2012

ROLE OF LYSOPHOSPHATIDIC ACID IN REGULATION OF CANCER CELL METABOLISM

Abir Mukherjee

Virginia Commonwealth University

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ROLE OF LYSOPHOSPHATIDIC ACID IN REGULATION OF CANCER CELL METABOLISM

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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Master of Science, the University of Sheffield, England, 2004

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August, 2012
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BRCA1</td>
<td>Breast cancer type 1 susceptibility protein</td>
</tr>
<tr>
<td>AC</td>
<td>Adenyl cyclase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ACL</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5′-triphosphate</td>
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<tr>
<td>ATX</td>
<td>Autotaxin</td>
</tr>
<tr>
<td>CAMKKβ</td>
<td>Calmodulin-dependent protein kinase kinase-beta</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cox-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Edg</td>
<td>Endothelial differentiation gene</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
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<td>Full Name</td>
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</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FDG-PET</td>
<td>Fluorodeoxyglucose positron emission tomography</td>
</tr>
<tr>
<td>FH</td>
<td>Fumarate hydratase</td>
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<td>Fru-2,6-BP</td>
<td>Fructose-2, 6-bisphosphate</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>Glutaminase 1</td>
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<td>GLUT1</td>
<td>Glucose transporter 1</td>
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<tr>
<td>GPAT</td>
<td>Glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
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<td>Hif-1</td>
<td>Hypoxia inducible factor -1</td>
</tr>
<tr>
<td>HK-2</td>
<td>Hexokinase 2</td>
</tr>
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<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>INSIG</td>
<td>Insulin-induced gene</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
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<tr>
<td>KLF7</td>
<td>Kruppel-like factor 7</td>
</tr>
<tr>
<td>KRAS</td>
<td>V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPAAT</td>
<td>LPA acyl transferase</td>
</tr>
<tr>
<td>LPC</td>
<td>lysophosphocholine</td>
</tr>
<tr>
<td>LPPs</td>
<td>Lipid phosphate phosphatases</td>
</tr>
<tr>
<td>LysoPLD</td>
<td>lysophospholipase D</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDH1B</td>
<td>Malate dehydrogenase 1B</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MO25</td>
<td>Mouse protein 25</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF1</td>
<td>Nuclear factor 1</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal human bronchial-epithelial</td>
</tr>
<tr>
<td>NPP</td>
<td>Nucleotide pyrophosphatase/phosphodiesterase</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PDK</td>
<td>Pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PFK1</td>
<td>Phosphofructokinase 1</td>
</tr>
<tr>
<td>PFKFB3</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzyme 3</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>PGAM1</td>
<td>Phosphoglyceric acid mutase 1</td>
</tr>
<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase-1</td>
</tr>
<tr>
<td>PHD2</td>
<td>Prolyl hydroxylase domain protein 2</td>
</tr>
<tr>
<td>PI3KCA</td>
<td>Phosphoinositide-3-kinase, catalytic, alpha polypeptide</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphoinositol biphosphate</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase muscle isozyme</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>PRPS1L1</td>
<td>Phosphoribosyl pyrophosphate synthetase 1-like 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Rock</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>RPIA</td>
<td>Ribose-5-phosphate isomerase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real time-quantitative PCR</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>S1P</td>
<td>Site 1 protease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S2P</td>
<td>Site 2 protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>Sterol regulatory element binding protein cleavage activating protein</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SMB</td>
<td>Somatomedin B</td>
</tr>
<tr>
<td>SP1</td>
<td>Specificity Protein 1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding proteins</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol regulator elements</td>
</tr>
<tr>
<td>STRAD</td>
<td>Ste20-related adaptor</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TIGAR</td>
<td>TP53-induced glycolysis and apoptosis regulator</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel–Lindau tumor suppressor protein</td>
</tr>
<tr>
<td>VZG-1</td>
<td>Ventricular zone gene-1</td>
</tr>
</tbody>
</table>
ABSTRACT

ROLE OF LYSOPHOSPHATIDIC ACID IN REGULATION OF CANCER CELL METABOLISM

By Abir Mukherjee, MSc.

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

Major Director: Xianjun Fang
Associate Professor, Biochemistry and Molecular Biology
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 de 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 LPA2, a receptor subtype overexpressed in multiple cancers, providing an explanation for the cancer specific regulation of FAS and ACC by LPA. Downstream of the LPA2 receptor, we identified the Gα12-Rho-Rock pathway to activate SREBPs and the Gαq-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 LPA2, leading to accumulation of intracellular triacylglycerol and phospholipids. Pharmacological and molecular inhibition of LPA2, 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-1$\alpha$ protein, an established activator of HK-2 and glycolysis, LPA-regulated HK-2 expression and glycolysis was largely independent of Hif-1$\alpha$. 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<sub>2</sub> 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

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

Table 1.2 Altered expressions of oncogenes in ovarian cancer

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

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 pyrophosphate (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

<table>
<thead>
<tr>
<th>Effect</th>
<th>Cell type/remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell proliferation and survival</strong></td>
<td>Many cell types, normal and transformed (51)</td>
</tr>
<tr>
<td><strong>Cell migration and invasion</strong></td>
<td>Many cell types, normal and transformed (51)</td>
</tr>
<tr>
<td><strong>Tumor progression</strong></td>
<td>Mouse xenografts (overexpression or knockdown of LPA receptors) (51,52)</td>
</tr>
<tr>
<td><strong>Wound healing in vivo</strong></td>
<td>Skin (53); intestinal epithelium (54)</td>
</tr>
<tr>
<td><strong>Cell contraction</strong></td>
<td>Smooth muscle cells; myofibroblasts (55)</td>
</tr>
<tr>
<td><strong>Platelet activation and aggregation</strong></td>
<td>LPA in atherosclerotic plaques; synergy with ADP (56,57)</td>
</tr>
<tr>
<td><strong>Cytokine production</strong></td>
<td>Fibroblasts; astrocytes; leukocytes; epithelial and endothelial cells; carcinoma cells (51)</td>
</tr>
<tr>
<td><strong>Stabilization of embryonic vessels</strong></td>
<td>Mouse allantois explant culture (58)</td>
</tr>
<tr>
<td><strong>Neurite retraction, collapse/turning of growth cones</strong></td>
<td>Neuroblastoma cells (28); primary neurons (59)</td>
</tr>
<tr>
<td><strong>Inhibition/reversal of differentiation</strong></td>
<td>Neuroblastoma and glioma cells (60); astrocytes (61) vascular smooth muscle cells (62); pre-adipocytes (63)</td>
</tr>
<tr>
<td><strong>Cerebral cortex growth and folding ex vivo</strong></td>
<td>Action on neural progenitor cells; not observed in LPA₁/LPA₂-deficient mice (64)</td>
</tr>
<tr>
<td><strong>Initiation of neuropathic pain and demyelination of the dorsal root in vivo</strong></td>
<td>Reduced in LPA₁-deficient mice (65)</td>
</tr>
<tr>
<td><strong>Demyelination of the dorsal root ex vivo</strong></td>
<td>Direct action on myelinating Schwann cells (66)</td>
</tr>
<tr>
<td><strong>Membrane depolarization (chloride efflux-mediated)</strong></td>
<td>Neuronal cells (67); fibroblasts (68)</td>
</tr>
<tr>
<td><strong>Blastocyst implantation (timing and spacing)</strong></td>
<td>LPA₃-mediated (69)</td>
</tr>
</tbody>
</table>
The Edg LPA receptors (LPA₁/Edg2, LPA₂/Edg4 and LPA₃/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 (LPA₄/GPR23/P2Y9, LPA₅/GPR92 and LPA₆/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).
LPA₁/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. LPA₁ 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 LPA₁ had been observed in the mouse brain (70,71). Hence the role of LPA₁ in the developing nervous system is a major focus of research. LPA₁ 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 LPA₁ was evident from studies using LPA₁ 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 LPA₁ knockout mice to pathophysiological conditions, its roles in initiation of neuropathic pain (65), and in pulmonary and renal fibrosis have been established (76,77).

LPA₂/Edg4 was identified from GenBank searches of orphan GPCRs and its high amino acid sequence similarity to LPA₁ (55%) (78). In humans, LPA₂ gene is located on chromosome 19 and encodes for a 39 kd protein (79). The expression of LPA₂ as compared to LPA₁ is relatively restricted. In adult mice, LPA₂ 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). LPA₂ knockout mice are viable, grossly normal and display no apparent breeding abnormality. Moreover the LPA₁ and LPA₂ double knockout mice did not show any phenotype additional to LPA₁ knockout mice, except for a minor increase in frontal hematomas (82). Thus it can be speculated that under physiological conditions, LPA₂ may not play a significant role and its functions might be redundant to LPA₁. However, LPA₂ has been found to be upregulated in various cancers and may contribute to pathogenesis of ovarian cancer, colorectal cancer and other malignancies (83,84).

LPA₃/Edg7 was initially identified as an orphan receptor using degenerate PCR-based cloning. Based on its responsiveness to LPA and its homology to LPA₁,₂ receptors (53.7 and 48.8% for LPA₁ and LPA₂ 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, LPA₃ expression is detectable in a number of tissues including heart, testis, prostate, pancreas, lung, ovary, and brain (85,86). In mice, LPA₃ 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, LPA₃ female knockout mice had a dramatic phenotype in the reproductive system. Loss of LPA₃ resulted in delayed implantation and an alteration in embryo spacing resulting in reduced litter size (69). It is, however, notable that LPA₃ knockout mice had no observable phenotype in the nervous system, in spite of its expression in brain.
LPA₄/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 LPA₄ 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 LPA₄ knockout mice. The LPA₄ null mice were viable and had no phenotypic abnormality. However, we demonstrated that the loss of LPA₄ 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 LPA₄ knockout mice and reported partial lethality and defects in blood vessel formation in their LPA₄ knockout mice (90). Although we did not observe any phenotypic defects in our LPA₄ null mice, the difference in phenotype could arise from difference in genetic backgrounds of mice used.

LPA₅/GPR97 is a recently identified LPA receptor that shares 35% homology with LPA₄ and is structurally distant from the Edg LPA receptors (91). The LPA₅ gene is located on chromosome 12 and codes for a 41 kd protein. Using mouse and human tissue samples, LPA₅ 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 LPA₅, 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 \textit{LPA6/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).

LPA7/GPR87 is the last known LPA receptor. The \textit{LPA7} gene is present on chromosome 3 in both mouse and humans. It has been shown that LPA7 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.

\subsection*{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 LPA1 is expressed in a wide range of human and mouse tissues, analysis of LPA1 mRNA expression data from tumor samples failed to conclusively prove overexpression of this receptor in major cancers (98). Moreover several groups have suggested LPA1 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 LPA1 is involved in oncogenic processes, especially in promotion of tumor cell invasion and metastasis. Overexpression
of LPA1 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 LPA1 in enhancing migratory and metastatic potential of cancer cells was also supported by the observation that, Nm23-H1 (a metastatic suppressor) inhibited expression of LPA1 (104,105).

Unlike LPA1, expression of LPA2 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 LPA2 in driving tumorigenesis. Overexpression of LPA2 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 LPA2 (driven by the MMTV promoter) in mice was found to increase the incidence of mammary tumors, as compared to wild type litter mates. Moreover, LPA2 knockout mice were found to be resistant to intestinal tumor formation in both the Adenomatous polyposis coli (APC)\textsuperscript{-/-} mouse model (112) and dextran sulfate sodium model (113). It is thus evident that LPA2 plays a
major role in tumor development. However, the exact mechanism by which LPA2 regulates oncogenic processes remains elusive.

The role of LPA3 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, LPA3 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 LPA3 contributes to LPA-driven proliferation of these cells (115). In addition, LPA3 negatively regulates LPA1-driven migration of rat lung cancer cells (116). It is evident that further studies are required in order to clarify the role of LPA3 in cancer.

As compared to the Edg LPA receptors, the contributions of non-Edg receptors in tumorigenesis have not been elaborately studied. LPA4, 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 LPA4 levels between cancers and their corresponding non-cancer tissues (Figure 1.3). Thus it is imperative to study LPA4 expression in individual cancer types separately. We have recently shown that LPA4 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 LPA5, LPA6 and LPA7 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>P-value</th>
<th>Fold Change</th>
<th>1.</th>
<th>2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPAR2</td>
<td>0.015</td>
<td>1.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR4</td>
<td>0.439</td>
<td>1.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR8</td>
<td>0.811</td>
<td>-0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR6</td>
<td>0.760</td>
<td>-1.09</td>
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<td></td>
</tr>
<tr>
<td>LPAR1</td>
<td>0.836</td>
<td>-1.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Lung (30)  
2. Lung Adenocarcinoma (27)

<table>
<thead>
<tr>
<th>Gene</th>
<th>P-value</th>
<th>Fold Change</th>
<th>1.</th>
<th>2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPAR5</td>
<td>9.73E-4</td>
<td>7.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR3</td>
<td>0.059</td>
<td>1.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR1</td>
<td>0.439</td>
<td>1.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR8</td>
<td>0.441</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR6</td>
<td>0.781</td>
<td>-1.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR4</td>
<td>0.920</td>
<td>-1.77</td>
<td></td>
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</tbody>
</table>

1. Breast (9)  
2. Invasive Ductal Breast Carcinoma (33)

<table>
<thead>
<tr>
<th>Gene</th>
<th>P-value</th>
<th>Fold Change</th>
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</tr>
</thead>
<tbody>
<tr>
<td>LPAR3</td>
<td>4.48E-6</td>
<td>1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR2</td>
<td>4.29E-4</td>
<td>1.12</td>
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<td></td>
</tr>
<tr>
<td>LPAR1</td>
<td>0.897</td>
<td>-0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR8</td>
<td>1.00E0</td>
<td>-0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR6</td>
<td>1.00E0</td>
<td>-0.26</td>
<td></td>
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</tr>
</tbody>
</table>

1. Ovary (4)  
2. Ovarian Serous Adenocarcinoma (41)

<table>
<thead>
<tr>
<th>Gene</th>
<th>P-value</th>
<th>Fold Change</th>
<th>1.</th>
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</thead>
<tbody>
<tr>
<td>LPAR2</td>
<td>4.28E-5</td>
<td>5.97</td>
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<tr>
<td>LPAR3</td>
<td>2.40E-4</td>
<td>1.61</td>
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<tr>
<td>LPAR1</td>
<td>0.580</td>
<td>-0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR8</td>
<td>0.705</td>
<td>-0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR6</td>
<td>0.895</td>
<td>-0.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Bladder (48)  
2. Superficial Bladder Cancer (28)

<table>
<thead>
<tr>
<th>Gene</th>
<th>P-value</th>
<th>Fold Change</th>
<th>1.</th>
<th>2.</th>
</tr>
</thead>
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<td>7.30E-5</td>
<td>1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR1</td>
<td>0.413</td>
<td>1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR8</td>
<td>0.434</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR6</td>
<td>0.441</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPAR5</td>
<td>0.726</td>
<td>-0.05</td>
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</tr>
<tr>
<td>LPAR1</td>
<td>1.000</td>
<td>-1.93</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Colon (10)  
2. Colon Carcinoma (5)

---

Least Expressed  
Most Expressed  
Not measured
1.1.4 LPA RECEPTOR MEDIATED SIGNALING

Like other GPCRs, LPA receptors couple to multiple G-proteins. G-proteins are heterotrimeric proteins (composed of α-β-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 $G_{ai}$, $G_{aq}$, $G_{a12/13}$ and in certain cases $G_{as}$. LPA$_1$ and LPA$_2$ receptors couple to $G_{ai}$, $G_{aq}$, and $G_{a12/13}$, whereas LPA$_3$ couples to $G_{ai}$ and $G_{aq}$. The non-Edg LPA receptors are not known to activate $G_{ai}$ but are known to activate $G_{as}$ instead. This is a significant difference as coupling to $G_{as}$ leads to activation of adenyl cyclase (AC) and hence leads to increase in cAMP (cyclic-adenosine monophosphate), whereas as $G_{ai}$ mediates inhibition of AC. LPA via $G_{ai}$ 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 $G_{aq}$ 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 $G_{a12/13}$ activates RhoA leading to cytoskeletal changes and cell rounding (122). Thus LPA via $G_{ai}$ regulates cell proliferation and survival, via $G_{aq}$ regulates the production of secondary messengers and by combined actions of $G_{ai}$ and $G_{a12/13}$ regulates cell migration and invasion.
Apart from the Gα-subunit, Gβγ-subunits have also been shown to activate signaling pathways. Upon LPA stimulation, Gβγ-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 G\(\alpha_i\) pathway (118,120), however possible contribution by RhoA signaling has also been suggested (124).

Although LPA activates the G\(\alpha_{qi}\)-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 G\(\alpha_i\) 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, HIF1\(\alpha\), 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* LIPOGENESIS

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).
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 \textit{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 increase 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).
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 I 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

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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 LPA₅, a LPA receptor subtype overexpressed in ovarian cancer and other malignancies. Downstream of LPA₂, the Gα₁₂/₁₃ and Gα₉ 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 LPA1/Edg2, LPA2/Edg4 and LPA3/Edg7 receptors are members of the endothelial differentiation gene (Edg) family, sharing 46 - 50% amino acid sequence identity (70). GPR23/P2Y9/LPA4 of the purinergic receptor family and the related GPR92/LPA5 and P2Y5/LPA6 have been identified as additional LPA receptors, which are structurally distant from the LPA1-3 receptors (70,249). The Edg LPA receptors, in particular LPA2, are overexpressed in many types of human malignancies including ovarian cancer (36,107). Strong evidence implicates LPA2 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 LPA2, 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\textsubscript{α12/13}-Rho signaling cascade is critical for LPA activation of the SREBP while G\textsubscript{αq}-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-\textsuperscript{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 LPA1, LPA2, LPA3, 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 LPA1, LPA2, LPA3, 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 LPA1,3 receptors were kind gifts from Dr. S. Huang (Medical College of Georgia) (253). The expression vector pcDNA3 expressing dominant negative form of Gαi was provided by Dr. P. Hylemon (Virginia Commonwealth University) (254,255). The Gαq and Gα12 cDNAs were provided by Dr. RD Ye (University of Illinois at Chicago). The dominant-negative mutants of Gαq (G208A) and Gα12 (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 NheI and HindIII 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 LPA₁, LPA₂, LPA₃, 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 KH₂PO₄ and 750 mM KH₂PO₄, 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.

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.
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.
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

![Figure 2.4 LPA induces dephosphorylation of AMPKα and ACC.](image-url)

*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.
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 14C acetic acid before lipid extraction as described in Experimental Procedures. The incorporation of 14C 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 LPA2 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 LPA₁, LPA₂, and LPA₃ (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 LPA₁, LPA₂, and LPA₃ in the regulation of lipogenesis. We used siRNA to knockdown expression of LPA₁, LPA₂, and LPA₃ in Caov-3 cells and examined SREBP activation and AMPKα dephosphorylation in response to LPA treatment. Interestingly, only knockdown of LPA₂ 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 LPA₁ or LPA₃ 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 LPA₂ receptor in LPA-dependent activation of SREBP-1 and inhibition of AMPKα. However, overexpression of LPA₂ 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 LPA₂ 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 LPA₂ (Fig. 2.7D). In contrast, knockdown of LPA₃ (Fig. 2.7D) or LPA₁ (data not shown) did not inhibit LPA-induced lipid synthesis.
We next examined the signaling effectors downstream of LPA2 responsible for cleavage of SREBP-1 and dephosphorylation of AMPKα. The LPA1-3 receptors couple to Gαi and Gαq, while only LPA1 and LPA2 couple to Gα12/13 (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 Gα12 attenuated LPA-induced SREBP-1 cleavage but not LPA-induced dephosphorylation of AMPKα. In contrast, expression of dominant negative Gαq 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 its inactive analog U73433, blocked AMPK\(\alpha\) dephosphorylation induced by LPA. The data supports a \( G_{\alpha q}\)-PLC-dependent mechanism to control phosphorylation and activity of AMPK\(\alpha\) 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 Gαi, Gαq, and Gα12 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 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 LPA₂ 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 LPA2 is required for cell proliferation and anchorage-independent growth. **A.** LPA2 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 LPA₂ are not well understood. Most previous studies have been focused on the ability of LPA₂ 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). LPA₂ 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 LPA₂ to the lipogenic phenotype of ovarian tumor cells. The role of LPA₂ in lipid metabolism provides a new avenue to explore the oncogenic role of LPA.

Different G proteins downstream of LPA₂ 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 G₁₂/₁₃-Rho pathway, while AMPK dephosphorylation/inhibition is mediated by the G₉-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 Gq-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 \( \alpha \)-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-1\( \alpha \)). 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 LPA2-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 enzyme 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). LPA1, LPA2 and LPA3 are LPA receptors that belong to the endothelial gene (Edg) subfamily of GPCRs. The purinergic family receptor LPA4 and related LPA5, LPA6 and LPA7 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, LPA2 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). LPA2 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 LPA2 has also been linked to proliferation of colon and breast cancer cells and mesothelioma cells (84,99,291). Although LPA2 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 LPA2 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-oleoyl, 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.
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 LPA1, LPA2, LPA3, 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 LPA1, LPA2, LPA3, 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 (CCAGTCGCCACACC and CACGCTCCCCACCA) within pGL2-1476-HK-2-Luc were converted into inactive (CCAGGTGTCTTACACC and CACGCGTCTTACCA) 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, RT² 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™ 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 LPA₂ 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 12th hour of LPA treatment, 5-³H (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>
<thead>
<tr>
<th>Table 3.1 Oligonucleotides used in study</th>
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<tr>
<td><strong>Luciferase primers</strong></td>
</tr>
<tr>
<td>-1476 fwd 5’-GCACTCGAGGGATTATGATTTTTCTTATTTTTCCT-3’</td>
</tr>
<tr>
<td>+73 rvs 5’-GCAAGAGCTTTCAATTCGATTTTTCTTATTTTTCCT-3’</td>
</tr>
<tr>
<td>-478 fwd 5’-GCACTCGAGCCGGCCGATGCTACAATAG-3’</td>
</tr>
<tr>
<td>-273 fwd 5’-GCACTCGAGCTCATGCGCCTTTCCGTC-3’</td>
</tr>
<tr>
<td>SRE1 Mut fwd 5’-CAGAGGGCCCGTTTCCAGGTGTCTTACACCCCGGGTCCGCGAT-3’</td>
</tr>
<tr>
<td>SRE1 Mut rvs 5’-ATCGCGGACCCGGGTGTAAGACACCTGGA AAA ACG GGC CTC TG-3’</td>
</tr>
<tr>
<td>SRE2 Mut fwd 5’-GGGTCGCGATCACCGTGCTCTTTACACCATAGGCCAGCGCT-3’</td>
</tr>
<tr>
<td>SRE2 Mut rvs 5’-CAGGCTCGGCTATGGGTGGGGGTGGAGCGTGATCGCGGACCCCG-3’</td>
</tr>
<tr>
<td><strong>DNA pull-down oligonucleotides</strong></td>
</tr>
<tr>
<td>HK-2 WT fwd 5’-CGTTTTTCCAGTCGCCACACACCCGGGTCCGCGATCAGCTCCCCCACCCTAG CCGA-3’</td>
</tr>
<tr>
<td>HK-2 WT rvs 5’-TCGGCTATGGGTGGGGGTGGAGCGTGATCGCGGACCCCGGGGTG TGGGCGACTGAAAAACG -3’</td>
</tr>
<tr>
<td>HK-2 Mut fwd 5’-CGTTTTTCCAGGTGTCTTTACACCACCCGGGTCCGCGATCAGCG TCTCTTACCCATAGCCG -3’</td>
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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 [3H]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).
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.

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

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).
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.](image)
3.3.4 LPA₂ IS THE MAJOR RECEPTOR THAT UPREGULATES HK-2

EXPRESSSION AND GLYCOLYSIS

Caov-3, OVCA-432 and other ovarian cancer cell lines express the Edg LPA receptors LPA₁, LPA₂, and LPA₃ (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 LPA₁, LPA₂, and LPA₃ in Caov-3 cells. Only knockdown of LPA₂ 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 LPA₂ is the major LPA receptor subtype accounting for LPA-driven HK-2 induction and glycolysis in ovarian cancer cells.

**Figure 3.6** LPA₂ is the major LPA receptor subtype responsible for HK-2 induction (**A**) and glycolysis (**B**). Each of LPA₁-₃ 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-1α 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-1α 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.

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

Since LPA₂, 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).
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
LPA₂ 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 LPA₂ mRNA was significantly increased in all stages of lung cancers including Stage I. Consistent with potential regulation of HK-2 via LPA₂ signaling, these lung cancer specimens also showed overexpression of HK-2 mRNA when compared with normal lung tissues. (Figure 3.12C).
Figure 3.12 LPA₂ and HK-2 are abnormally overexpressed in lung cancer. A. RT-qPCR was carried out to determine the relative levels of LPA₂ in lung cancer cell lines and NHBE cells. Expression of LPA₂ (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 LPA₂ 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-LPA2-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 LPA2, a receptor subtype overexpressed in many malignancies including ovarian cancer (106,108-111), was the major receptor promoting glycolysis. LPA1, 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 LPA2 completely inhibited LPA-dependent glycolysis, suggesting a primary role of LPA2 in the process. Given the importance of LPA2 in cancer cell metabolism and the non-essential physiological role of this receptor in mice, inhibition of LPA2 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 through 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 LPA2 and LPA3 in ovarian cancer (107,120), and subsequently several labs have provided evidence for overexpression of LPA2 in various other malignancies. LPA2 couples to \( \alpha_i \), \( \alpha_q \) and \( \alpha_{12/13} \) G-proteins. Pertussis toxin sensitive \( \alpha_i \)-driven signaling has been shown to regulate LPA-mediated cell proliferation (118,120). Downstream of \( \alpha_i \), 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 LPA2 as the major receptor regulating these processes. LPA2 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 LPA2 than non-transformed IOSE-29 cells. This could explain the LPA2-specific activation of SREBP proteins and lipogenesis in ovarian cancer cells. It was interesting that LPA2-mediated these processes selectively in the presence of other co-expressed receptors. In particular, although highly expressed in OVCA-432 cells, LPA3 was not involved in LPA-mediated lipogenesis. On the contrary, downregulation of LPA3 was consistently associated with slight potentiation of LPA-induced lipogenesis, indicating possible crosstalk between LPA2 and potentially negative LPA3 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 LPA2, providing yet another piece of evidence for coupling of glycolytic and lipogenic processes via a co-regulator.

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Interestingly, LPA$_2$ and to some extent LPA$_1$, contributed to stabilization of Hif-1$\alpha$ proteins (data not shown), yet the mechanism involved in activation of glycolysis was found to be independent of Hif-1$\alpha$ protein. Our lab has previously demonstrated that LPA-mediated upregulation of VEGF an established Hif-1$\alpha$ target, in ovarian cancer cells is independent of Hif-1$\alpha$ (260). These studies point to alternative Hif-1$\alpha$-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 \textit{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.
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**ABSTRACTS AND PRESENTATIONS**


**AWARDS AND HONORS**

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