Y., Teranishi, M., Honoki, K., Mori, T., Fukushima, N., and Tsujiuchi, T. (2011) Loss of lysophosphatidic acid receptor-3 enhances cell migration in rat lung tumor cells. *Biochem Biophys Res Commun* **405**, 450-454

117. Abir Mukherjee, J. W., Yongling Gong, Xianjun Fang. (2012) Lysophosphatidic Acid Receptors in Cancer. in *Lysophospholipid Receptors: Signaling and Biochemistry.* (Hla, S., Moolenaar, and Chun ed., John Wiley & Sons, Inc

118. van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenaar, W. H. (1989) Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* **59**, 45-54

119. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1993) Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc Natl Acad Sci U S A* **90**, 1257-1261

120. Fang, X., Yu, S., LaPushin, R., Lu, Y., Furui, T., Penn, L. Z., Stokoe, D., Erickson, J. R., Bast, R. C., Jr., and Mills, G. B. (2000) Lysophosphatidic acid prevents apoptosis in fibroblasts via G(i)-protein-mediated activation of mitogen-activated protein kinase. *Biochem J* **352 Pt 1**, 135-143

121. Takeda, H., Matozaki, T., Takada, T., Noguchi, T., Yamao, T., Tsuda, M., Ochi, F., Fukunaga, K., Inagaki, K., and Kasuga, M. (1999) PI 3-kinase gamma and protein

kinase C-zeta mediate RAS-independent activation of MAP kinase by a Gi protein-coupled receptor. *Embo J* **18**, 386-395

122. Kranenburg, O., Poland, M., van Horck, F. P., Drechsel, D., Hall, A., and Moolenaar, W. H. (1999) Activation of RhoA by lysophosphatidic acid and Galpha12/13 subunits in neuronal cells: induction of neurite retraction. *Mol Biol Cell* **10**, 1851-1857

123. Garcia-Regalado, A., Guzman-Hernandez, M. L., Ramirez-Rangel, I., Robles-Molina, E., Balla, T., Vazquez-Prado, J., and Reyes-Cruz, G. (2008) G protein-coupled receptor-promoted trafficking of Gbeta1gamma2 leads to AKT activation at endosomes via a mechanism mediated by Gbeta1gamma2-Rab11a interaction. *Mol Biol Cell* **19**, 4188-4200

124. Hilal-Dandan, R., Means, C. K., Gustafsson, A. B., Morissette, M. R., Adams, J. W., Brunton, L. L., and Heller Brown, J. (2004) Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho. *J Mol Cell Cardiol* **36**, 481-493

125. Cook, S. J., and McCormick, F. (1996) Kinetic and biochemical correlation between sustained p44ERK1 (44 kDa extracellular signal-regulated kinase 1) activation and lysophosphatidic acid-stimulated DNA synthesis in Rat-1 cells. *Biochem J* **320 (Pt 1)**, 237-245

126. Cantley, L. C. (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657

127. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-1362

128. Fang, X., Yu, S., Bast, R. C., Liu, S., Xu, H. J., Hu, S. X., LaPushin, R., Claret, F. X., Aggarwal, B. B., Lu, Y., and Mills, G. B. (2004) Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J Biol Chem* **279**, 9653-9661

129. Jeong, K. J., Park, S. Y., Seo, J. H., Lee, K. B., Choi, W. S., Han, J. W., Kang, J. K., Park, C. G., Kim, Y. K., and Lee, H. Y. (2008) Lysophosphatidic acid receptor 2 and Gi/Src pathway mediate cell motility through cyclooxygenase 2 expression in CAOV-3 ovarian cancer cells. *Exp Mol Med* **40**, 607-616

130. Zhao, Y., and Natarajan, V. (2009) Lysophosphatidic acid signaling in airway epithelium: role in airway inflammation and remodeling. *Cell Signal* **21**, 367-377

131. Huang, M. C., Lee, H. Y., Yeh, C. C., Kong, Y., Zaloudek, C. J., and Goetzl, E. J. (2004) Induction of protein growth factor systems in the ovaries of transgenic mice

overexpressing human type 2 lysophosphatidic acid G protein-coupled receptor (LPA2). *Oncogene* **23**, 122-129

132. Goppelt-Struebe, M., Fickel, S., and Reiser, C. O. (2000) The platelet-derived-growth-factor receptor, not the epidermal-growth-factor receptor, is used by lysophosphatidic acid to activate p42/44 mitogen-activated protein kinase and to induce prostaglandin G/H synthase-2 in mesangial cells. *Biochem J* **345 Pt 2**, 217-224

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

134. Zhao, Y., He, D., Stern, R., Usatyuk, P. V., Spannhake, E. W., Salgia, R., and Natarajan, V. (2007) Lysophosphatidic acid modulates c-Met redistribution and hepatocyte growth factor/c-Met signaling in human bronchial epithelial cells through PKC delta and E-cadherin. *Cell Signal* **19**, 2329-2338

135. Warburg, O. (1956) On the origin of cancer cells. *Science* **123**, 309-314

136. Ookhtens, M., Kannan, R., Lyon, I., and Baker, N. (1984) Liver and adipose tissue contributions to newly formed fatty acids in an ascites tumor. *Am J Physiol* **247**, R146-153

137. Wang, Q., Li, S., Jiang, L., Zhou, Y., Li, Z., Shao, M., Li, W., and Liu, Y. (2010) Deficiency in hepatic ATP-citrate lyase affects VLDL-triglyceride mobilization and liver fatty acid composition in mice. *J Lipid Res* **51**, 2516-2526

138. Fukuda, H., Katsurada, A., and Iritani, N. (1992) Effects of nutrients and hormones on gene expression of ATP citrate-lyase in rat liver. *Eur J Biochem* **209**, 217-222

139. Migita, T., Narita, T., Nomura, K., Miyagi, E., Inazuka, F., Matsuura, M., Ushijima, M., Mashima, T., Seimiya, H., Satoh, Y., Okumura, S., Nakagawa, K., and Ishikawa, Y. (2008) ATP citrate lyase: activation and therapeutic implications in non-small cell lung cancer. *Cancer Res* **68**, 8547-8554

140. Yancy, H. F., Mason, J. A., Peters, S., Thompson, C. E., 3rd, Littleton, G. K., Jett, M., and Day, A. A. (2007) Metastatic progression and gene expression between breast cancer cell lines from African American and Caucasian women. *J Carcinog* **6**, 8

141. Yahagi, N., Shimano, H., Hasegawa, K., Ohashi, K., Matsuzaka, T., Najima, Y., Sekiya, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Nagai, R., Ishibashi, S., Kadowaki, T., Makuuchi, M., Ohnishi, S., Osuga, J., and Yamada, N. (2005) Co-ordinate

activation of lipogenic enzymes in hepatocellular carcinoma. *Eur J Cancer* **41**, 1316-1322

142. Varis, A., Wolf, M., Monni, O., Vakkari, M. L., Kokkola, A., Moskaluk, C., Frierson, H., Jr., Powell, S. M., Knuutila, S., Kallioniemi, A., and El-Rifai, W. (2002) Targets of gene amplification and overexpression at 17q in gastric cancer. *Cancer Res* **62**, 2625-2629

143. Turyn, J., Schlichtholz, B., Dettlaff-Pokora, A., Presler, M., Goyke, E., Matuszewski, M., Kmiec, Z., Krajka, K., and Swierczynski, J. (2003) Increased activity of glycerol 3-phosphate dehydrogenase and other lipogenic enzymes in human bladder cancer. *Horm Metab Res* **35**, 565-569

144. Szutowicz, A., Kwiatkowski, J., and Angielski, S. (1979) Lipogenetic and glycolytic enzyme activities in carcinoma and nonmalignant diseases of the human breast. *Br J Cancer* **39**, 681-687

145. Halliday, K. R., Fenoglio-Preiser, C., and Sillerud, L. O. (1988) Differentiation of human tumors from nonmalignant tissue by natural-abundance 13C NMR spectroscopy. *Magn Reson Med* **7**, 384-411

146. Bauer, D. E., Hatzivassiliou, G., Zhao, F., Andreadis, C., and Thompson, C. B. (2005) ATP citrate lyase is an important component of cell growth and transformation. *Oncogene* **24**, 6314-6322

147. Hatzivassiliou, G., Zhao, F., Bauer, D. E., Andreadis, C., Shaw, A. N., Dhanak, D., Hingorani, S. R., Tuveson, D. A., and Thompson, C. B. (2005) ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* **8**, 311-321

148. Abu-Elheiga, L., Almarza-Ortega, D. B., Baldini, A., and Wakil, S. J. (1997) Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping, and evidence for two isoforms. *J Biol Chem* **272**, 10669-10677

149. Swinnen, J. V., Vanderhoydonc, F., Elgamal, A. A., Eelen, M., Vercaeren, I., Joniau, S., Van Poppel, H., Baert, L., Goossens, K., Heyns, W., and Verhoeven, G. (2000) Selective activation of the fatty acid synthesis pathway in human prostate cancer. *Int J Cancer* **88**, 176-179

150. Milgraum, L. Z., Witters, L. A., Pasternack, G. R., and Kuhajda, F. P. (1997) Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma. *Clin Cancer Res* **3**, 2115-2120

151. Brusselmans, K., De Schrijver, E., Verhoeven, G., and Swinnen, J. V. (2005) RNA interference-mediated silencing of the acetyl-CoA-carboxylase-alpha gene induces growth inhibition and apoptosis of prostate cancer cells. *Cancer Res* **65**, 6719-6725

152. Chajes, V., Cambot, M., Moreau, K., Lenoir, G. M., and Joulin, V. (2006) Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. *Cancer Res* **66**, 5287-5294

153. Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternack, G. R. (1994) Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc Natl Acad Sci U S A* **91**, 6379-6383

154. Kuhajda, F. P. (2000) Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. *Nutrition* **16**, 202-208

155. Swinnen, J. V., Brusselmans, K., and Verhoeven, G. (2006) Increased lipogenesis in cancer cells: new players, novel targets. *Curr Opin Clin Nutr Metab Care* **9**, 358-365

156. Menendez, J. A., and Lupu, R. (2006) Oncogenic properties of the endogenous fatty acid metabolism: molecular pathology of fatty acid synthase in cancer cells. *Curr Opin Clin Nutr Metab Care* **9**, 346-357

157. Swinnen, J. V., Heemers, H., Deboel, L., Foufelle, F., Heyns, W., and Verhoeven, G. (2000) Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. *Oncogene* **19**, 5173-5181

158. Kumar-Sinha, C., Ignatoski, K. W., Lippman, M. E., Ethier, S. P., and Chinnaiyan, A. M. (2003) Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis. *Cancer Res* **63**, 132-139

159. Oskouian, B. (2000) Overexpression of fatty acid synthase in SKBR3 breast cancer cell line is mediated via a transcriptional mechanism. *Cancer Lett* **149**, 43-51

160. Van de Sande, T., De Schrijver, E., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2002) Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. *Cancer Res* **62**, 642-646

161. Yang, Y. A., Han, W. F., Morin, P. J., Chrest, F. J., and Pizer, E. S. (2002) Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Exp Cell Res* **279**, 80-90

162. Menendez, J. A., Ropero, S., Mehmi, I., Atlas, E., Colomer, R., and Lupu, R. (2004) Overexpression and hyperactivity of breast cancer-associated fatty acid synthase (oncogenic antigen-519) is insensitive to normal arachidonic fatty acid-induced suppression in lipogenic tissues but it is selectively inhibited by tumoricidal alpha-linolenic and gamma-linolenic fatty acids: a novel mechanism by which dietary fat can alter mammary tumorigenesis. *Int J Oncol* **24**, 1369-1383

163. Graner, E., Tang, D., Rossi, S., Baron, A., Migita, T., Weinstein, L. J., Lechpammer, M., Huesken, D., Zimmermann, J., Signoretti, S., and Loda, M. (2004) The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer Cell* **5**, 253-261

164. Priolo, C., Tang, D., Brahamandan, M., Benassi, B., Sicinska, E., Ogino, S., Farsetti, A., Porrello, A., Finn, S., Zimmermann, J., Febbo, P., and Loda, M. (2006) The isopeptidase USP2a protects human prostate cancer from apoptosis. *Cancer Res* **66**, 8625-8632

165. Tontonoz, P., Kim, J. B., Graves, R. A., and Spiegelman, B. M. (1993) ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol Cell Biol* **13**, 4753-4759

166. Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L., and Brown, M. S. (1993) SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell* **75**, 187-197

167. Hua, X., Wu, J., Goldstein, J. L., Brown, M. S., and Hobbs, H. H. (1995) Structure of the human gene encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and SREBF2 to chromosomes 17p11.2 and 22q13. *Genomics* **25**, 667-673

168. Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L., and Brown, M. S. (1997) Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J Clin Invest* **99**, 838-845

169. Miserez, A. R., Cao, G., Probst, L. C., and Hobbs, H. H. (1997) Structure of the human gene encoding sterol regulatory element binding protein 2 (SREBF2). *Genomics* **40**, 31-40

170. Eberle, D., Hegarty, B., Bossard, P., Ferre, P., and Foufelle, F. (2004) SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie* **86**, 839-848

171. Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S., and Goldstein, J. L. (1996) Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* **98**, 1575-1584

172. Foretz, M., Guichard, C., Ferre, P., and Foufelle, F. (1999) Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc Natl Acad Sci U S A* **96**, 12737-12742

173. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., and Shimano, H. (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest* **101**, 2331-2339

174. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc Natl Acad Sci U S A* **95**, 5987-5992

175. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* **101**, 1-9

176. Bizeau, M. E., MacLean, P. S., Johnson, G. C., and Wei, Y. (2003) Skeletal muscle sterol regulatory element binding protein-1c decreases with food deprivation and increases with feeding in rats. *J Nutr* **133**, 1787-1792

177. Commerford, S. R., Peng, L., Dube, J. J., and O'Doherty, R. M. (2004) In vivo regulation of SREBP-1c in skeletal muscle: effects of nutritional status, glucose, insulin, and leptin. *Am J Physiol Regul Integr Comp Physiol* **287**, R218-227

178. Ducluzeau, P. H., Perretti, N., Laville, M., Andreelli, F., Vega, N., Riou, J. P., and Vidal, H. (2001) Regulation by insulin of gene expression in human skeletal muscle and adipose tissue. Evidence for specific defects in type 2 diabetes. *Diabetes* **50**, 1134-1142

179. Heemers, H., Maes, B., Foufelle, F., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2001) Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway. *Mol Endocrinol* **15**, 1817-1828

180. Lacasa, D., Le Liepvre, X., Ferre, P., and Dugail, I. (2001) Progesterone stimulates adipocyte determination and differentiation 1/sterol regulatory element-binding protein 1c gene expression. potential mechanism for the lipogenic effect of progesterone in adipose tissue. *J Biol Chem* **276**, 11512-11516

181. Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J. M., Shimomura, I., Shan, B., Brown, M. S., Goldstein, J. L., and Mangelsdorf, D. J. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* **14**, 2819-2830

182. Radhakrishnan, A., Ikeda, Y., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc Natl Acad Sci U S A* **104**, 6511-6518

183. Sun, L. P., Seemann, J., Goldstein, J. L., and Brown, M. S. (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. *Proc Natl Acad Sci U S A* **104**, 6519-6526

184. Espenshade, P. J., Cheng, D., Goldstein, J. L., and Brown, M. S. (1999) Autocatalytic processing of site-1 protease removes propeptide and permits cleavage of sterol regulatory element-binding proteins. *J Biol Chem* **274**, 22795-22804

185. Duncan, E. A., Brown, M. S., Goldstein, J. L., and Sakai, J. (1997) Cleavage site for sterol-regulated protease localized to a leu-Ser bond in the lumenal loop of sterol regulatory element-binding protein-2. *J Biol Chem* **272**, 12778-12785

186. Rawson, R. B., Zelenski, N. G., Nijhawan, D., Ye, J., Sakai, J., Hasan, M. T., Chang, T. Y., Brown, M. S., and Goldstein, J. L. (1997) Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. *Mol Cell* **1**, 47-57

187. Roth, G., Kotzka, J., Kremer, L., Lehr, S., Lohaus, C., Meyer, H. E., Krone, W., and Muller-Wieland, D. (2000) MAP kinases Erk1/2 phosphorylate sterol regulatory element-binding protein (SREBP)-1a at serine 117 in vitro. *J Biol Chem* **275**, 33302-33307

188. Kotzka, J., Lehr, S., Roth, G., Avci, H., Knebel, B., and Muller-Wieland, D. (2004) Insulin-activated Erk-mitogen-activated protein kinases phosphorylate sterol regulatory element-binding Protein-2 at serine residues 432 and 455 in vivo. *J Biol Chem* **279**, 22404-22411

189. Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., Guertin, D. A., Madden, K. L., Carpenter, A. E., Finck, B. N., and Sabatini, D. M. (2011) mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell* **146**, 408-420

190. Hirano, Y., Murata, S., Tanaka, K., Shimizu, M., and Sato, R. (2003) Sterol regulatory element-binding proteins are negatively regulated through SUMO-1 modification independent of the ubiquitin/26 S proteasome pathway. *J Biol Chem* **278**, 16809-16819

191. Hirano, Y., Yoshida, M., Shimizu, M., and Sato, R. (2001) Direct demonstration of rapid degradation of nuclear sterol regulatory element-binding proteins by the ubiquitin-proteasome pathway. *J Biol Chem* **276**, 36431-36437

192. Rolland, F., Winderickx, J., and Thevelein, J. M. (2002) Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Res* **2**, 183-201

193. Lunt, S. Y., and Vander Heiden, M. G. (2011) Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* **27**, 441-464

194. Christen, S., and Sauer, U. (2011) Intracellular characterization of aerobic glucose metabolism in seven yeast species by 13C flux analysis and metabolomics. *FEMS Yeast Res* **11**, 263-272

195. Valgepea, K., Adamberg, K., Nahku, R., Lahtvee, P. J., Arike, L., and Vilu, R. (2010) Systems biology approach reveals that overflow metabolism of acetate in Escherichia coli is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Syst Biol* **4**, 166

196. Swietach, P., Vaughan-Jones, R. D., and Harris, A. L. (2007) Regulation of tumor pH and the role of carbonic anhydrase 9. *Cancer Metastasis Rev* **26**, 299-310

197. Fischer, K., Hoffmann, P., Voelkl, S., Meidenbauer, N., Ammer, J., Edinger, M., Gottfried, E., Schwarz, S., Rothe, G., Hoves, S., Renner, K., Timischl, B., Mackensen, A., Kunz-Schughart, L., Andreesen, R., Krause, S. W., and Kreutz, M. (2007) Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* **109**, 3812-3819

198. Pouyssegur, J., Dayan, F., and Mazure, N. M. (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* **441**, 437-443

199. Rivenzon-Segal, D., Boldin-Adamsky, S., Seger, D., Seger, R., and Degani, H. (2003) Glycolysis and glucose transporter 1 as markers of response to hormonal therapy in breast cancer. *Int J Cancer* **107**, 177-182

200. Artemov, D., Bhujwalla, Z. M., Pilatus, U., and Glickson, J. D. (1998) Two-compartment model for determination of glycolytic rates of solid tumors by in vivo 13C NMR spectroscopy. *NMR Biomed* **11**, 395-404

201. Kunkel, M., Reichert, T. E., Benz, P., Lehr, H. A., Jeong, J. H., Wieand, S., Bartenstein, P., Wagner, W., and Whiteside, T. L. (2003) Overexpression of Glut-1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma. *Cancer* **97**, 1015-1024

202. Baer, S. C., Casaubon, L., and Younes, M. (1997) Expression of the human erythrocyte glucose transporter Glut1 in cutaneous neoplasia. *J Am Acad Dermatol* **37**, 575-577

203. Smith, T. A. (1999) Facilitative glucose transporter expression in human cancer tissue. *Br J Biomed Sci* **56**, 285-292

204. Macheda, M. L., Rogers, S., and Best, J. D. (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol* **202**, 654-662

205. Yamamoto, T., Seino, Y., Fukumoto, H., Koh, G., Yano, H., Inagaki, N., Yamada, Y., Inoue, K., Manabe, T., and Imura, H. (1990) Over-expression of facilitative glucose transporter genes in human cancer. *Biochem Biophys Res Commun* **170**, 223-230

206. Nishioka, T., Oda, Y., Seino, Y., Yamamoto, T., Inagaki, N., Yano, H., Imura, H., Shigemoto, R., and Kikuchi, H. (1992) Distribution of the glucose transporters in human brain tumors. *Cancer Res* **52**, 3972-3979

207. Brown, R. S., and Wahl, R. L. (1993) Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study. *Cancer* **72**, 2979-2985

208. Cantuaria, G., Fagotti, A., Ferrandina, G., Magalhaes, A., Nadji, M., Angioli, R., Penalver, M., Mancuso, S., and Scambia, G. (2001) GLUT-1 expression in ovarian carcinoma: association with survival and response to chemotherapy. *Cancer* **92**, 1144-1150

209. Flier, J. S., Mueckler, M. M., Usher, P., and Lodish, H. F. (1987) Elevated levels of glucose transport and transporter messenger RNA are induced by ras or src oncogenes. *Science* **235**, 1492-1495

210. Younes, M., Brown, R. W., Mody, D. R., Fernandez, L., and Laucirica, R. (1995) GLUT1 expression in human breast carcinoma: correlation with known prognostic markers. *Anticancer Res* **15**, 2895-2898

211. Rempel, A., Bannasch, P., and Mayer, D. (1994) Differences in expression and intracellular distribution of hexokinase isoenzymes in rat liver cells of different transformation stages. *Biochim Biophys Acta* **1219**, 660-668

212. Mathupala, S. P., Rempel, A., and Pedersen, P. L. (1997) Aberrant glycolytic metabolism of cancer cells: a remarkable coordination of genetic, transcriptional, post-translational, and mutational events that lead to a critical role for type II hexokinase. *J Bioenerg Biomembr* **29**, 339-343

213. Mayer, D., Klimek, F., Rempel, A., and Bannasch, P. (1997) Hexokinase expression in liver preneoplasia and neoplasia. *Biochem Soc Trans* **25**, 122-127

214. Mathupala, S. P., Ko, Y. H., and Pedersen, P. L. (2009) Hexokinase-2 bound to mitochondria: cancer's stygian link to the "Warburg Effect" and a pivotal target for effective therapy. *Semin Cancer Biol* **19**, 17-24

215. Wolf, A., Agnihotri, S., Micallef, J., Mukherjee, J., Sabha, N., Cairns, R., Hawkins, C., and Guha, A. (2011) Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme. *J Exp Med* **208**, 313-326

216. Nakashima, R. A., Mangan, P. S., Colombini, M., and Pedersen, P. L. (1986) Hexokinase receptor complex in hepatoma mitochondria: evidence from N,N'-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore-forming protein VDAC. *Biochemistry* **25**, 1015-1021

217. Pastorino, J. G., and Hoek, J. B. (2003) Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr Med Chem* **10**, 1535-1551

218. Vyssokikh, M. Y., Zorova, L., Zorov, D., Heimlich, G., Jurgensmeier, J. J., and Brdiczka, D. (2002) Bax releases cytochrome c preferentially from a complex between porin and adenine nucleotide translocator. Hexokinase activity suppresses this effect. *Mol Biol Rep* **29**, 93-96

219. Mathupala, S. P., Ko, Y. H., and Pedersen, P. L. (2006) Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene* **25**, 4777-4786

220. Mazurek, S., Boschek, C. B., Hugo, F., and Eigenbrodt, E. (2005) Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin Cancer Biol* **15**, 300-308

221. Christofk, H. R., Vander Heiden, M. G., Harris, M. H., Ramanathan, A., Gerszten, R. E., Wei, R., Fleming, M. D., Schreiber, S. L., and Cantley, L. C. (2008) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* **452**, 230-233

222. Zwerschke, W., Mazurek, S., Massimi, P., Banks, L., Eigenbrodt, E., and Jansen-Durr, P. (1999) Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. *Proc Natl Acad Sci U S A* **96**, 1291-1296

223. Christofk, H. R., Vander Heiden, M. G., Wu, N., Asara, J. M., and Cantley, L. C. (2008) Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* **452**, 181-186

224. Vander Heiden, M. G., Locasale, J. W., Swanson, K. D., Sharfi, H., Heffron, G. J., Amador-Noguez, D., Christofk, H. R., Wagner, G., Rabinowitz, J. D., Asara, J. M., and Cantley, L. C. (2010) Evidence for an alternative glycolytic pathway in rapidly proliferating cells. *Science* **329**, 1492-1499

225. Semenza, G. L. (2010) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* **29**, 625-634

226. Wang, G. L., and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* **270**, 1230-1237

227. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* **92**, 5510-5514

228. Kaelin, W. G., Jr., and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* **30**, 393-402

229. Kapitsinou, P. P., and Haase, V. H. (2008) The VHL tumor suppressor and HIF: insights from genetic studies in mice. *Cell Death Differ* **15**, 650-659

230. Kaelin, W. G., Jr. (2008) The von Hippel-Lindau tumour suppressor protein: O2 sensing and cancer. *Nat Rev Cancer* **8**, 865-873

231. Selak, M. A., Armour, S. M., MacKenzie, E. D., Boulahbel, H., Watson, D. G., Mansfield, K. D., Pan, Y., Simon, M. C., Thompson, C. B., and Gottlieb, E. (2005) Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell* **7**, 77-85

232. King, A., Selak, M. A., and Gottlieb, E. (2006) Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene* **25**, 4675-4682

233. Marin-Hernandez, A., Gallardo-Perez, J. C., Ralph, S. J., Rodriguez-Enriquez, S., and Moreno-Sanchez, R. (2009) HIF-1alpha modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. *Mini Rev Med Chem* **9**, 1084-1101

234. Dang, C. V., and Semenza, G. L. (1999) Oncogenic alterations of metabolism. *Trends Biochem Sci* **24**, 68-72

235. Elstrom, R. L., Bauer, D. E., Buzzai, M., Karnauskas, R., Harris, M. H., Plas, D. R., Zhuang, H., Cinalli, R. M., Alavi, A., Rudin, C. M., and Thompson, C. B. (2004) Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* **64**, 3892-3899

236. Robey, R. B., and Hay, N. (2009) Is Akt the "Warburg kinase"?-Akt-energy metabolism interactions and oncogenesis. *Semin Cancer Biol* **19**, 25-31

237. Kim, J. W., Gao, P., Liu, Y. C., Semenza, G. L., and Dang, C. V. (2007) Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol* **27**, 7381-7393

238. Menendez, J. A., and Lupu, R. (2007) Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer* **7**, 763-777

239. Wang, C., Rajput, S., Watabe, K., Liao, D. F., and Cao, D. (2010) Acetyl-CoA carboxylase-a as a novel target for cancer therapy. *Front Biosci (Schol Ed)* **2**, 515-526

240. Gansler, T. S., Hardman, W., 3rd, Hunt, D. A., Schaffel, S., and Hennigar, R. A. (1997) Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival. *Hum Pathol* **28**, 686-692

241. Kuhajda, F. P., Piantadosi, S., and Pasternack, G. R. (1989) Haptoglobin-related protein (Hpr) epitopes in breast cancer as a predictor of recurrence of the disease. *N Engl J Med* **321**, 636-641

242. Li, J. N., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F., and Pizer, E. S. (2001) Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. *Cancer Res* **61**, 1493-1499

243. Pizer, E. S., Thupari, J., Han, W. F., Pinn, M. L., Chrest, F. J., Frehywot, G. L., Townsend, C. A., and Kuhajda, F. P. (2000) Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. *Cancer Res* **60**, 213-218

244. Zhou, W., Simpson, P. J., McFadden, J. M., Townsend, C. A., Medghalchi, S. M., Vadlamudi, A., Pinn, M. L., Ronnett, G. V., and Kuhajda, F. P. (2003) Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. *Cancer Res* **63**, 7330-7337

245. Menendez, J. A., Colomer, R., and Lupu, R. (2004) Inhibition of tumor-associated fatty acid synthase activity enhances vinorelbine (Navelbine)-induced cytotoxicity and apoptotic cell death in human breast cancer cells. *Oncol Rep* **12**, 411-422

246. Little, J. L., Wheeler, F. B., Fels, D. R., Koumenis, C., and Kridel, S. J. (2007) Inhibition of fatty acid synthase induces endoplasmic reticulum stress in tumor cells. *Cancer Res* **67**, 1262-1269

247. Baker, D. L., Morrison, P., Miller, B., Riely, C. A., Tolley, B., Westermann, A. M., Bonfrer, J. M., Bais, E., Moolenaar, W. H., and Tigyi, G. (2002) Plasma lysophosphatidic acid concentration and ovarian cancer. *Jama* **287**, 3081-3082

248. Westermann, A. M., Havik, E., Postma, F. R., Beijnen, J. H., Dalesio, O., Moolenaar, W. H., and Rodenhuis, S. (1998) Malignant effusions contain lysophosphatidic acid (LPA)-like activity. *Ann Oncol* **9**, 437-442

249. Yanagida, K., Masago, K., Nakanishi, H., Kihara, Y., Hamano, F., Tajima, Y., Taguchi, R., Shimizu, T., and Ishii, S. (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. *J Biol Chem* **284**, 17731-17741

250. Ha, J., Daniel, S., Broyles, S. S., and Kim, K. H. (1994) Critical phosphorylation sites for acetyl-CoA carboxylase activity. *J Biol Chem* **269**, 22162-22168

251. Fang, X., Jin, X., Xu, H. J., Liu, L., Peng, H. Q., Hogg, D., Roth, J. A., Yu, Y., Xu, F., Bast, R. C., Jr., and Mills, G. B. (1998) Expression of p16 induces transcriptional downregulation of the RB gene. *Oncogene* **16**, 1-8

252. Maines-Bandiera, S. L., Kruk, P. A., and Auersperg, N. (1992) Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenetic responses to extracellular matrix. *Am J Obstet Gynecol* **167**, 729-735

253. Chen, H., Wu, X., Pan, Z. K., and Huang, S. (2010) Integrity of SOS1/EPS8/ABI1 tri-complex determines ovarian cancer metastasis. *Cancer Res* **70**, 9979-9990

254. Dent, P., Han, S. I., Mitchell, C., Studer, E., Yacoub, A., Grandis, J., Grant, S., Krystal, G. W., and Hylemon, P. B. (2005) Inhibition of insulin/IGF-1 receptor signaling enhances bile acid toxicity in primary hepatocytes. *Biochem Pharmacol* **70**, 1685-1696

255. Dent, P., Fang, Y., Gupta, S., Studer, E., Mitchell, C., Spiegel, S., and Hylemon, P. B. (2005) Conjugated bile acids promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism in murine and human hepatocytes. *Hepatology* **42**, 1291-1299

256. Soede, R. D., Wijnands, Y. M., Kamp, M., van der Valk, M. A., and Roos, E. (2000) Gi and Gq/11 proteins are involved in dissemination of myeloid leukemia cells to the liver and spleen, whereas bone marrow colonization involves Gq/11 but not Gi. *Blood* **96**, 691-698

257. Stanners, J., Kabouridis, P. S., McGuire, K. L., and Tsoukas, C. D. (1995) Interaction between G proteins and tyrosine kinases upon T cell receptor.CD3-mediated signaling. *J Biol Chem* **270**, 30635-30642

258. Gohla, A., Offermanns, S., Wilkie, T. M., and Schultz, G. (1999) Differential involvement of Galpha12 and Galpha13 in receptor-mediated stress fiber formation. *J Biol Chem* **274**, 17901-17907

259. Aktories, K., and Hall, A. (1989) Botulinum ADP-ribosyltransferase C3: a new tool to study low molecular weight GTP-binding proteins. *Trends Pharmacol Sci* **10**, 415-418

260. Song, Y., Wu, J., Oyesanya, R. A., Lee, Z., Mukherjee, A., and Fang, X. (2009) 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

261. Briggs, M. R., Yokoyama, C., Wang, X., Brown, M. S., and Goldstein, J. L. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence. *J Biol Chem* **268**, 14490-14496

262. Chatterjee, S., Szustakowski, J. D., Nanguneri, N. R., Mickanin, C., Labow, M. A., Nohturfft, A., Dev, K. K., and Sivasankaran, R. (2009) Identification of novel genes and pathways regulating SREBP transcriptional activity. *PLoS One* **4**, e5197

263. Lee, Z., Swaby, R. F., Liang, Y., Yu, S., Liu, S., Lu, K. H., Bast, R. C., Jr., Mills, G. B., and Fang, X. (2006) Lysophosphatidic acid is a major regulator of growth-regulated oncogene alpha in ovarian cancer. *Cancer Res* **66**, 2740-2748

264. Brown, M. S., and Goldstein, J. L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331-340

265. Yang, T., Espenshade, P. J., Wright, M. E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J. L., and Brown, M. S. (2002) Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* **110**, 489-500

266. Hardie, D. G. (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**, 774-785

267. Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, D. G. (1996) Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J Biol Chem* **271**, 27879-27887

268. Kishi, Y., Okudaira, S., Tanaka, M., Hama, K., Shida, D., Kitayama, J., Yamori, T., Aoki, J., Fujimaki, T., and Arai, H. (2006) Autotaxin is overexpressed in glioblastoma multiforme and contributes to cell motility of glioblastoma by converting lysophosphatidylcholine to lysophosphatidic acid. *J Biol Chem* **281**, 17492-17500

269. Noguchi, K., Herr, D., Mutoh, T., and Chun, J. (2009) Lysophosphatidic acid (LPA) and its receptors. *Curr Opin Pharmacol* **9**, 15-23

270. Kamrava, M., Simpkins, F., Alejandro, E., Michener, C., Meltzer, E., and Kohn, E. C. (2005) Lysophosphatidic acid and endothelin-induced proliferation of ovarian cancer cell lines is mitigated by neutralization of granulin-epithelin precursor (GEP), a prosurvival factor for ovarian cancer. *Oncogene* **24**, 7084-7093

271. Goldsmith, Z. G., Ha, J. H., Jayaraman, M., and Dhanasekaran, D. N. (2011) Lysophosphatidic Acid Stimulates the Proliferation of Ovarian Cancer Cells via the gep Proto-Oncogene Galpha(12). *Genes Cancer* **2**, 563-575

272. Goetzl, E. J., Dolezalova, H., Kong, Y., Hu, Y. L., Jaffe, R. B., Kalli, K. R., and Conover, C. A. (1999) Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res* **59**, 5370-5375

273. Halvorson, D. L., and McCune, S. A. (1984) Inhibition of fatty acid synthesis in isolated adipocytes by 5-(tetradecyloxy)-2-furoic acid. *Lipids* **19**, 851-856

274. Kuhajda, F. P., Pizer, E. S., Li, J. N., Mani, N. S., Frehywot, G. L., and Townsend, C. A. (2000) Synthesis and antitumor activity of an inhibitor of fatty acid synthase. *Proc Natl Acad Sci U S A* **97**, 3450-3454

275. Rashid, A., Pizer, E. S., Moga, M., Milgraum, L. Z., Zahurak, M., Pasternack, G. R., Kuhajda, F. P., and Hamilton, S. R. (1997) Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. *Am J Pathol* **150**, 201-208

276. Swinnen, J. V., Esquenet, M., Goossens, K., Heyns, W., and Verhoeven, G. (1997) Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. *Cancer Res* **57**, 1086-1090

277. Menendez, J. A., Oza, B. P., Colomer, R., and Lupu, R. (2005) The estrogenic activity of synthetic progestins used in oral contraceptives enhances fatty acid synthase-dependent breast cancer cell proliferation and survival. *Int J Oncol* **26**, 1507-1515

278. Zhao, Y., Tong, J., He, D., Pendyala, S., Evgeny, B., Chun, J., Sperling, A. I., and Natarajan, V. (2009) Role of lysophosphatidic acid receptor LPA2 in the development of allergic airway inflammation in a murine model of asthma. *Respir Res* **10**, 114

279. Rawson, R. B. (2003) The SREBP pathway--insights from Insigs and insects. *Nat Rev Mol Cell Biol* **4**, 631-640

280. Yellaturu, C. R., Deng, X., Park, E. A., Raghow, R., and Elam, M. B. (2009) Insulin enhances the biogenesis of nuclear sterol regulatory element-binding protein (SREBP)-1c by posttranscriptional down-regulation of Insig-2A and its dissociation from SREBP cleavage-activating protein (SCAP).SREBP-1c complex. *J Biol Chem* **284**, 31726-31734

281. Kim, E. K., Park, J. M., Lim, S., Choi, J. W., Kim, H. S., Seok, H., Seo, J. K., Oh, K., Lee, D. S., Kim, K. T., Ryu, S. H., and Suh, P. G. (2011) Activation of AMP-activated protein kinase is essential for lysophosphatidic acid-induced cell migration in ovarian cancer cells. *J Biol Chem* **286**, 24036-24045

282. Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame Hardie, D., Ashworth, A., and Alessi, D. R. (2005) Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *Embo J* **24**, 1810-1820

283. Sakamoto, K., Zarrinpashneh, E., Budas, G. R., Pouleur, A. C., Dutta, A., Prescott, A. R., Vanoverschelde, J. L., Ashworth, A., Jovanovic, A., Alessi, D. R., and Bertrand, L. (2006) Deficiency of LKB1 in heart prevents ischemia-mediated activation of AMPKalpha2 but not AMPKalpha1. *Am J Physiol Endocrinol Metab* **290**, E780-788

284. Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., Depinho, R. A., Montminy, M., and Cantley, L. C. (2005) The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* **310**, 1642-1646

285. Shackelford, D. B., and Shaw, R. J. (2009) The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer* **9**, 563-575

286. Cairns, R. A., Harris, I. S., and Mak, T. W. (2011) Regulation of cancer cell metabolism. *Nat Rev Cancer* **11**, 85-95

287. Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033

288. Scatena, R., Bottoni, P., Pontoglio, A., Mastrototaro, L., and Giardina, B. (2008) Glycolytic enzyme inhibitors in cancer treatment. *Expert Opin Investig Drugs* **17**, 1533-1545

289. Wilson, J. E. (2003) Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol* **206**, 2049-2057

290. Arora, K. K., and Pedersen, P. L. (1988) Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP. *J Biol Chem* **263**, 17422-17428

291. Yamada, T., Yano, S., Ogino, H., Ikuta, K., Kakiuchi, S., Hanibuchi, M., Kanematsu, T., Taniguchi, T., Sekido, Y., and Sone, S. (2008) Lysophosphatidic acid stimulates the proliferation and motility of malignant pleural mesothelioma cells through lysophosphatidic acid receptors, LPA1 and LPA2. *Cancer Sci* **99**, 1603-1610

292. Vander Heiden, M. G., Plas, D. R., Rathmell, J. C., Fox, C. J., Harris, M. H., and Thompson, C. B. (2001) Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol* **21**, 5899-5912

293. Ashcroft, S. J., Weerasinghe, L. C., Bassett, J. M., and Randle, P. J. (1972) The pentose cycle and insulin release in mouse pancreatic islets. *Biochem J* **126**, 525-532

294. Mills, G. B., May, C., McGill, M., Roifman, C. M., and Mellors, A. (1988) A putative new growth factor in ascitic fluid from ovarian cancer patients: identification, characterization, and mechanism of action. *Cancer Res* **48**, 1066-1071

295. Mukherjee, A., Wu, J., Barbour, S., and Fang, X. (2012) Lysophosphatidic acid activates lipogenic pathways and de novo lipid synthesis in ovarian cancer cells. *J Biol Chem*

296. Locasale, J. W., and Cantley, L. C. (2010) Altered metabolism in cancer. *BMC Biol* **8**, 88

297. Fang, M., Shen, Z., Huang, S., Zhao, L., Chen, S., Mak, T. W., and Wang, X. (2010) The ER UDPase ENTPD5 promotes protein N-glycosylation, the Warburg effect, and proliferation in the PTEN pathway. *Cell* **143**, 711-724

298. Barthel, A., Okino, S. T., Liao, J., Nakatani, K., Li, J., Whitlock, J. P., Jr., and Roth, R. A. (1999) Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *J Biol Chem* **274**, 20281-20286

299. Yun, J., Rago, C., Cheong, I., Pagliarini, R., Angenendt, P., Rajagopalan, H., Schmidt, K., Willson, J. K., Markowitz, S., Zhou, S., Diaz, L. A., Jr., Velculescu, V. E., Lengauer, C., Kinzler, K. W., Vogelstein, B., and Papadopoulos, N. (2009) Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* **325**, 1555-1559

300. Gatenby, R. A., and Gillies, R. J. (2004) Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* **4**, 891-899

301. Govindarajan, B., Sligh, J. E., Vincent, B. J., Li, M., Canter, J. A., Nickoloff, B. J., Rodenburg, R. J., Smeitink, J. A., Oberley, L., Zhang, Y., Slingerland, J., Arnold, R. S., Lambeth, J. D., Cohen, C., Hilenski, L., Griendling, K., Martinez-Diez, M., Cuezva, J. M., and Arbiser, J. L. (2007) Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. *J Clin Invest* **117**, 719-729

302. Jones, R. G., and Thompson, C. B. (2009) Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev* **23**, 537-548

303. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**, 646-674

304. Yalcin, A., Clem, B. F., Simmons, A., Lane, A., Nelson, K., Clem, A. L., Brock, E., Siow, D., Wattenberg, B., Telang, S., and Chesney, J. (2009) Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. *J Biol Chem* **284**, 24223-24232

305. Colombo, S. L., Palacios-Callender, M., Frakich, N., Carcamo, S., Kovacs, I., Tudzarova, S., and Moncada, S. (2011) Molecular basis for the differential use of glucose and glutamine in cell proliferation as revealed by synchronized HeLa cells. *Proc Natl Acad Sci U S A* **108**, 21069-21074

306. Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E., and Kuhajda, F. P. (1996) Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. *Cancer Res* **56**, 2745-2747

307. De Schrijver, E., Brusselmans, K., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2003) RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. *Cancer Res* **63**, 3799-3804

308. Nieman, K. M., Kenny, H. A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M. R., Romero, I. L., Carey, M. S., Mills, G. B., Hotamisligil, G. S., Yamada, S. D., Peter, M. E., Gwin, K., and Lengyel, E. (2011) Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med* **17**, 1498-1503

309. Shi, P., Wang, M., Zhang, Q., and Sun, J. (2008) Lipid-rich carcinoma of the breast. A clinicopathological study of 49 cases. *Tumori* **94**, 342-346

310. Sijens, P. E., Levendag, P. C., Vecht, C. J., van Dijk, P., and Oudkerk, M. (1996) 1H MR spectroscopy detection of lipids and lactate in metastatic brain tumors. *NMR Biomed* **9**, 65-71

311. Le, T. T., Huff, T. B., and Cheng, J. X. (2009) Coherent anti-Stokes Raman scattering imaging of lipids in cancer metastasis. *BMC Cancer* **9**, 42

312. Metser, U., Miller, E., Lerman, H., Lievshitz, G., Avital, S., and Even-Sapir, E. (2006) 18F-FDG PET/CT in the evaluation of adrenal masses. *J Nucl Med* **47**, 32-37

313. Herber, D. L., Cao, W., Nefedova, Y., Novitskiy, S. V., Nagaraj, S., Tyurin, V. A., Corzo, A., Cho, H. I., Celis, E., Lennox, B., Knight, S. C., Padhya, T., McCaffrey, T. V., McCaffrey, J. C., Antonia, S., Fishman, M., Ferris, R. L., Kagan, V. E., and Gabrilovich, D. I. (2010) Lipid accumulation and dendritic cell dysfunction in cancer. *Nat Med* **16**, 880-886

314. Osthus, R. C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L. A., and Dang, C. V. (2000) Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem* **275**, 21797-21800

315. Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**, 721-732

316. Mathupala, S. P., Rempel, A., and Pedersen, P. L. (2001) Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *J Biol Chem* **276**, 43407-43412

317. Rome, S., Lecomte, V., Meugnier, E., Rieusset, J., Debard, C., Euthine, V., Vidal, H., and Lefai, E. (2008) Microarray analyses of SREBP-1a and SREBP-1c target genes identify new regulatory pathways in muscle. *Physiol Genomics* **34**, 327-337

318. Gosmain, Y., Lefai, E., Ryser, S., Roques, M., and Vidal, H. (2004) Sterol regulatory element-binding protein-1 mediates the effect of insulin on hexokinase II gene expression in human muscle cells. *Diabetes* **53**, 321-329

319. Gosmain, Y., Dif, N., Berbe, V., Loizon, E., Rieusset, J., Vidal, H., and Lefai, E. (2005) Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and refeeding in rat tissues. *J Lipid Res* **46**, 697-705

320. Plas, D. R., and Thompson, C. B. (2005) Akt-dependent transformation: there is more to growth than just surviving. *Oncogene* **24**, 7435-7442

321. Inoki, K., Corradetti, M. N., and Guan, K. L. (2005) Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* **37**, 19-24

322. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., and Simons, J. W. (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* **59**, 5830-5835

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PUBLICATIONS

- **Mukherjee A**, Wu J, Barbour S, Fang X (2012). Lysophosphatidic acid activates lipogenic pathways and de novo lipid synthesis in ovarian cancer cells. J Biol Chem. 2012 Jun 3. [Epub ahead of print].

- Wu J, **Mukherjee A**, Lebman DA, Fang X (2012). The Lysophosphatidic acid receptor 1 is a novel target of transforming growth factor beta. Oncogene, In press, 2012

- Wu J, **Mukherjee A**, Lebman DA, Fang X (2011). Lysophosphatidic acid-induced p21Waf1 expression mediates the cytostatic response of breast and ovarian cancer cells to transforming growth factor beta. Mol Cancer Res. 9(11); 1562-70.

- Oyesanya RA, Greenbaum S, Dang D, Lee Z, **Mukherjee A**, Wu J, Dent P, Fang X (2010). Differential requirement of the epidermal growth factor receptor for G protein-mediated activation of transcription factors by lysophosphatidic acid. Mol Cancer 14; 9(1):8.

- Song Y, Wu J, Oyesanya RA, Lee Z, **Mukherjee A**, Fang X (2009). 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;15(2):492-5

- Lee Z, Cheng CT, Zhang H, Subler MA, Wu J, **Mukherjee A**, Windle JJ, Chen CK, Fang X (2008). Role of LPA4/p2y9/GPR23 in negative regulation of cell motility. Mol Biol Cell; 19(12):5435-45.

- Oyesanya RA, Lee ZP, Wu J, Chen J, Song Y, **Mukherjee A**, Dent P, Kordula T, Zhou H, Fang X (2008). Transcriptional and post-transcriptional mechanisms for lysophosphatidic acid-induced cyclooxygenase-2 expression in ovarian cancer cells. FASEB J; 22 (8):2639-51.

- Rehman I, Cross SS, Catto JW, Leiblich Aaron, **Abir Mukherjee**, Azzouzi AR, Leung Hing, Hamdy, Freddie C (2005). Promoter Hyper-methylation of Calcium binding Protein S100A6 and S100A2 in Human Prostate Cancer. The Prostate; 65(4):322-30.

ABSTRACTS AND PRESENTATIONS

- **Abir Mukherjee**, Jinhua Wu, Suzanne Barbour, Frank Fang. Lysophophatidic acid is a novel regulator of de novo lipogenesis in ovarian cancer. Metabolism and Cancer. American Association for Cancer Research, Baltimore, Maryland, October 16-19, 2011

- **Abir Mukherjee**, Jinhua Wu, Suzanne Barbour, Frank Fang. Activation of Lipogenic Pathways by Lysophosphatidic Acid in Ovarian Cancer. Forty Fifth South Eastern Regional Lipid Conference, Cashiers North Carolina, November 12 2010.

- **Abir Mukherjee**, Zendra Lee, Ching-Ting Chen, Helen Zhang, Mark A Subler, Jinhua Wu, Jolene J. Windle, Ching-Kang Chen and Xianjun Fang. Role of LPA4/P2Y9/Gpr23 in negative regulation of cell motility, The twenty fifth annual Daniel T. Watts research poster symposium ,Virginia Commonwealth University, October 28 2008

- **Abir Mukherjee**, Jinhua Wu, Regina Oyesanya, Zendra Lee, Frank Fang. "Essential role of Bcl10 in lysophosphatidic acid-induced NF-kB activation and cytokine production in ovarian cancer cells", Forty Second South Eastern Regional Lipid Conference, Cashiers North Carolina, November 12 2007.

AWARDS AND HONORS

- Excellence in Cancer Research Award, 2nd place poster, Massey Cancer Center Retreat, VA, 2011
- Phi Kappa Phi Honor Society, 2011
- The Herbert John Evans's Award, VCU, 2011
- Integrated Cellular and Molecular Biology Symposium, Outstanding Poster, Medical College of Virginia, VCU, 2010
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