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**DIFFERENTIAL EFFECTS OF GROWTH FACTORS ON GLYCOLYSIS  
IN OVARIAN CANCER CELLS**

A Dissertation submitted in partial fulfillment of the requirements for the degree of Master of  
Science at Virginia Commonwealth University.

By

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## LIST OF ABBREVIATIONS

2-DG	2-Deoxy-D-glucose
3-BrPA	3-Bromo-2-oxopropanoic acid
AC	Adenyl cyclase
ACC	Acetyl-CoA carboxylase
ACLY	ATP citrate lyase
AGL	Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase
AKT/PKB	Protein kinase B
ALDOA	Aldolase A, fructose-bisphosphate
ALDOB	Aldolase B, fructose-bisphosphate
ALDOC	Aldolase C, fructose-bisphosphate
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
BPGM	2,3-bisphosphoglycerate mutase

cAMP	Cyclic-adenosine monophosphate
CS	Citrate synthase
DLAT	Dihydrolipoamide S-acetyltransferase
DLST	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)
Edg	Endothelial differentiation gene
EGF	Epidermal growth factor
ENO1	Enolase 1 (alpha)
ENO2	Enolase 2 (gamma, neuronal)
ENO3	Enolase 3 (beta, muscle)
Erk	Extracellular signal-regulated kinases
FAS	Fatty acid synthase
FBP1	Fructose-1,6-bisphosphatase 1
FBP2	Fructose-1,6-bisphosphatase 2
FH	Fumarate hydratase
G6PC	Glucose-6-phosphatase, catalytic subunit
G6PC3	Glucose-6-phosphatase, catalytic 3
G6PD	Glucose-6-phosphatase dehydrogenase
GALM	Galactose mutarotase (aldose 1-epimerase)
GBE1	Glucan (1,4-alpha-), branching enzyme 1
GCK	Glucokinase (hexokinase 4)
GPCRs	G-protein coupled receptors
GPI	Glucose-6-phosphate isomerase

GSK3A	Glycogen synthase kinase 3 alpha
GSK3B	Glycogen synthase kinase 3 beta
GYS1	Glycogen synthase 1 (muscle)
GYS2	Glycogen synthase 2 (liver)
H6PD	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
HB-EGF	Heparin-binding EGF
HIF	Hypoxia-inducible transcription factor
HK2	Hexokinase 2
HK3	Hexokinase 3 (white cell)
IDH1	Isocitrate dehydrogenase 1 (NADP <sup>+</sup> ), soluble
IDH2	Isocitrate dehydrogenase 2 (NADP <sup>+</sup> ), mitochondrial
IDH3A	Isocitrate dehydrogenase 3 (NADP <sup>+</sup> ) alpha
IDH3B	Isocitrate dehydrogenase 3 (NADP <sup>+</sup> ) beta
IDH3G	Isocitrate dehydrogenase 3 (NADP <sup>+</sup> ) gamma
IGF	Insulin-like growth factor
IGFBPs	IGF-binding proteins
IR	Insulin receptor
LDH	Lactate dehydrogenase
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPA	Lysophosphatidic acid

LPS	Lysophosphatidylserine
LPAAT	LPA acyl transferases
LPPs	Lipid phosphate phosphatases
lysoPLD/ATX	Lysophospholipase D
MAG	Monoacylglycerol
MAPK	Mitogen activated protein kinase
MDH1	Malate dehydrogenase 1, NAD (soluble)
MDH1B	Malate dehydrogenase 1B, NAD (soluble)
MDH2	Malate dehydrogenase 2, NAD (mitochondrial)
MMTV	Mouse mammary tumor virus
OGDH	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)
PA	Phosphatidic acid
PC	Pyruvate carboxylase
PCK1	Phosphoenolpyruvate carboxykinase 1 (soluble)
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
PDHA1	Pyruvate dehydrogenase (lipoamide) alpha 1
PDHB	Pyruvate dehydrogenase (lipoamide) beta
PDK1	Pyruvate dehydrogenase kinase, isozyme 1
PDK2	Pyruvate dehydrogenase kinase, isozyme 2

PDK3	Pyruvate dehydrogenase kinase, isozyme 3
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
PDP2	Pyruvate dehydrogenase phosphatase catalytic subunit 2
PDPR	Pyruvate dehydrogenase phosphatase regulatory subunit
PFK	Phosphofructokinase
PGAM2	Phosphoglycerate mutase 2 (muscle)
PGK1	Phosphoglycerate kinase 1
PGK2	Phosphoglycerate kinase 2
PGLS	6-phosphogluconolactonase
PGM1	Phosphoglucomutase 1
PGM2	Phosphoglucomutase 2
PGM3	Phosphoglucomutase 3
PHKA1	Phosphorylase kinase, alpha 1 (muscle)
PHKB	Phosphorylase kinase, beta
PHKG1	Phosphorylase kinase, gamma 1 (muscle)
PHKG2	Phosphorylase kinase, gamma 2 (testis)
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKLR	Pyruvate kinase, liver and RBC
PLA1	Phospholipase A1
PRPS1	Phosphoribosyl pyrophosphate synthetase 1

PRPS1L1	Phosphoribosyl pyrophosphate synthetase 1-like 1
PRPS2	Phosphoribosyl pyrophosphate synthetase 2
PTX	Pertussis toxin
PYGL	Phosphorylase, glycogen, liver
PYGM	Phosphorylase, glycogen, muscle
Ras	Rat sarcoma
RBKS	Ribokinase
RPE	Ribulose-5-phosphate-3-epimerase
RPIA	Ribose 5-phosphate isomerase A
S1P	Sphingosine-1-phosphate
SCO2	Synthesis of cytochrome <i>c</i> oxidase protein
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SDHB	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
SDHC	Succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa
SDHD	Succinate dehydrogenase complex, subunit D, integral membrane protein
sPLA2	Secretory phospholipase A2
SREBP	Sterol regulatory element binding protein
SUCLA2	Succinate-CoA ligase, ADP-forming, beta

	subunit
SUCLG1	Succinate-CoA ligase, alpha subunit
SUCLG2	Succinate-CoA ligase, GDP-forming, beta subunit
TALDO1	Transaldolase 1
TCA cycle	Tricarboxylic acid cycle
TGF	Transforming growth factor
TIGAR	TP53-induced glycolysis and apoptosis regulator
TKT	Transketolase
TPI1	Triosephosphate isomerase 1
UGP2	UDP-glucose pyrophosphorylase 1
VEGF	Vascular endothelial growth factor



## **ABSTRACT**

# **DIFFERENTIAL EFFECTS OF GROWTH FACTORS ON GLYCOLYSIS IN OVARIAN CANCER CELLS**

By Fang Yuan, MS

A Dissertation submitted in partial fulfillment of the requirements for the degree of Master of  
Science at Virginia Commonwealth University.

Virginia Commonwealth University School of Medicine, 2013

Major Director: Xianjun Fang

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## ABSTRACT

Lysophosphatidic acid (LPA), a naturally-occurring, simple phospholipid, is present at elevated levels in the blood and ascites of ovarian cancer patients. LPA is a ligand of seven cell surface G protein-coupled receptors. It has been known as an oncogenic growth factor in ovarian cancer and other types of human malignancies. However, the precise biological functions of LPA in ovarian oncogenesis remain to be fully elucidated. Our laboratory is interested in studying the potential role of LPA, as a tumor microenvironment factor, in regulation of cancer cell metabolism. A fundamental change associated with most cancer is the switch of glucose metabolism from mitochondrial oxidative phosphorylation to aerobic glycolysis, a phenomenon described by Otto Warburg nearly a century ago. This seems to be necessary to meet bioenergetic and biosynthetic demands of rapidly dividing tumor cells. However, the mechanism underlying the switch from aerobic respiration to aerobic glycolysis in cancer cells remains poorly understood.

In this thesis project, my goal was to explore the effect of LPA on glycolysis and to compare LPA with other important growth factors in their capability to promote the glycolytic pathway in ovarian cancer cells. We demonstrated that LPA stimulated aerobic glycolysis as

well as cell proliferation in ovarian cancer cell lines. The two parallel responses were LPA dose dependent. To determine whether LPA is unique in driving glycolysis, we compared the effect of LPA with other growth factors, including EGF, insulin and IGF-1 which are all involved in pathogenesis of ovarian cancer. While doses of these growth factors could be adjusted to achieve similar levels of cell proliferation, LPA and EGF were much more potent than insulin and IGF-1 in stimulation of glycolytic flux and lactate production. Therefore, we identified LPA and EGF as highly glycolytic factors relevant to the development and maintenance of the glycolytic phenotype of ovarian cancer cells.

The next part of my study was focused on the molecular mechanism for the differential effects of LPA, EGF, insulin and IGF-1 on glycolytic metabolism. We used the glucose metabolism RT-PCR array to profile expression of glycolytic genes. The most remarkable change induced by LPA and EGF was the robust induction of hexokinase 2 (HK2) that stimulates irreversible entry of glucose to the glycolytic pathway. However, insulin and IGF-1 only weakly induced HK2 expression. Further experimental evidence using HK2 inhibitors indicated that HK2 up-regulation was the critical mediator of LPA-induced glycolysis. Further, the cells grown in LPA and EGF-stimulated conditions appear to show larger volume compared to insulin and IGF-1-treated cells, consistent with the hypothesis that active glycolysis contributes to biosynthetic processes to maintain cell sizes.

Taken together, these findings of the current study revealed high glycolytic effects of LPA and EGF in ovarian cancer and the underlying HK2-mediated mechanism that distinguishes LPA and EGF from other growth factors such as insulin and IGF-1.

## CHAPTER 1

### INTRODUCTION

#### 1.0 Overview

Ovarian cancer begins in the ovary and the most common type of ovarian cancer is ovarian epithelial carcinoma (PubMed Health 2012). The American Cancer Society estimates that about 21,990 new cases of ovarian cancer are diagnosed in the United States in 2012. It is the 8<sup>th</sup> most common cancer in women but is the most lethal gynecological malignancy (American Cancer Society Ovarian Cancer Overview 2012).

Ovarian cancer is classified into four stages (Stages I-IV). At stage I, the tumor is limited to one or both ovaries. The tumor has pelvic extension or implants at stage II. At the advanced stages III and IV, ovarian cancer often spreads beyond pelvis, to the peritoneal cavity, and becomes distant metastases in the liver and other parts of the body. When the tumor is still restricted to the primary site, the 5-year survival rate is more than 90% (American Cancer Society Ovarian Cancer Overview 2012). However, the American cancer survival rate survey showed that ovarian cancer is the 5<sup>th</sup> cause of cancer death among

women and the overall 5-year survival rate is only 28% largely due to the inability to detect the ovarian cancer at early curable stages and the lack of effective therapies for advanced disease (American Cancer Society Ovarian Cancer Overview 2012). The standard management of ovarian cancer remains surgical debulking of tumor mass followed by chemotherapeutic treatment. Relapse with drug-resistant phenotype usually occurs, leading to eventual deaths of ovarian cancer patients (Armstrong D. 2002). For these reasons, it is critical to identify ovarian cancer at early stages or to develop effective therapies against late-stage ovarian cancer. These missions, however, rely on better understanding of pathogenic processes underlying the initiation and progression of ovarian cancer.

Genetic and epigenetic alterations in tumor suppressors and oncogenes play pivotal roles in malignant transformation of ovarian epithelial cells (Herceg Z., Hainaut P. 2007). However, accumulating evidence supports the involvement of tumor micro-environmental factors including interacting stromal cells, immune cells and diffusible molecules act in concert with intracellular genetic mutation and activation to mediate oncogenic processes (Zhang X., Nie D., Chakrabarty S. 2010). Our lab has a long-standing interest in the biological contributions of lysophosphatidic acid (LPA) to the development of human ovarian cancer. LPA is present at high levels in the ascites of ovarian cancer patients and other malignant effusions (Mills G. B. et al. 1988 & 1990; Xu Y. et al. 1995; Westermann A. M. et al. 1998). Previous studies from others as well as our laboratory have demonstrated that LPA promotes proliferation, survival, drug resistance, migration and invasion of ovarian and other cancer cells. Specific LPA receptors, such as LPA2, are overexpressed in ovarian cancer cells and ovarian cancer cell lines (Kitayama J, et al. 2004). However, the exact mechanism by which LPA drives tumor cell growth remains poorly understood. Recently we have evaluated

the role of LPA in regulation of tumor cell metabolism to ask whether LPA drives cell growth via up-regulating energy and biomass production. This is a new concept that has not been studied in the field of LPA biology. In this thesis, I focused on the effect of LPA on glycolytic metabolism and compared the effect with those of other prominent growth factors relevant to ovarian cancer.

## **1.1 LPA**

### **1.1.1 Metabolism of LPA**

LPA (1-acyl-2-hydroxy-sn-glycero-3-phosphate) is a naturally-occurring simple phospholipid. It contains a single fatty acyl chain, a glycerol backbone and a phosphate group. LPA could be produced by many different types of cells, for example, activated platelets, endothelial cells, fibroblasts, adipocytes, prostate and ovarian cancer cells. LPA is present in various body fluids. For instance, the concentrations of LPA in serum are about 1-5 $\mu$ M. LPA is present in high levels in ascites of ovarian cancer ascites and other malignant effusions. In human blood, most LPA forms are 16:0, 18:2, and 18:1-LPA (Sano T, et al. 2002) and the last form is the most commonly used laboratory reagent for LPA signaling studies. LPA is an extracellular mediator that evokes growth-factor-like responses in many cell types, both normal and transformed cells. The major biological actions of LPA include promotion of cell proliferation, survival, migration, and invasion (Moolenaar W. H. 1989).

Two major synthetic pathways are involved in the production of LPA (Mills GB,

Moolenaar WH, 2003). The first route is mediated by the action of phospholipases, secretory phospholipase A2 (sPLA2) and phospholipase A1 (PLA1). These phospholipases cleave the fatty acyl chain of phospholipids at the sn-2 or sn-1 position. The resulting lysophospholipids, such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylserine (LPS), can be converted to LPA by lysophospholipase D (lysoPLD/ATX), which has the ability to hydrolyze LPC, LPE and LPS. This route has been identified to be the primary synthetic pathway of LPA in the blood involving activated platelets. The other route of LPA production involves the action of monoacylglycerol kinase that phosphorylates monoacylglycerol (MAG) to form LPA.

On the other hand, extracellular LPA levels are controlled by lipid phosphate phosphatases (LPPs), a family of enzymes located on cell membranes with the catalytic sites facing extracellular space (Mills GB, Moolenaar WH, 2003). LPPs dephosphorylate LPA to form MAG which loses the biological activity of LPA. Thus overexpression of LPPs in ovarian cancer cells is associated with inhibition of LPA signaling, leading to increased cellular apoptosis and inhibition of tumor cell growth both in vitro and in vivo (Tanyi, J. L. et al. 2003). Besides, LPA could be also catabolized via acylation to phosphatidic acid (PA) by the action of LPA acyl transferases (LPAAT) (Mills GB, Moolenaar WH, 2003).

### 1.1.2 LPA receptors

In addition to cell proliferation, survival, migration, and tumor cell invasion, LPA had been also shown to regulate wound healing, cell contraction, neurogenesis, angiogenesis and immunity (Moolenaar WH, 1995; Mills GB, Moolenaar WH, 2003). These biological functions of LPA are mediated by a number of G-protein coupled LPA receptors (GPCRs). So far, seven GPCRs have been identified to be receptors for LPA (Noguchi K, et al. 2009; Yanagida K, et al. 2009). The best-characterized are LPA1/Edg2, LPA2/Edg4 and LPA3/Edg7. They are members of the endothelial differentiation gene (Edg) super-family, where receptors for another related lysophospholipids, sphingosine-1-phosphate (S1P), are found (Noguchi K, et al. 2009). The three Edg LPA receptors share about 50-60% amino acid identity. In addition, newly identified LPA receptors, LPA4/GPR23/P2Y9, LPA5/GPR92/GPR93, LPA6/P2Y5 and LPA7/GPR87 belong to the purinergic receptor family, sharing little sequence homology with the Edg LPA receptors (Noguchi K, et al. 2009).

LPA1 is the first identified high-affinity GPCR for LPA (Fukushima N, et al. 2001; Ishii I, et al. 2004). It is widely expressed in the embryonic cells, colon, small intestine, placenta, brain, uterus, testis, lung, stomach, kidney, spleen, thymus, skeletal muscle and heart tissues (Hecht, J.H. et al. 1996). LPA1 couples with three types of G proteins,  $G_{\alpha q}$ ,  $G_{\alpha i}$  and  $G_{\alpha 12/13}$ . These G proteins can activate further downstream signaling pathways, leading to activation of Ras-MAPK, PI3K-AKT, PLC and Rho to regulate cell proliferation, survival, migration, and invasion (Ishii et al., 2000; An et al., 1998b; Erickson et al., 1998). However, there is no consensus evidence that this LPA receptor is overexpressed in ovarian cancer or



other malignancies (Fang X. 2002).

LPA2 was originally identified from GenBank searches of orphan GPCRs. It shares about 60% amino acid similarity to LPA1 (Contos J., et al. 2000). The expression of LPA2 is relatively restricted compared to LPA1. In mouse, LPA2 is highly expressed in kidney, uterus and testis and lung; but lower levels of expressions are discovered in stomach, spleen, thymus, brain and heart (Contos JJ, et al. 2000). Over-expression of LPA2 has been reported in many types of cancer including ovarian, breast, colon, gastric, and thyroid cancer, suggesting a tumor-promoting role for LPA2 (Choi J, et al. 2010). Indeed, transgenic expression of LPA2 leads to mammary tumorigenesis when the LPA2 gene is driven by the MMTV (mouse mammary tumor virus) promoter (Liu S, et al. 2009). Ovarian specific transgenic expression of LPA2 is linked to enhanced expression of oncogenic VEGF (vascular endothelial growth factor) and uPA but is insufficient for ovarian oncogenesis (Huang MC, et al. 2004). The most direct evidence to implicate LPA2 in cancer is derived from the recent studies by Yun's group using the LPA2 null mice (Yun CC et al. 2005). Mice lacking LPA2 do not show any significant abnormalities in physiology. However, compared to wild type mice, the LPA2-deficient mice were more resistant to intestinal tumor formation induced by colitis or by ApcMin mutation.

LPA3 was originally identified as an orphan GPCR gene using degenerate PCR-based cloning and homology searches. (Bandoh et al. 1999; Im et al. 2000) It has about 50% amino acid similarities with the first two LPA receptors. It is expressed in many tissues, such as heart, lung, kidney and exclusively in the luminal endometrial epithelium (Ye X, et al. 2005). Like LPA1 and LPA2, LPA3 activates G $\alpha$ q and G $\alpha$ i to mediate LPA induced activation of phospholipase C and calcium mobilization, and inhibition of adenylyl cyclase (AC) (Ishii et

al., 2000). However, LPA3 does not couple with  $G\alpha_{12/13}$ , and therefore does not activate Rho GTPase.

LPA4 is closely related to the purinergic (P2Y) GPCR family (Choi J, et al. 2010). It was first identified through the analysis of the expressed sequence tag database. LPA4 gene is located on chromosome X and has a specific binding affinity to LPA ligand, but not to other lysophospholipids (Noguchi et al. 2003). Overexpression of LPA4 induces changes in cell morphology, such as stress fiber formation and neurite retraction through the activation of  $G\alpha_{12/13}$  and the Rho/Rho kinase pathway (Lee et al. 2007; Yanagida et al. 2007).

LPA5 was identified as an orphan GPCR which belongs to the rhodopsin GPCR family and structurally different from other LPA receptors (Lee C, et al. 2007). Functional studies showed LPA5 induces neurite retraction and stress fiber formation by activating  $G\alpha_{12/13}$ , and triggered calcium signaling by coupling to  $G\alpha_q$  (Lee et al. 2006). Furthermore, it increases cAMP levels and PKA activation in LPA5-expressed cells (Kotarsky et al. 2006).

### **1.1.3 LPA and Cell Proliferation**

LPA as a growth factor was first reported in fibroblasts (Van der Bend et al., 1992). Subsequently, the mitogenic activity of LPA was found in many other cell types, including normal and transformed cells (Huang et al. 2004). LPA drives cell proliferation through the pertussis toxin (PTX) sensitive G $\alpha$ i pathway (Fang et al. 2000; Van Corven et al. 1992). G $\alpha$ i induces the inhibition of intracellular adenylyl cyclase, activating the downstream Ras-Erk and PI3K-AKT pathways which are essential for cell proliferation (Fang et al. 2000; Kingsbury et al. 2003). The active Erk translocates to the nucleus where it activates the expression of proliferation-associated genes. In addition, both Erk and PI3K promote cell survival and inhibit apoptosis (Hafner A. et al. 2012; Ostrakhovitch EA, Cherian MG. 2005; Bondar V. et al. 2002).

### **1.2 Receptor Tyrosine Kinases**

In addition to the GPCR family, the largest group of cell surface receptors (Fredriksson R. et al. 2003), receptor tyrosine kinases constitutes another receptor family important in the regulation of cell proliferation and cancer development (Schlessinger J and Ullrich A. 1992). The prototype example is the receptor for epidermal growth factor (EGF). The ligands for

EGFR include EGF, transforming growth factor (TGF) and heparin-binding EGF (HB-EGF) (Hubbard SR and Till JH. 2000). Upon activation by its ligands, EGFR undergoes a transition from an inactive monomeric form to an active homodimer. EGFR dimerization stimulates its intrinsic intracellular tyrosine kinase activity, causing auto-phosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR (Lemmon MA and Schlessinger J. 2010). These phosphorylated tyrosine residues will serve as sites for interacting with downstream signaling molecules to initiate several signal transduction cascades, leading to cell proliferation and migration (Schlessinger J and Ullrich A. 1992).

It has become clear that EGFR and other members of EGFR family such as ErbB2 are overexpressed in many types of malignancies including ovarian and breast cancer (Walker F. et al. 2009). Moreover, activating mutations in the C-terminal kinase of EGFR are common in colon, lung and other major types of cancer, affecting tumor progression and drug resistance to chemotherapies (Lynch TJ, et al. 2004). Therefore EGFR-targeted therapies using anti-EGFR antibodies or small molecule inhibitors have been in clinical applications for cancer treatment (Jackman DM. et al. 2009; El-Sayed IH. Et al. 2006).

Insulin-like growth factor (IGF) is a potent growth factor present in the blood to stimulate growth of multiple cell types (Laron Z. 2001). Insulin, in addition to its metabolic role, also possesses mitogenic activity (Ish-Shalom D. et al. 1997). Insulin and IGF have been implicated in ovarian oncogenesis (Beauchamp MC. 2009). Ample evidence supports a role for IGF-1 in ovarian cancer through increased cell growth (Laron Z. 2001). The signal transduction of insulin and IGF-1 receptor tyrosine kinases is similar to that of EGFR. But, they are stronger activators of PI3K/AKT pathway than EGFR (Duncan MD. et al. 1994; Harrington LS. Et al. 2004). In addition, the activities of IGF-1/receptor systems are

regulated both positively and negatively by a series of IGF-binding proteins (IGFBPs) present in the blood (Hwa V, Oh Y, Rosenfeld RG. 1999). Intracellularly, insulin receptor (IR) and IGFR differ from EGFR in the presence of IR substrate 1 that transmits signals from activated IR or IGFR to downstream signaling cascades.

### 1.3 Cancer Cell Metabolism

Cancer cells are characterized by lipogenic and glycolytic phenotypes. Different from non-transformed cells, malignant cells display heightened *de novo* lipid synthesis, as well as, hyperactive aerobic glycolysis. Our lab has recently reported a role of LPA in activation and maintenance of lipogenic activity in ovarian cancer cells (Mukherjee A. et al. 2012). However, it is unknown whether LPA has a more profound effect on glucose utilization.

The glycolysis pathway converts one molecule of glucose into two molecules of pyruvate in association with formation of two ATPs (Figure 1.2). Under normoxia, most normal cells produce energy by glycolysis followed by oxidation of pyruvate in mitochondria (TCA cycle). The TCA cycle coupled to the respiration chain eventually generates a total of 36 ATPs from 1 molecule of glucose. Alternatively, pyruvate can be converted to in the absence of oxygen. Cancer cells prefer glycolysis even in the presence of oxygen, a phenomenon first observed by Dr. Otto Warburg nearly a century ago (Warburg, O. 1956). Most cancer cells produce energy by a high rate of glycolysis followed by lactic acid

formation in the cytosol, rather than by a comparatively low rate of glycolysis followed by complete oxidation of pyruvate in mitochondria as in most normal cells (Figure 1.1).

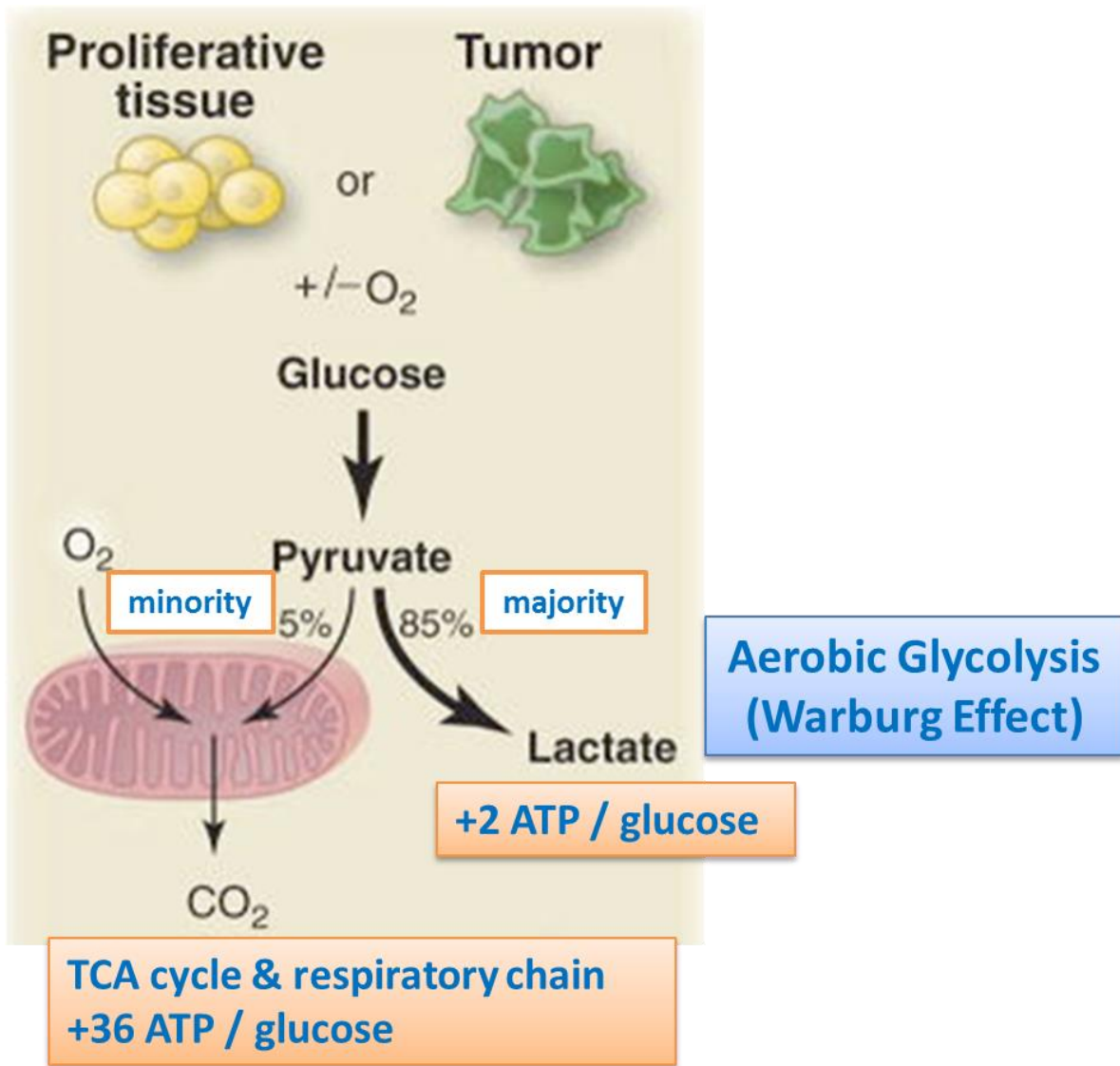


Figure 1.1 Aerobic glycolysis (Warburg Effect). Redraw figure and include in legend

“adapted from Vander Heiden, et al. 2009”

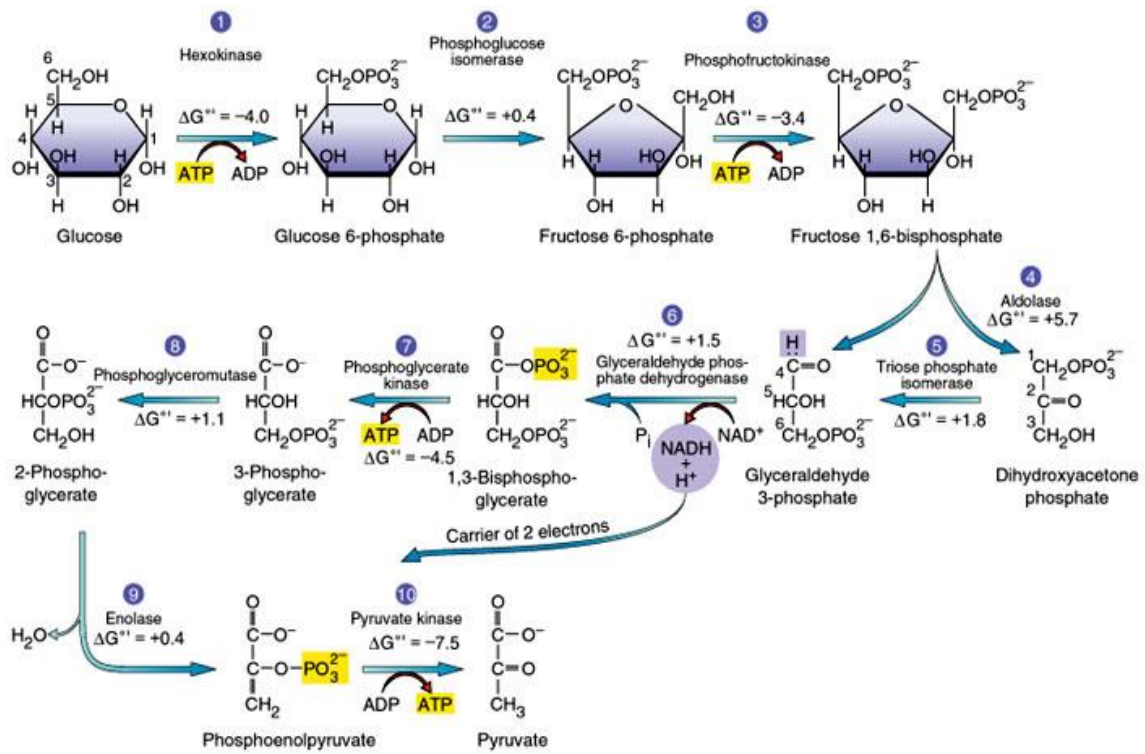
A high rate of glycolysis seems to be advantageous to meet bioenergetic and biosynthetic demands of rapidly dividing tumor cells (Lnt, S. et al. 2011). Cancer cells rely on the glycolytic metabolism for growth, survival and chemo-resistance. The mechanism underlying the switch from aerobic respiration to aerobic glycolysis in cancer cells remains poorly understood. Hypoxia and acidic pH stimulate glycolysis through stabilization of the hypoxia-inducible transcription factor (HIF) to activate transcription of a number of glycolytic genes (Kim JW. et al. 2006). However, hypoxia and acidic microenvironment are not causal factors of the glycolytic phenotype that occurs in both hypoxic and oxygenated regions of a tumor (Onnis B. et al. 2009; Wang GL. et al. 1995). Tumor cells *in vitro* also utilize glycolysis when cultured in normoxic and neutral conditions. Ras, AKT, and c-Myc have been reported to up-regulate expression of various glycolytic enzymes (Riddle SR. et al. 2000; Allen CB. et al. 1998). Loss of the tumor suppressor p53 inhibits the mitochondrial respiratory chain via suppression of SCO2 (the synthesis of cytochrome *c* oxidase protein) and promotes glycolysis via TIGAR, a TP53-induced glycolysis and apoptosis regulator (Kim JW. et al. 2006). In addition, the mitochondrial respiratory function can be impaired by mutations in mitochondrial DNA. However, it is not clear whether these genetic defects are sufficient for the development and maintenance of the glycolytic phenotype seen in a wide spectrum of cancers.

Previous studies of cancer-associated glycolysis have overlooked potential inputs from abnormal growth-promoting signals present in the tumor microenvironment that could trigger a glycolytic response in cooperation with the above-mentioned genetic aberrations in tumor cells. This differs from self-controlled glycolysis associated with proliferation of normal cells



where the growth activities are transient and restrained by the normal regulatory machinery.

In this thesis work, we hypothesize that LPA, a lysophospholipid mediator present at high levels in ascitic fluids of ovarian cancer patients, is a pathophysiological factor to drive glycolysis in ovarian cancer. I tested the hypothesis by comparing the effect of LPA on glycolysis with those of other peptide growth factors implicated in ovarian cancer pathogenesis, namely EGF, IGF-1 and insulin. Our results indicate that LPA and EGF are potent stimuli of glycolysis in ovarian cancer lines, while insulin and IGF-1 only modestly increased the cellular glycolytic activity although they showed similar mitogenic activities. I performed the glucose metabolic arrays covering all glycolytic enzymes in order to understand the molecular mechanism underlying the differential effects of LPA, EGF, insulin and IGF-1 on glycolysis. The array results indicate that LPA and EGF strongly induce expression of hexokinase 2 (HK2) that catalyzes the first rate-limiting step of glycolytic reactions. In contrast, insulin only slightly activated expression of HK2 compared to LPA and EGF. The data highlights the importance of HK2 as a major mediator of hyperactive glycolysis in ovarian cancer cells. Thus, targeting LPA-HK2 or EGF-HK2 could offer therapeutic opportunities to disrupt glycolysis in a cancer cell-specific manner. The differential effects of LPA, EGF, insulin and IGF-1 also open a new research avenue to explore the relationship of glycolysis and cell growth.



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Glycolysis Part 2)

Figure 1.2 The glycolytic pathway

## CHAPTER 2

### MATERIALS AND METHODS

#### 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 Applied Science (Indianapolis, IN). Glucose (D-[5-<sup>3</sup>H(N)]-) was obtained from Perkin Elmer (Boston, MA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). All other cell culture reagents were obtained from Invitrogen Inc. (Carlsbad, CA). Scintillation solution, Complete Counting Cocktail Budget-Solve was purchased from Research Products International Corp. (Mount Prospect, IL). Reverse transcription kit, TaqMan gene expression assays, Universal PCR Master Mix and qPCR probes were obtained from Applied Biosystems (Carlsbad, CA).

#### Cell Culture

The sources of Caov3, OVCA432 and other ovarian cancer cell lines were described previously (Fang et al. 1998). These cells were cultured in RPMI 1640 supplemented with 10%

FBS, L-Glutamine (1 mM) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

All cell lines were frozen at early passages and used for <10 weeks in continuous culture.

### **Analysis of Cell Proliferation**

Caov3 and OVCA432 cells were cultured in 6-well plates, starved for 24 hours and treated with LPA, EGF, insulin or IGF-1 at concentrations indicated. Approximately 36-40 hours later, the cells were trypsinized with 0.25% Trypsin-EDTA and neutralized with complete culture medium. The cell viabilities were determined by trypan blue exclusion assessed with the Bio-Rad TC<sup>10</sup> automatic cell counter, which was usually more than 95%. The cell numbers were quantified with the Z1 Coulter counter (Beckman Coulter, FA). The results were presented as net increases induced by indicated growth factors (the numbers of cells prior to treatment were subtracted). All results were mean ± S.D. of replicates.

### **Analysis of Glycolytic Rate**

The glycolytic rates were determined using a modified version of the Elstrom et al. (2004) method, measuring conversion of 5-<sup>3</sup>H-glucose to <sup>3</sup>H-H<sub>2</sub>O, presented as percentage (%) conversion of glucose. The details of the method are summarized in Figure 2.1. Caov3, OVCA432 and other ovarian cancer cell lines were plated in 6-well plates, starved in serum-free medium for 24 hours and then stimulated with LPA, EGF, insulin, or IGF-1 at indicated concentrations. After 12 hours, D-[5-<sup>3</sup>H(N)]-glucose was added to the medium at a concentration of 2 µCi/well and incubated for another 24 hours before collection of 0.5 ml culture medium into a 15 ml tube. Hydrochloric acid (1.2 M) was added to a final concentration of 0.2 M to terminate any further biological reactions. A 0.5 ml micro

centrifuge tube without cap containing 0.25 ml distilled water was inserted into the 15 ml tube. Precautions were taken to ensure that the two liquid phases remained separate during incubation. The 15 ml tube was capped tightly to allow vapor diffusion of the water component between the two liquid phases for more than 36 hours. At the end of incubation, the radioactivity present in 0.25 ml water in the 0.5 ml tube (reflecting  $^3\text{H-H}_2\text{O}$ ) and radioactivity present in 0.25 ml solution taken from the 15 ml tube (representing both  $^3\text{H-H}_2\text{O}$  and  $^3\text{H-glucose}$ ) were quantified by Scintillation counting using complete Counting Cocktail Budget-Solve from Research Products International Corp. (Mount Prospect, IL) and the LS 6500 Multi-Purpose Scintillation Counter. The ratios of the radioactivities present in water and in medium were presented as glycolytic rates. All results were mean  $\pm$  S.D. of replicates

During the glycolysis, one molecule of water is released from one molecule of 2-phosphoglycerate, so that 2-phosphoglycerate could convert to phosphoenolpyruvate which then produces pyruvate (see Figure 1.2). In our study, we utilized glucose at a tritium on  $\text{C}^5$ . In the glycolysis pathway, this labeled glucose would release one molecule of tritium labeled water to the media. By measuring the ratio of radioactive  $^3\text{H-H}_2\text{O}$  versus total radioactivity ( $^3\text{H-glucose} + ^3\text{H-H}_2\text{O}$ ), we are able to quantitate the glycolytic rate in the cells.

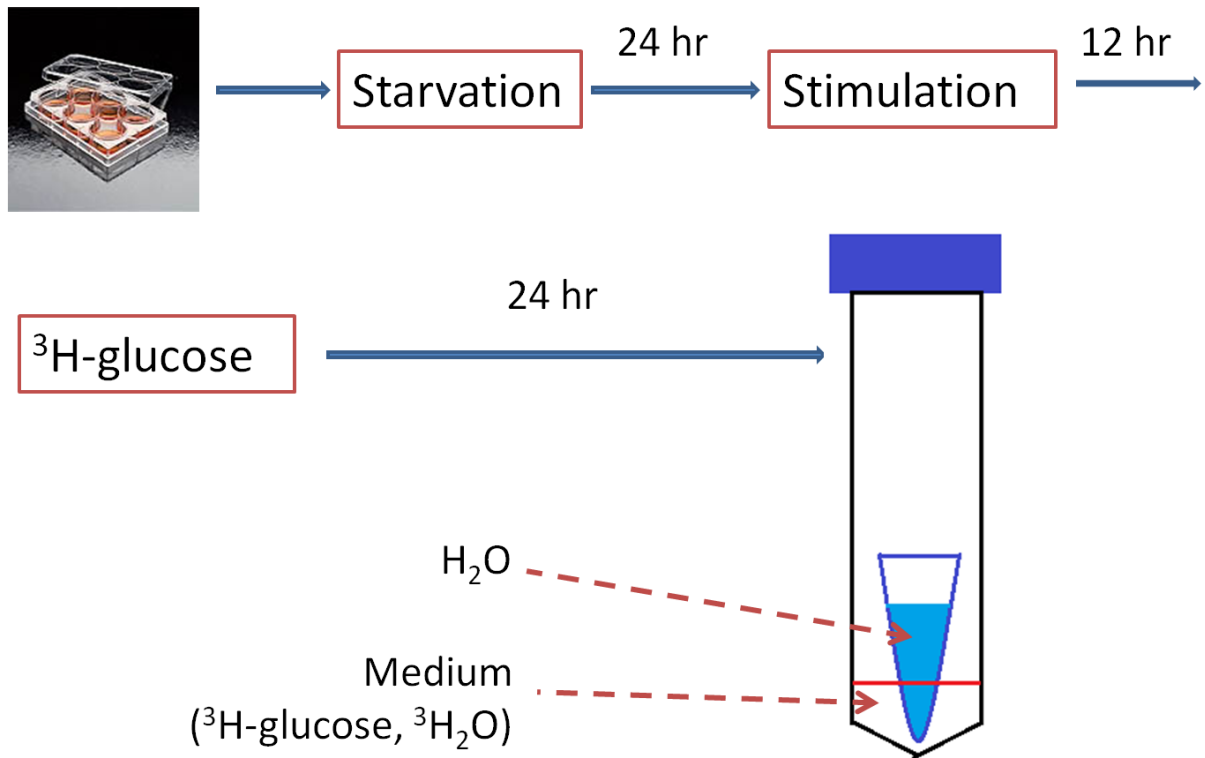


Figure 2.1 Experimental protocol for measurement of glycolysis rate

## **Lactate Measurement**

Cells were plated in 6-well plates, starved in serum-free medium for 24 hours and then treated with BSA, LPA, EGF, insulin, or IGF-1 for 36-40 hours before the culture supernatants were collected. The culture medium was centrifuged at 1500g to remove any floating cells. The lactate concentrations in the medium were determined using the L-Lactate Assay Kit I purchased from Eton Bioscience (San Diego, CA) following the manufacturer's protocol.

## **PCR array**

The Human glucose metabolism, RT<sup>2</sup> profiler PCR Arrays (PAHS-006Z) were obtained from SABiosciences. Caov3 cells in 60 mm dishes were starved for 24 hours in serum-free medium and treated with LPA, EGF, insulin or BSA (vehicle) for 12 hours before RNA isolation using RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized using RT<sup>2</sup> First Strand Kit (Qiagen) and qPCR was carried out using RT<sup>2</sup> SYBR® Green qPCR Master Mix (Qiagen) according to the manufacturer's protocol.

## **Analysis of cell morphology and cell size**

After treatment for 36 hours with LPA, EGF, insulin or IGF-1, the morphology of Caov3 and OVCA432 cells in 6-well plates was examined with phase-contrast microscope. The microscopic photographs were taken at varying magnifications. After trypsinization, the size ranges of cells in suspension were analyzed with the Bio-RAD TC<sup>10</sup> Automated Cell Counter.

## Statistics

All numerical data were presented as mean  $\pm$  SD of triplicates. The results were confirmed by three independent experiments. The statistical significance of differences was 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  $p < 0.01$ .



## CHAPTER 3

### RESULTS

#### 3.1 LPA promotion of glycolysis and cell proliferation in ovarian cancer cells

LPA has been long been known as a mediator of ovarian cancer and other types of cancers (Mills G. B. et al. 1988). It is present in the blood and ascitic fluids of ovarian cancer patients (Mills G. B. et al. 1990). LPA stimulates proliferation, survival, migration and invasion of ovarian cancer cells. These effects are mediated by several GPCR receptors of LPA and specific downstream signaling cascades such as Gi-linked Erk and PI3K signaling cascades (Moolenaar W. H. et al. 1995). However, mechanistic studies to elucidate further molecular programs leading to these events such as cell proliferation are lacking. Recent studies suggest that metabolic reprogramming is implicated in driving cell proliferation (Fang X. et al. 2002). For example, dividing cells need to gain sufficient precursors to synthesize DNA, proteins and phospholipids in completion of mitosis. A few studies have shown that LPA signaling modifies metabolic features of cancer cells (Fang X. et al. 2002). The only clue in the literature that LPA may be capable of activating glycolysis is derived from

Bernhart et al. (2010). This study reported that LPA increased expression of glycolytic enzymes in the C13NJ microglia cell line. We and others have previously demonstrated that LPA treatment of ovarian cancer cells leads to stabilization of hypoxia-inducible factor (HIF), a transcription factor potentially involved in activation of glycolytic genes (Allen CB. et al. 1998; Kim JW. et al. 2006).

Therefore, we have examined whether LPA can promote glycolysis in ovarian cancer cell lines. As shown in Figure 3.1, LPA stimulated multi-fold increases in glycolytic flux in both Caov3 and OVCA432 cells. This effect was also seen in other cell ovarian cancer cell lines (data not shown). We compared the effects of LPA on glycolysis as well as cell proliferation and found that the two responses occur in parallel, both in a LPA-dose dependent manner. These effects reached a plateau at 10  $\mu$ M LPA. In addition to the dose-dependent effects of LPA, the detected glycolytic flux increased with the incubation time of  $^3\text{H}$ -glucose. When incubated for only 6 hours, we observed a less than 2.5 fold increased in glycolysis. However, when this was extended to 24 hours of incubation, the reading increased to 3 fold (Figure 3.2), indicating that the effect of LPA on glycolysis was sustained.

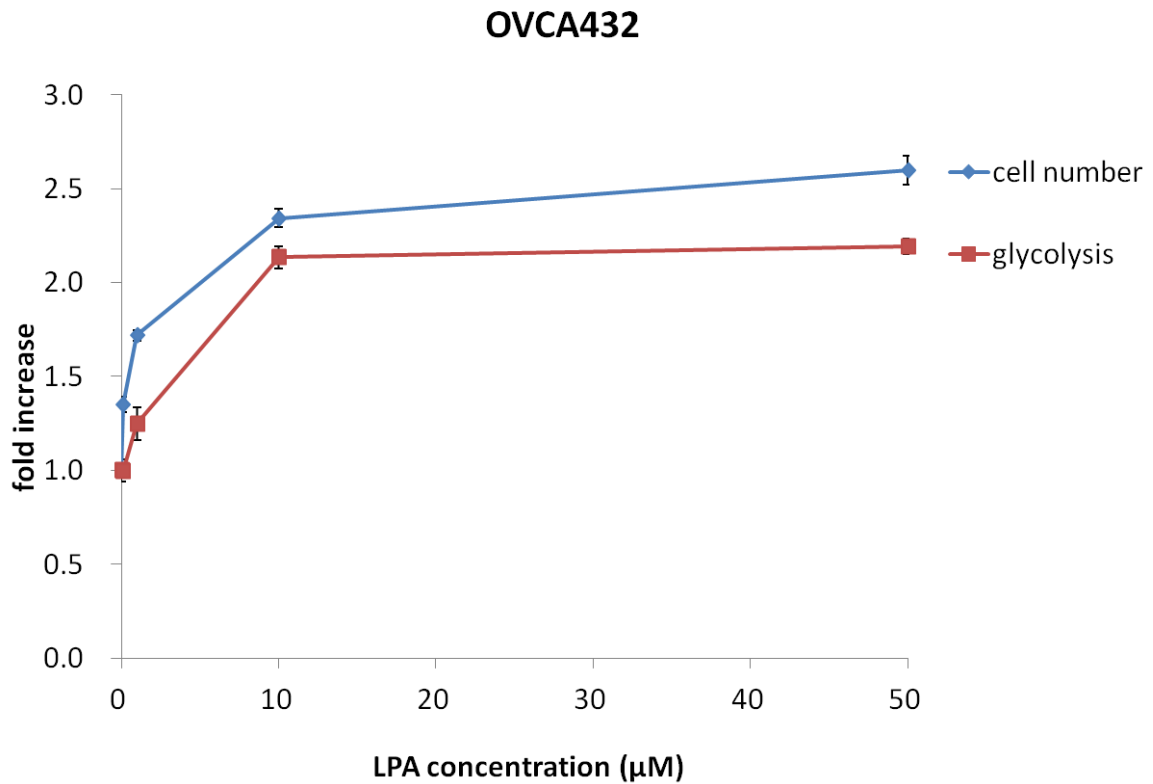
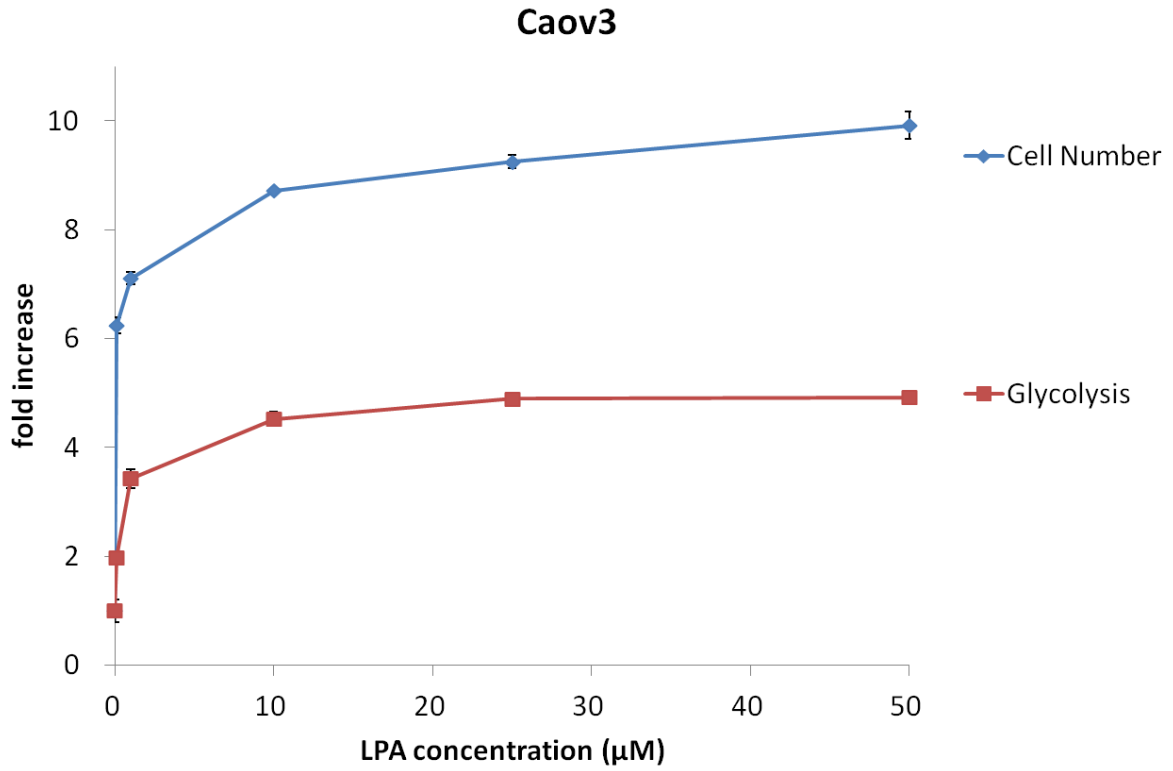


Figure 3.1 LPA stimulated multi-fold increases in glycolytic rate and cell numbers in both Caov3 and OVCA432 cells.

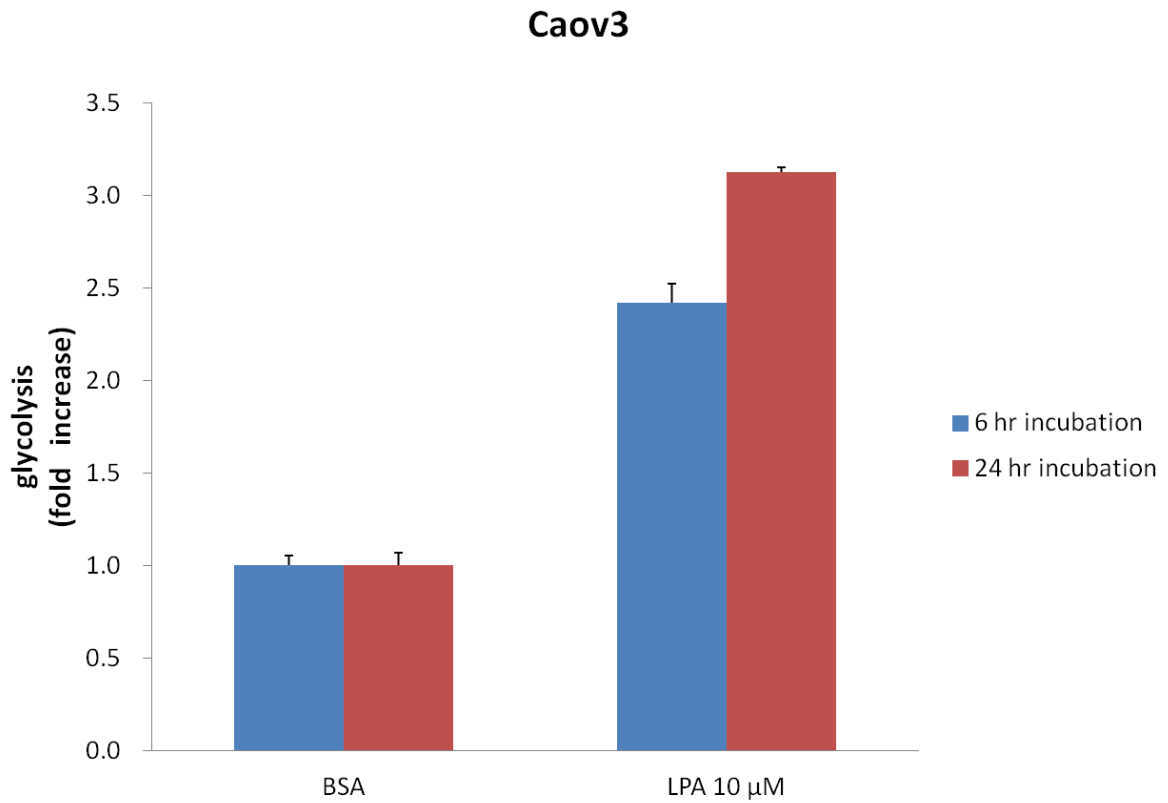


Figure 3.2 Time dependence of LPA-stimulated glycolysis.  $^3\text{H}$ -glucose was incubated with Caov3 cells for the last 6 hours or last 24 hours before analysis of glycolysis.

### 3.2. Differential effects of LPA and other growth factors on glycolysis

To determine whether LPA is unique in driving glycolysis, we compared the effect of LPA with other growth factors involved in pathogenesis of ovarian cancer. These include EGF, insulin and IGF-1. We first tested different concentrations of EGF, insulin and IGF-1 in order to identify the doses that elicited mitogenic responses at levels similar to 10  $\mu\text{M}$  LPA. As shown in Figure 3.3, in Caov3 cells, EGF (40 ng/ml), insulin (10  $\mu\text{g/ml}$ ), IGF-1 (20 ng/ml) and LPA (10  $\mu\text{M}$ ) triggered comparable levels of proliferation. In OVCA432 cells, similar cell proliferation was achieved at 2 ng/ml EGF, 0.125  $\mu\text{g/ml}$  insulin, 25 ng/ml IGF-1 and 10  $\mu\text{M}$  LPA. These results suggest that these growth factors are all potent mitogenic factors in ovarian cancer cells.

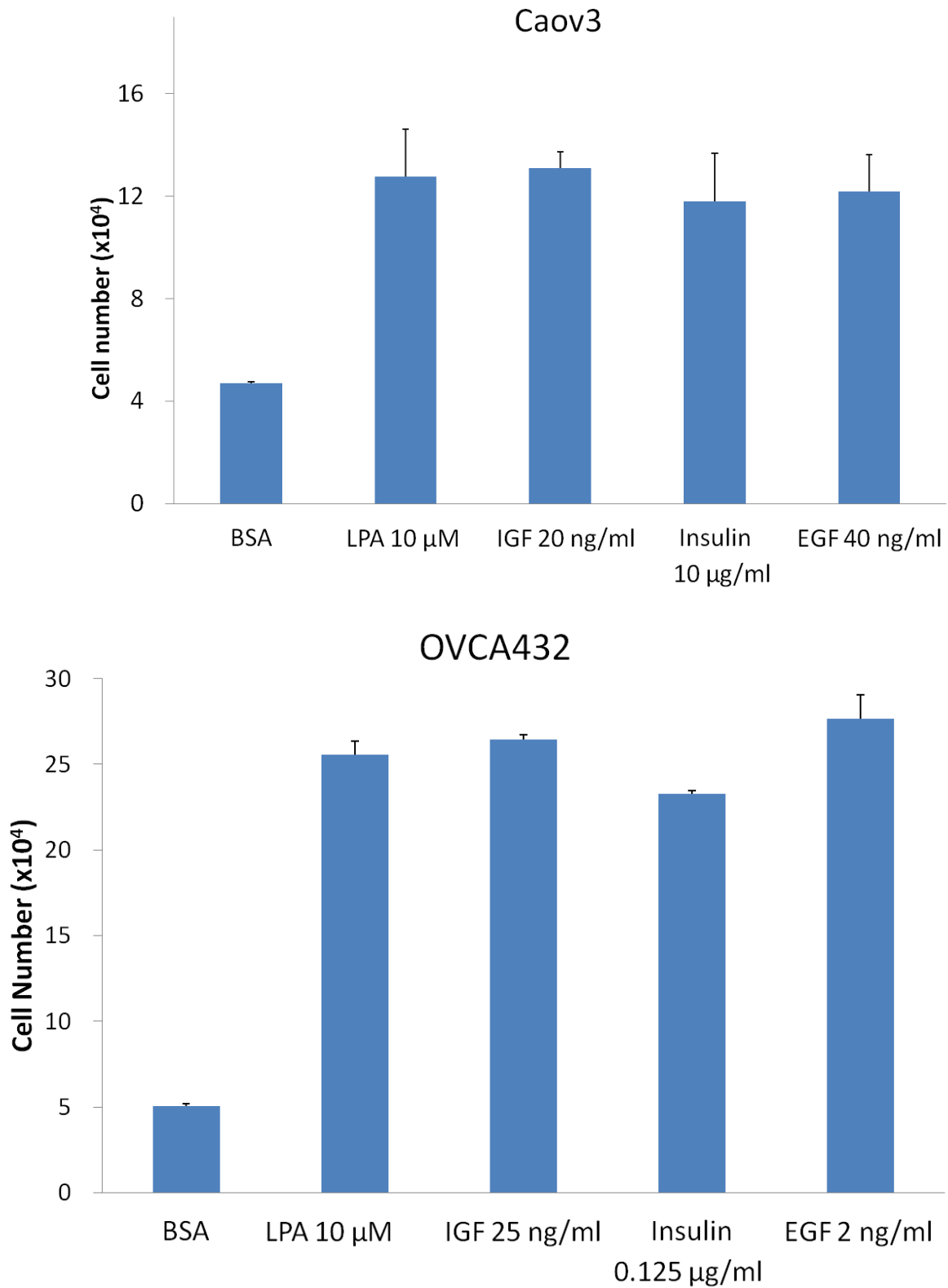


Figure 3.3 Caov3 and OVCA432 cells were stimulated with LPA, EGF, insulin or IGF-1 at the indicated concentrations for 36-40 hours. The cell numbers were determined with Coulter counter and presented as net increases induced by the growth factors (the numbers of cells prior to treatment were subtracted).

We next examined whether these other growth factors also stimulated glycolytic metabolism while promoting similar levels of cell proliferation. The glycolytic flux was measured as in Fig. 2.1 (Chapter 2) and concentrations of lactate present in culture supernatants were quantified using the Lactate Assay Kit I. These results in Caov3 and OVCA432 are presented in Fig. 3.4, and Fig. 3.5, respectively.

LPA and EGF stimulated more glycolysis than insulin and IGF-1. In Caov3 cells, glycolytic conversion of glucose was increased from 4.6% in the unstimulated control cells to 12.5% by LPA, 15.3% by EGF. In contrast, insulin and IGF-1 stimulated statistically significant but much weaker increases (from 4.6% basal control to 6.8 % by insulin, and to 7.1% by IGF-1) (Figure 3.4, *left panel*). Similarly, LPA and EGF stimulated more lactate production as reflected by much higher lactate concentrations in the culture medium than in insulin or IGF-1 conditioned medium (Figure 3.4, *right panel*).

We observed similar effects of LPA, EGF, insulin and IGF-1 on glycolysis and lactate production in OVCA432 cells (Figure 3.5). The comparisons suggest that the growth factors are not equivalently glycolytic in spite of showing similar mitogenic activities. In other words, there exist two types of growth factors: highly glycolytic, such as LPA and EGF, or weakly glycolytic, represented by insulin and IGF-1.

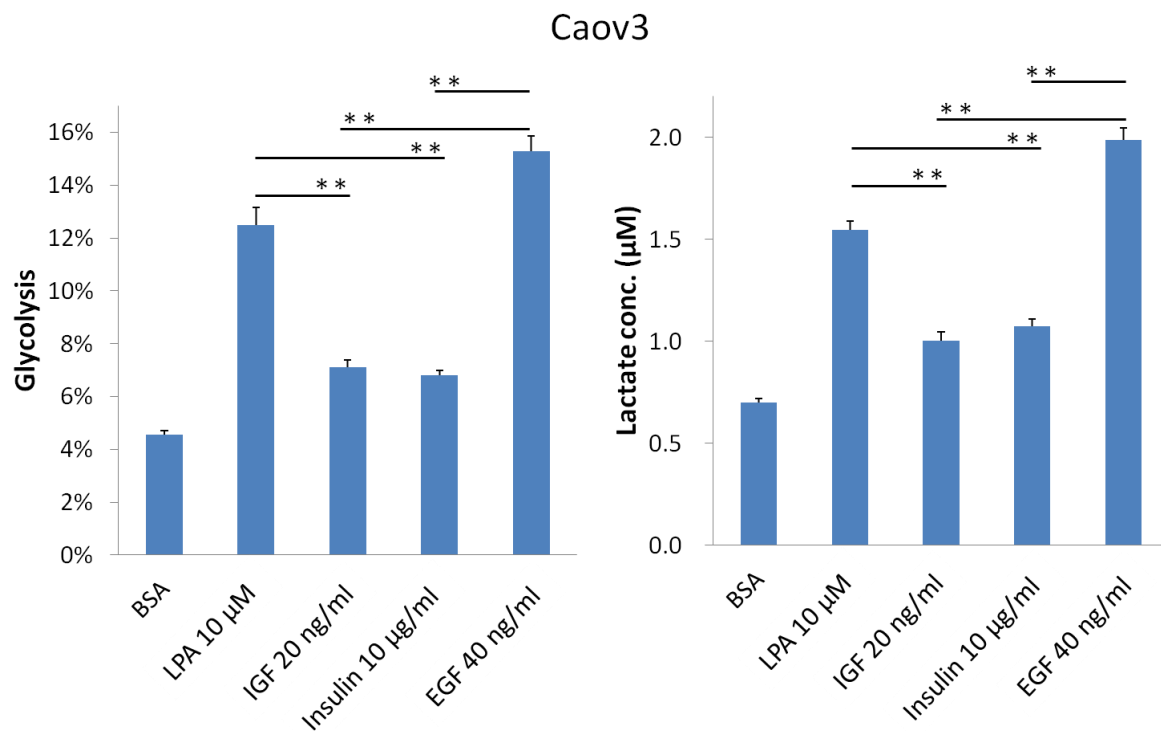


Figure 3.4 Differential effects of LPA, EGF, insulin and IGF-1 on glycolysis and lactate production in Caov3 cells. Values for LPA, EGF, IGF and insulin are significantly higher than BSA ( $p < 0.1$ ).



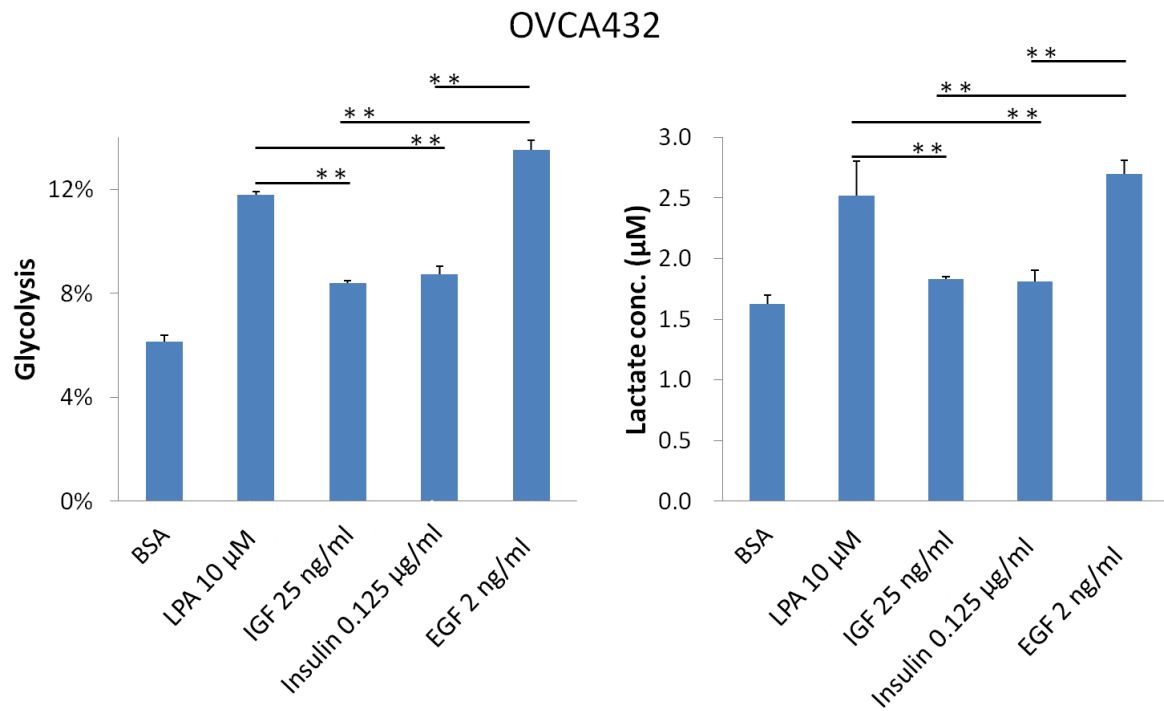


Figure 3.5 Differential effects of LPA, EGF, insulin and IGF-1 on glycolysis and lactate production in OVCA432 cells. Values for LPA, EGF, IGF and insulin are significantly higher than BSA ( $p < 0.1$ ).

### 3.3. Differential effects of LPA and other growth factors on expression of glycolytic genes

To determine the molecular mechanism for the differential effects of growth factors on glycolytic metabolism, we performed glucose metabolism arrays. We treated Caov3 cells with LPA (10  $\mu$ M), EGF (40 ng/ml), or insulin (10  $\mu$ g/ml) for 12 hours, isolated the RNA for micro RT-qPCR array to determine mRNA expression changes of genes involved in glucose metabolism (SABioscience). As shown in Figure 3.6, the array covers 84 key genes involved in glucose metabolism including all glycolytic enzymes. As shown in Figure 3.7, there was significant overlap of up and down-regulated genes responsive to LPA and EGF. HK2, the enzyme catalyzing the irreversible first rate-limiting step of glucose metabolism was found to be most markedly up-regulated by LPA (39 fold) and by EGF (22 fold) (see Figure 3.8).

HK2 overexpression or hyperactivity is common in malignant cells, which constitutes the physiological basis for cancer detection by 2'-fluoro-2'-deoxy-D-glucose positron emission tomography (FDG-PET) scans. A number of glucose metabolic enzymes were repressed by LPA and EGF. Among them, PDK4 was inhibited by 20 and 10 fold in LPA- and EGF-treated cells, respectively. PDK4 and its isozyme PDK2 (also significantly down-regulated by LPA and EGF) are key regulatory kinases that phosphorylate and inactivate PDH complex and thus control the metabolic fate of pyruvate (Grassian AR, et al. 2011; Jeong JY, et al. 2012). HK2 was also the most up-regulated gene in response to insulin. However, the magnitude of induction (4.2 fold up) was much weaker than those of LPA and EGF, consistent with the less active glycolysis in insulin-treated cells. In addition, the effects

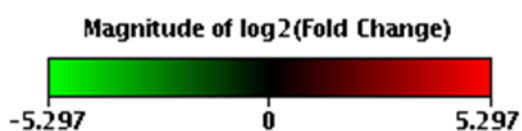
of insulin on PDK4 (2.2 fold down) and PDK2 (1.4 fold down) were marginal. Instead, the most down-regulated glycolytic enzyme by insulin is triosephosphate isomerase 1 (TPI1) (16.7 fold down) that catalyzes the reversible interconversion of the triosephosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate of the glycolytic pathway (Nagano N, et al. 2002). The depletion of TPI1 could counteract the effect of increased HK2 expression/activity, providing further explanation for the limited glycolysis seen in insulin-treated cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACLY	ACO1	ACO2	AGL	ALDOA	ALDOB	ALDOC	BPGM	CS	DLAT	DLD	DLST
B	ENO1	ENO2	ENO3	FBP1	FBP2	FH	G6PC	G6PC3	G6PD	GALM	GBE1	GCK
C	GPI	GSK3A	GSK3B	GYS1	GYS2	H6PD	HK2	HK3	IDH1	IDH2	IDH3A	IDH3B
D	IDH3G	MDH1	MDH1B	MDH2	OGDH	PC	PCK1	PCK2	PDHA1	PDHB	PDK1	PDK2
E	PDK3	PDK4	PDP2	PDPR	PFKL	PGAM2	PGK1	PGK2	PGLS	PGM1	PGM2	PGM3
F	PHKA1	PHKB	PHKG1	PHKG2	PKLR	PRPS1	PRPS1L1	PRPS2	PYGL	PYGM	RBKS	RPE
G	RPIA	SDHA	SDHB	SDHC	SDHD	SUCLA2	SUCLG1	SUCLG2	TALDO1	TKT	TP11	UGP2

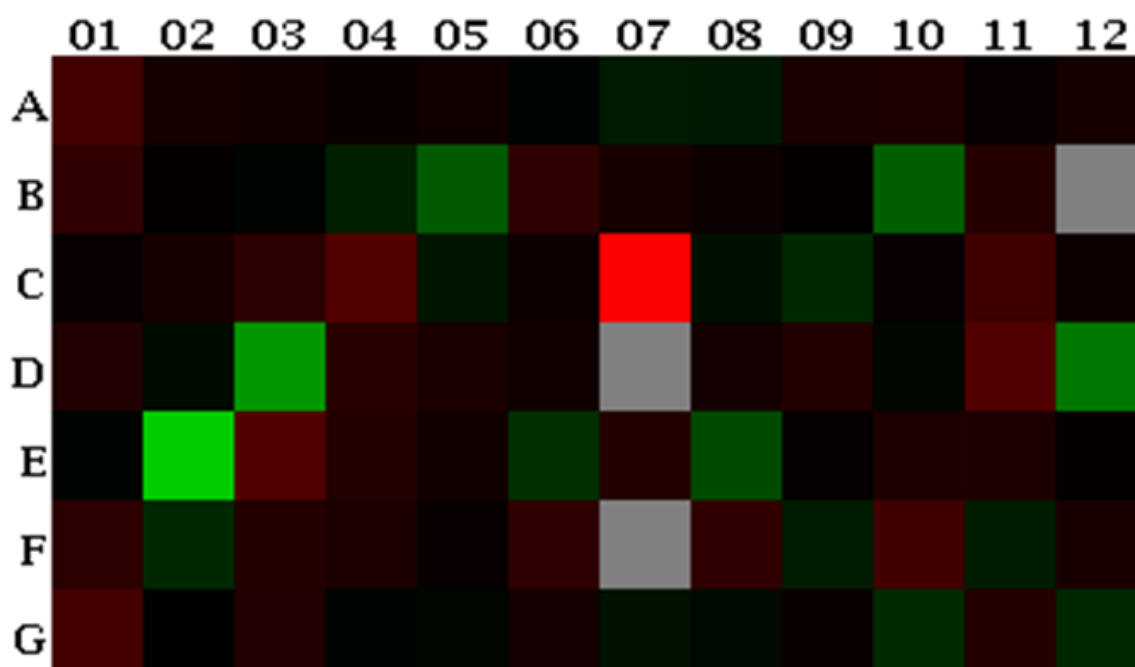
Figure 3.6 List of glucose metabolic genes included in the glucose metabolism array

## (A) LPA

3D Profile	1	2	3	4	5	6	7	8	9	10	11	12
A	2.58	1.36	1.31	1.15	1.26	0.94	0.68	0.72	1.45	1.51	1.11	1.36
B	1.99	1.05	0.93	0.63	0.27	1.95	1.38	1.18	1.05	0.26	1.7	0.94
C	1.13	1.37	1.9	3.25	0.72	1.22	39.32	0.79	0.57	1.12	2.42	1.23
D	1.58	0.84	0.11	1.78	1.45	1.27	0.94	1.33	1.63	0.92	3.18	0.18
E	0.95	0.05	3.19	1.66	1.3	0.5	1.62	0.35	1.08	1.56	1.49	1.07
F	1.87	0.5	1.63	1.49	1.13	1.92	0.94	1.97	0.66	2.49	0.66	1.41
G	2.62	0.96	1.57	0.93	0.93	1.35	0.78	0.86	1.17	0.54	1.68	0.58

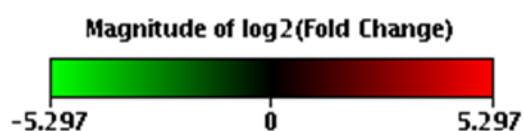


## LPA

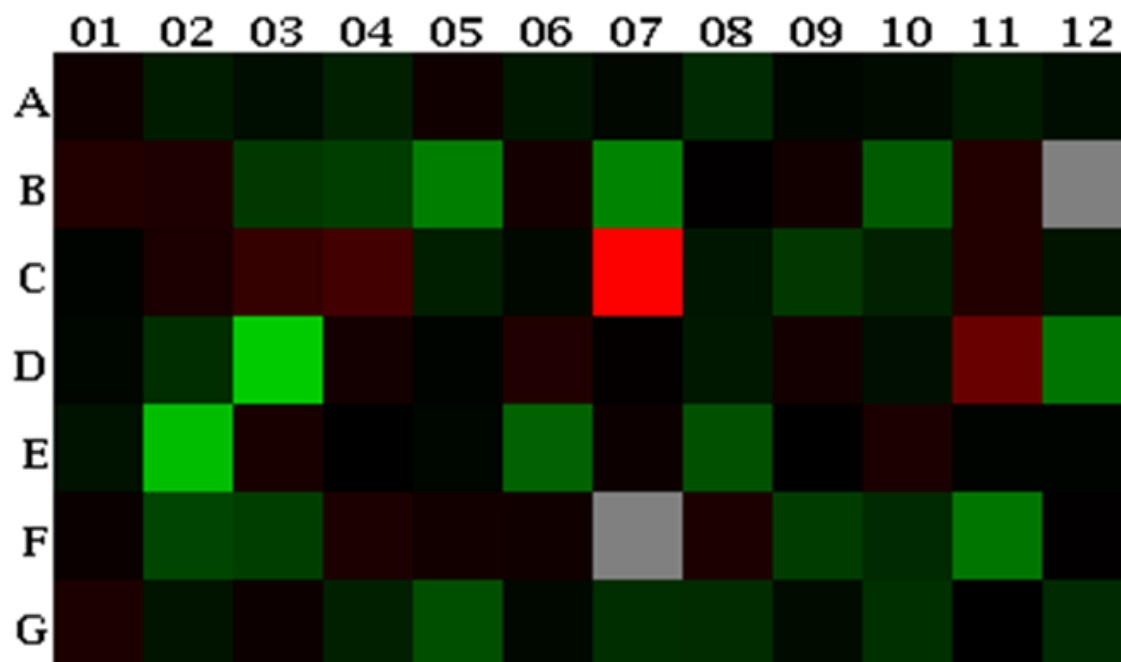


## (B) EGF

3D Profile	1	2	3	4	5	6	7	8	9	10	11	12
A	1.22	0.71	0.85	0.68	1.21	0.76	0.9	0.59	0.93	0.86	0.71	0.85
B	1.51	1.43	0.51	0.47	0.22	1.27	0.2	1.03	1.25	0.33	1.51	0.91
C	0.94	1.38	1.87	2.25	0.7	0.9	22.3	0.77	0.51	0.66	1.51	0.78
D	0.93	0.56	0.08	1.28	0.95	1.47	1.05	0.75	1.27	0.82	3.52	0.24
E	0.8	0.1	1.34	1.01	0.94	0.3	1.15	0.38	1.01	1.4	0.96	0.95
F	1.12	0.42	0.47	1.41	1.25	1.21	0.91	1.4	0.49	0.6	0.24	1.02
G	1.4	0.78	1.16	0.68	0.38	0.91	0.58	0.58	0.86	0.55	0.99	0.6

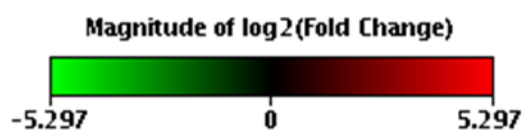


## EGF



### (C) Insulin

3D Profile	1	2	3	4	5	6	7	8	10	11	12
A	1.76	1.16	1.39	0.94	1.33	0.8	1.32	1	1.31	1.06	0.86
B	1.43	1.38	1.51	1.02	0.47	1.53	1.06	1.47	0.8	1.17	0.97
C	1.17	1.23	1.27	1.53	2.73	1.65	4.2	2.23	1.13	1.67	1.17
D	1.1	1.09	0.99	1.63	1.32	1.52	0.97	0.96	1.17	1.97	0.69
E	1.34	0.45	0.96	1.26	1.15	1.71	1.11	0.41	1.28	0.99	0.25
F	1.11	0.94	0.79	0.74	0.75	1.23	0.97	0.97	0.91	1.3	1.01
G	1.17	1.08	1.27	0.88	1.05	1.16	0.95	1	0.67	0.06	0.2



## Insulin

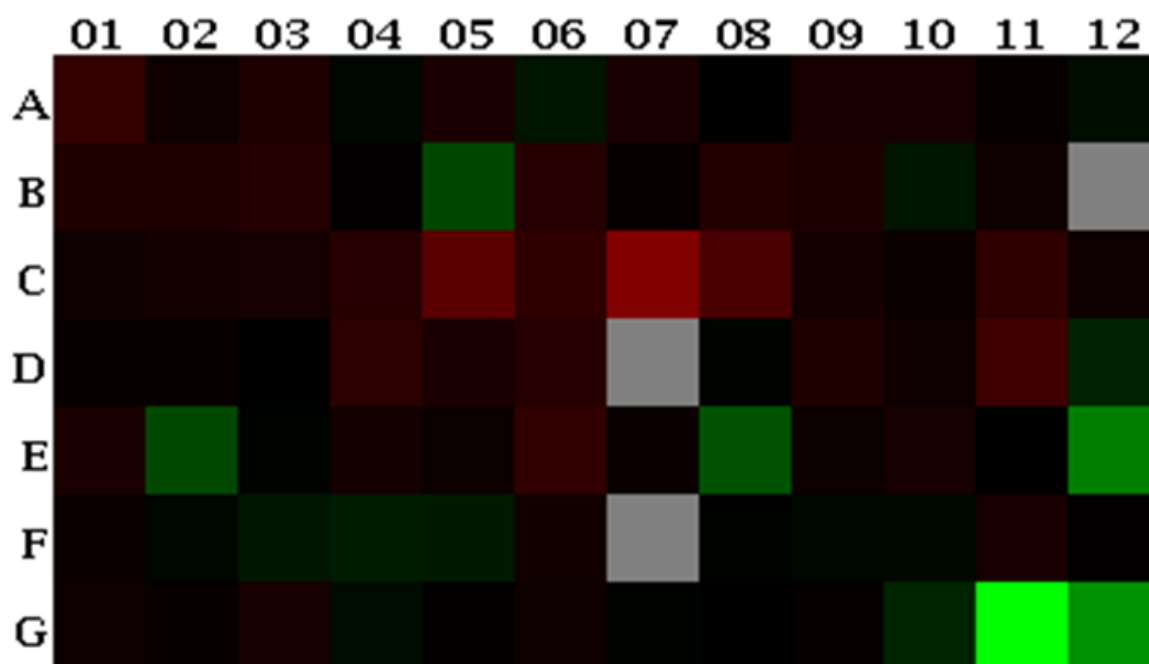


Figure 3.7 Regulation of glucose metabolism gene expression by LPA (A), EGF (B), and insulin (C) in Caov3 cells. Shown were fold changes in gene expression (*upper panel*) and heatmap of the data (*lower panel*) of the glucose metabolism array.

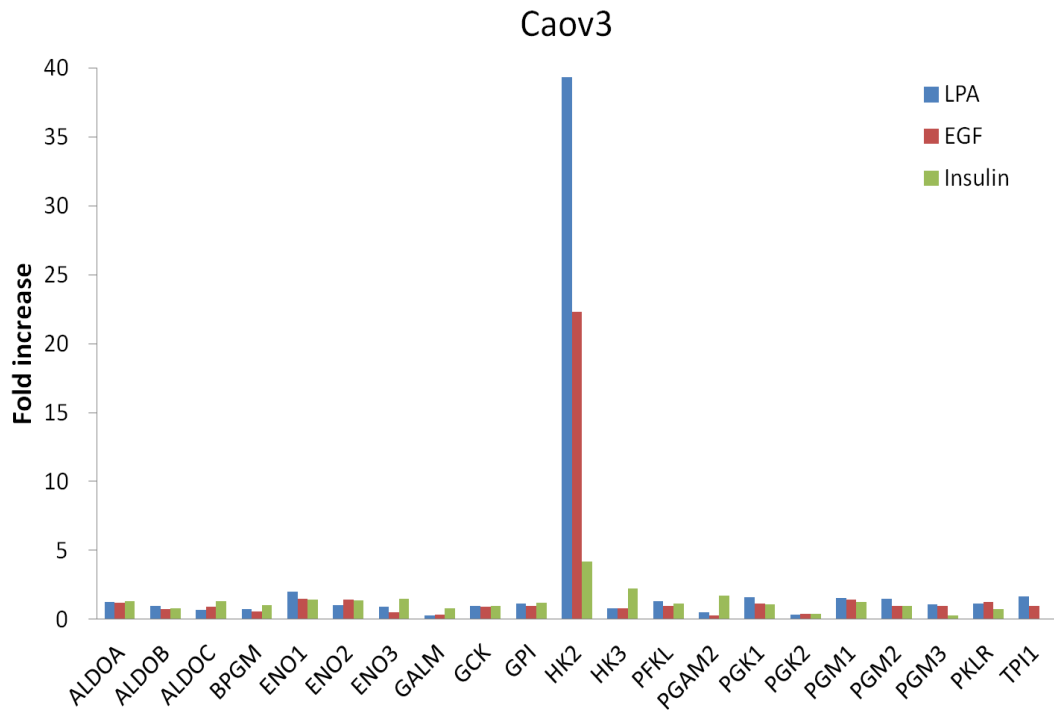


Figure 3.8 Super induction of HK2 by LPA and EGF in Caov3 cells.



### 3.4 HK2 as a key mediator of the glycolytic response

To further confirm the importance of HK2 in LPA-driven glycolysis, we examined effects of HK2 inhibitors on LPA-induced glycolysis. The 2-Deoxy-D-glucose (2-DG) is a glucose analog which can be converted to phosphorylated 2-DG (2-DG-P) by Hexokinase, but it cannot undergo remaining steps of glycolysis because of its 2-hydroxyl group. Consequently, HK2 was trapped with this analog of glucose. As shown in Figure 3.9, LPA strongly stimulated the glycolysis rate. However, the rate was decreased significantly when 2-DG (10 mM) was present (Figure 3.9 *upper panel*). Moreover, we utilized another HK2 inhibitor, 3-Bromo-2-oxopropanoic acid (3-BrPA) to confirm the role of up-regulated HK2 in activation of glycolysis by LPA. This inhibitor is a pyruvate analog. It directly binds glyceraldehyde 3-phosphate-dehydrogenase. However, most studies show that 3-BrPA is a potent irreversible inhibitor of HK2 and effectively blocks glycolysis (Geschwind JF et al. 2004; Ko YH et al. 2001). Figure 3.9 (*bottom panel*) showed that the glycolysis rate was significantly reduced by 3-BrPA (75  $\mu$ M) in both Caov3 and OVCA432 cell lines. These results indicated that HK2 is the important glycolytic enzyme required for promoting glycolytic response to LPA.

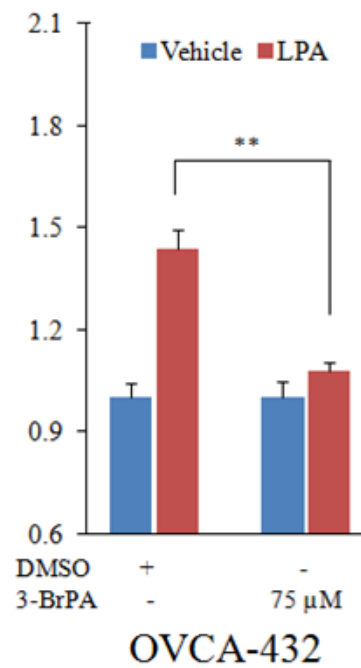
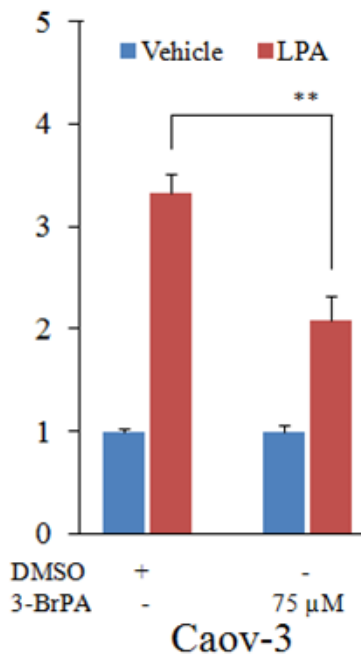
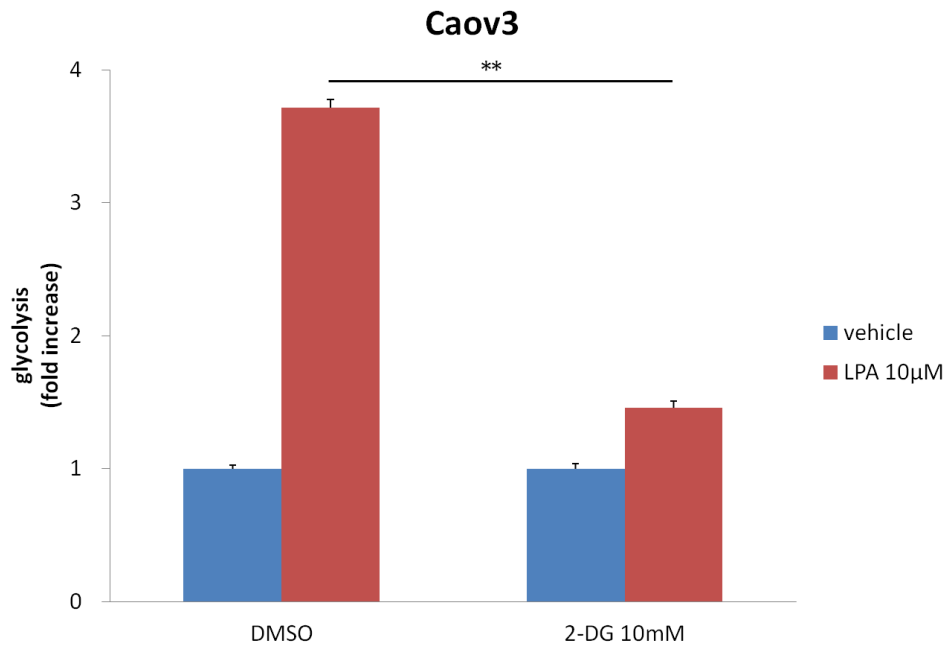
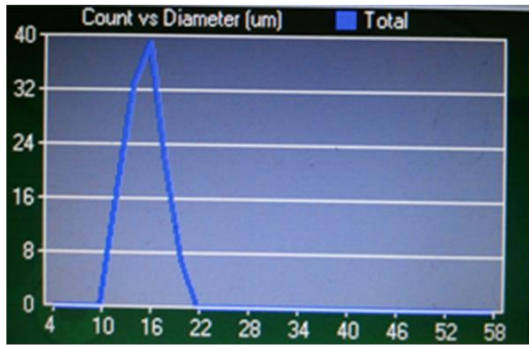


Figure 3.9 Effects of the HK2 inhibitors on LPA-induced glycolysis in Caov3 and OVCR432 cells.

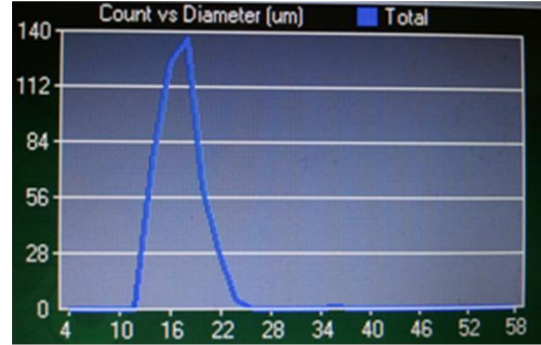
### 3.5. Cellular impacts of hyperactive glycolysis

If ovarian cancer cells display similar proliferation in association with different extents of glycolysis, one would expect different phenotypes of the cells grown under these growth factors. An advantage of hyperactive glycolysis is believed to provide precursors for biosynthesis of macromolecules essential for formation of daughter cells. Thus we examined the morphology and size of cells treated with LPA, EGF, insulin or IGF-1. The LPA and EGF-treated cells looked larger and more flat than insulin and IGF-1-treated cells under microscope (data not shown). To measure cell size, we trypsinized the cells and scanned with Bio-Rad TC<sup>10</sup> automatic cell counter. A population of more than  $1 \times 10^6$  cells was analyzed. As shown in Figure. 3.10, both peak value and cell size range were right shifted in LPA and EGF stimulated cells compared to insulin and IGF-1-treated cells. These results suggest that the cells grown with LPA or EGF had larger volumes than insulin and IGF-1-treated cells, consistent with the hypothesis that active glycolysis contributes to biosynthetic processes to maintain cell size.

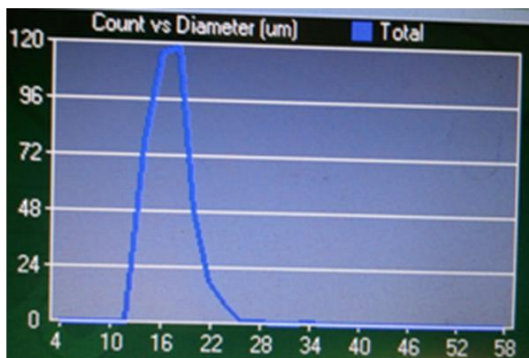
## BSA



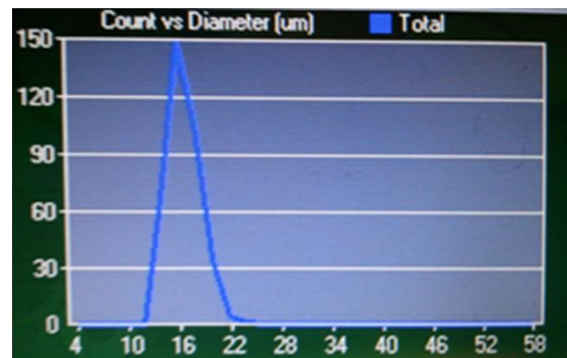
## LPA



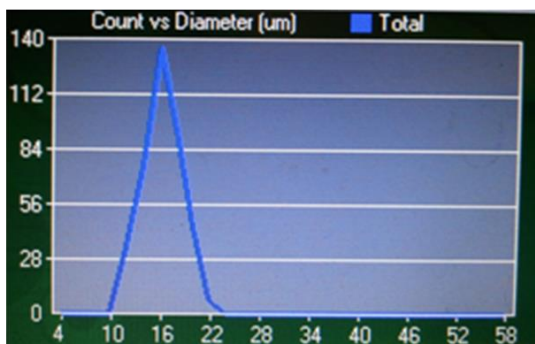
## EGF



## IGF



## Insulin



Caov3	Peak Value (μM)	Cell Size Range (μ M)
BSA	16	10-22
LPA	18	12-25
EGF	18	12-25
IGF	16	11-22
Insulin	16	10-23

Figure 3.10 Differences in volumes of Caov3 cells grown with LPA, EGF, insulin and IGF-1

as determined by analysis of cells in suspension with the TC<sup>10</sup> automated cell counter.

### 3.6 Potential role of EGFR in LPA-driven glycolysis

There was significant overlap in up and down-regulation of glucose metabolic genes responsive to LPA and EGF (Figure 3.7), suggesting certain common mechanisms or signaling networks shared by LPA and EGF. It has been shown previously that LPA could “trans-activate” EGFR or other RTKs to exert its biological functions. To test this possibility, we used a commercial compound, Dacomitinib (PF00299804), which is an irreversible EGFR tyrosine kinase inhibitor. As shown in Figure 3.11, LPA-induced glycolysis and cell proliferation were both strongly inhibited by 1.25  $\mu$ M PF00299804, suggesting that these actions of LPA may involve transactivation of EGFR.

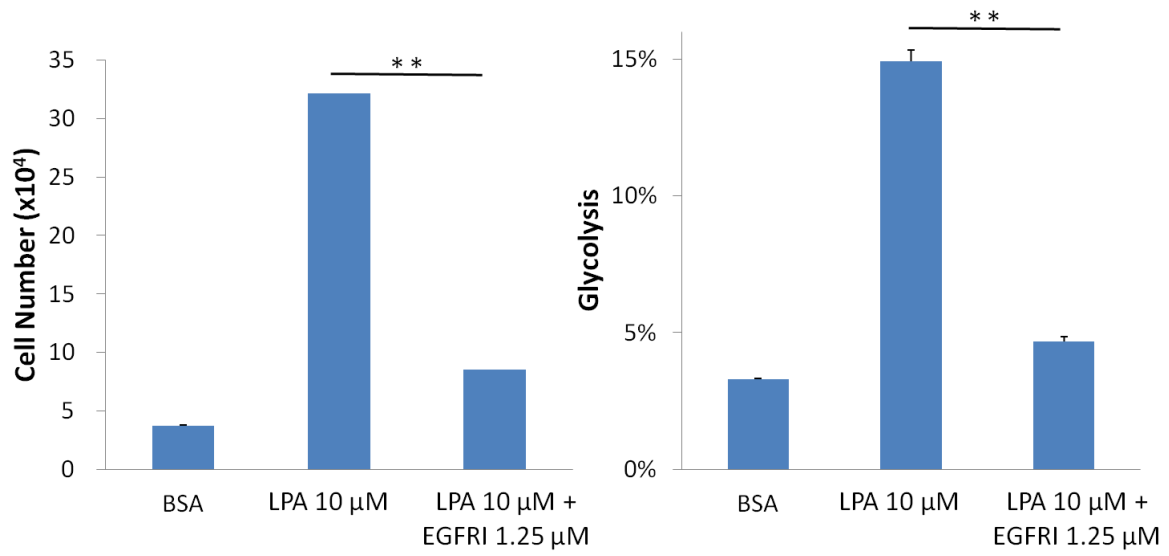


Figure 3.11 Blockade of LPA-induced glycolysis (*left, A*) and cell proliferation (*right, B*) by EGFR inhibitor PF00299804 in Caov3 cells. (the numbers of cells prior to treatment were subtracted).

## CHAPTER 4

### DISCUSSION

An altered metabolic pattern was discovered by Dr. Otto Warburg nearly a century ago (Warburg O. 1956). Most tumor cells prefer producing energy by a high rate of aerobic glycolysis, an inefficient pathway for ATP production (2 ATPs/glucose), compared to a low rate of complete oxidation of pyruvate through the TCA cycle and mitochondrial respiration, a much more efficient way to generate bioenergy (36 ATPs/glucose). This seems to be a paradox for actively proliferative tumor cells. However, substantial evidence supports the importance for active glycolysis in rapidly growing tumor cells (Lnt SY. et al. 2011). The process provides not only a quick way to generate ATP from substrate-based phosphorylation, but also glycolytic intermediates for biosynthesis of amino acids, lipids and other macromolecules (Lnt, SY, et al. 2011; Cairns, RA, et al. 2011). Inhibition of glycolytic enzymes such as HK2, phosphofructokinase (PFK), GADPH, and lactate dehydrogenase (LDH) has been shown to prevent cell growth, survival, drug resistance and tumorigenesis in various types of cancer (Scatena, R, et al. 2008; Chen, Z, et al. 2007), thus offering a potential therapeutic opportunity. However, these inhibitors are non-selective blockers of

glucose metabolism in both normal and cancer cells (Chen, Z, et al. 2007), a major concern with their application to cancer patients. An innovative approach is to target hyperactive glycolysis specifically in tumor cells. This goal will depend on identification of etiological determinants that promote glycolysis in cancer cells.

However, the etiological mechanism that governs the glycolytic switch from aerobic respiration in cancer cells remains poorly understood. Hypoxia and acidic pH could stimulate glycolysis through stabilization of the hypoxia-inducible transcription factor (HIF) to activate transcription of glycolytic enzymes. However, hypoxia and acidic microenvironments are not the causal factors underlying the glycolytic phenotype that occurs in both hypoxic and oxygenated regions of a tumor (Onnis B. et al. 2009; Wang GL. et al. 1995). Tumor cells *in vitro* also prefer glycolysis when cultured in normoxic and neutral conditions. When activated, the Ras, AKT, and c-Myc oncogenes could upregulate expression of a variety of glycolytic genes (Riddle SR. et al. 2000; Allen CB. et al. 1998). In contrast, loss of the tumor suppressor p53 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 (Kim JW. et al. 2006). Finally, the mitochondrial respiratory function can be negatively affected by mutations in mitochondrial DNA. However, these defects are present only in fractions 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.

Our laboratory is interested in studying the molecular mechanisms behind metabolic abnormalities in cancer cells including the lipogenic and glycolytic phenotypes. It is expected



that identification of specific cancer-associated mechanisms could lead to new strategies to target cancer in a cancer cell-specific manner without toxic effects on normal cells. The lab has recently discovered that LPA, an ovarian cancer mediator found at high levels in tumor microenvironments, promotes *de novo* lipid synthesis via the sterol regulatory element binding protein (SREBP)-FAS and AMP-activated protein kinase (AMPK)-ACC pathways in ovarian cancer cells (Mukherjee, A, et al. 2012). Following this work, the lab has further extended to study whether LPA has a more profound influence on glucose metabolism. The objective of my thesis project was to explore the effect of LPA on glycolysis and the comparison of LPA with other important growth factors in their capability to regulate the glycolytic pathway in ovarian cancer cells.

This work represents a new direction in the study of cancer-associated glycolysis. There has been no systemic analysis of the potential of growth factors in driving glycolysis in cancer cells. Previous studies have focused on roles of genetic alterations in the determination of the Warburg effect. Based on our results, the cells incubated in the absence of growth factors did not undergo excessive glycolysis (Figure 3.4 and Figure 3.5). These ovarian cancer cell lines, such as Caov3 and OVCA432, are p53 deficient and overexpress c-Myc and other oncogenes, suggesting that intracellular genetic mutations are insufficient to cause hyperactive glycolysis. When growth factors were supplemented, ovarian cancer cell lines increased glycolytic flux, mimicking the glycolytic phenotype seen *in vivo*. Thus extracellular environmental factors are involved in cooperation with intracellular genetic defects in the full-scale activation of glycolysis in cancer cells. So it is important in the future to study the role of tumor environments in the development and maintenance of hyperactive glycolysis in cancer cells.

By comparing the effects of LPA, EGF, insulin and IGF-1 on cellular glycolysis and lactate efflux, an interesting finding we made is that these growth factors are not equally glycolytic. LPA and EGF are significantly more potent than insulin and IGF-1 although they could stimulate comparable levels of cell proliferation. This suggests that the cell proliferation is not directly proportional to the glycolytic rate. The finding will open up a new avenue for us to analyze the quantitative contribution of glycolysis to cell proliferation. For example, if we reduce glycolytic levels partially in LPA and EGF-stimulated cells to that observed in insulin or IGF-1-treated cells, can we maintain the proliferative response to LPA or EGF? If not, there exist certainly two types of growth factors that rely upon different levels of glycolytic reactions to support cell proliferation. LPA and EGF represent the first type that is highly glycolytic while insulin and IGF-1 are the second type demonstrating weakly pro-glycolytic action. If this is also true *in vivo*, LPA and EGF are more likely to be endogenous factors contributing to the glycolytic phenotype of tumors.

Substantial evidence supports the importance of LPA and EGFR in ovarian cancer. They are both potent growth, survival and metastatic factors in ovarian cancer. It has been known that abnormal LPA metabolism and receptor expression contribute to ovarian oncogenesis (Yu, S, et al. 2008; Tanyi, JL, et al. 2003; Li, H, et al. 2010) although the precise role of LPA in ovarian oncogenesis is not understood. On the other hand, EGFR and other members of EGFR family are overexpressed in ovarian cancer (Walker F. et al. 2009). Overexpression of these RTKs of EGFR family correlates with the aggressiveness and poor prognosis of ovarian cancer (Lynch TJ. et al. 2004). The current observation that LPA and EGF are strong glycolytic promoters provides a novel biological function of these factors to mediate the Warburg effect of aerobic glycolysis.

To examine the molecular mechanism underlying the differential effects of LPA, EGF, insulin and IGF-1 on glycolysis, we carried out glucose metabolism arrays to look for a gene signature potentially responsible for driving the high degree of glycolysis in LPA and EGF-stimulated cells. Among the glycolytic genes, the most remarkable change induced by LPA and EGF was the dramatic induction of HK2, the first key enzyme in the glycolytic pathway that stimulates the irreversible step of glucose phosphorylation. In Caov3 cells, 39 fold up-regulation by LPA, a 22 fold up-regulation by EGF, and a much more modest 4.2 fold induction by insulin were observed. If HK2 is critical for driving glycolysis, the differential induction of HK2 by LPA, EGF and insulin may explain their differential abilities to activate glycolysis.

To confirm that HK2 is indeed a critical player in LPA-induced glycolysis, we took advantage of two specific HK2 inhibitors: 2-DG and 3-BrPA. Both significantly reduced LPA-driven glycolysis. We also performed siRNA to knockdown expression of HK2 to seek molecular evidence that the HK2 induction is involved in activation of glycolysis by LPA. We observed partial inhibition of LPA-induced glycolysis (data not shown). The partial inhibition was understandable because it was difficult for siRNA to efficiently knockdown 39 fold induction of HK2 mRNA by LPA.

It is worth noting that HK2 is overexpressed in majority of human malignancies (Wolf A. et al. 2008). Although no study has been conducted specifically to analyze HK2 expression in ovarian cancer, the microarray data publically available from the Oncomine database indicate that HK2 expression is generally increased in ovarian cancer compared to normal ovary tissues (data not shown). However, the mechanism for up-regulation of HK2 in ovarian cancer is unknown. LPA and EGF may be important factors responsible for HK2

overexpression in primary tumors.

In addition to striking induction of HK2 by LPA and EGF, we also observed significant overlap in expression profile of other glucose metabolic genes such as down-regulation of PDK2 and PDK4 in LPA and EGF-treated cells. Although the significance of the regulation of these genes is beyond the scope of the current study, the overlap itself suggests common mechanisms or signaling networks shared by LPA and EGF. It has been shown previously that LPA could “trans-activate” EGFR or other RTKs in LPA mediated biological functions (Zhao Y. et al. 2006). This crosstalk between LPA EGFR may act through production of EGFR ligands or through EGFR ligand-independent mechanisms. When the EGFR small molecule inhibitor PF00299804 was added to culture, we found that LPA-induced glycolysis and cell proliferation were attenuated, suggesting that these actions of LPA involve activation of EGFR and probably EGFR downstream signals. However, the nature of the interactions between LPA GPCR and EGFR signal transduction in regulation of glucose metabolic gene expression and the subsequent glycolysis and cell proliferation remains to be investigated.

Another interesting finding in this thesis work was the differences in morphology and size ranges of cells grown in LPA or EGF-treated conditions from those grown with insulin and IGF-1. We observed that LPA or EGF-stimulated cells showed more flat morphology and greater size compared to insulin or IGF-1-treated cells. The observation is consistent with the hypothesis that hyperactive glycolysis might serve to provide biosynthetic precursors. This is important for duplication of cellular contents, including DNA, proteins and membrane lipids, to maintain mass and dimensions of daughter cells. In this context, ATP generation by glycolysis may become secondary to the main aim, biosynthesis in rapidly growing tumor cells.

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