Lysophosphatidic Acid as a Target for Molecular Diagnosis and Therapy of Ovarian Cancer

Ph.D. Dissertation

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TABLE OF CONTENT

1. Abbreviations……..... 4

2. Introduction……. 5

2.1. Physiologic role of LPA…….. 7

2.2. LPA production and metabolism…… 8

2.3. LPA receptors …… 12

2.4. Pathophysiology of LPA in ovarian cancer…… 13

3. Aim of the Study and Hypothesis…… 16

4. Materials and Methods

4.1. Analysis of gene expression profiles…….. 17

4.2. Tumor samples and RNA preparation…… 17

4.3 Gene expression and data analysis…….. 18

4.4. Cell cultures…… 18

4.5. G418 selection…… 18

4.6. First strand cDNA synthesis and RT-PCR…….. 19

4.7. Analysis of total LPP and LPP-1 activity…… 20

4.8. Preparation of Affinity-purified LPP Anti-Peptide Antibodies……... 20

4.9. Indirect Immunofluorescence……. 21

4.10. Cell preparation for analysis of LPP activity…….. 22

4.11. Preparation of (32P) LPA as substrate……. 22

4.12. LPP enzyme assay and immunoprecipitation…….. 23

4.13. Intracellular calcium mobilization assay…… 24

4.14. Evaluation of ERK activation in LPP-3 transfected ovarian cancer cells…….. 24

4.15. Western blot analysis…….. 25

4.16. LPA determination in cell supernatants…….. 25

4.17. The LPP-1 and LPP-3 cDNA containing vectors…... 26

4.18. Transient and stable transfection……..... 27
4.19. Colony formation assay…… 28
4.20. GFP targeted cell cycle progression assay…….. 28
4.21. Analysis of bystander effect…… 29
4.22. Migration assay……... 30
4.23. Semi-quantitative RT-PCR of LPP-3……. 30
4.24. Tumor growth in nude mice…… 31
4.25. Evaluation of Autotaxin/LysoPLD effect…… 31
4.26. Statistical analysis……... 32

5. Results

5.1. Decreased LPP-1 mRNA expression…….. 33
5.2. Semi-quantitative RT-PCR analysis……... 35
5.3. LPP-1 activity is decreased in ovarian cancer cells… 35
5.4. Functionally competent expression of LPP-3…… 39
5.5. ERK activation and intracellular Ca2+ mobilization... 43
5.6. LPP-1 and LPP-3 decrease colony forming ability…. 45
5.7. LPP-1 and LPP-3 increase the apoptosis rate……. 51
5.8. LPP-3 decreases growth through LPA hydrolysis….. 55
5.9. LPPs decrease the growth of bystander cells…….. 55
5.10. LPP-1 overexpression decreases cell migration……. 58
5.11. LPP-3 decreases tumor growth in vivo…… 62
5.12. ATX/LysoPLD opposes the effect of LPP-3…… 63

6. Discussion…… 70
7. Conclusions……. 77
8. Summary……. 79
9. References…… 83
10. My publication list - associated with the thesis……. 98
11. My publication list - not associated with the thesis……..... 101
12. Acknowledgements……. 104

1. ABBREVIATIONS

LPA	Lysophosphatidic acid
Edg	Endothelial differentiation gene
LPP	Lipid phosphate phosphohydrolase
PAP	Phosphatidic acid phosphatase
hTERT	Human telomerase reverse transcriptase
OMPT	1-Oleoyl-Sn-2-O-Methyl-Rac-Glycero-3-Phosphothionate
CMV	Cytomegalovirus
SV40	Simian virus T 40 antigen
GFP	Green fluorescent protein
RT-PCR	Reverse transcription-PCR
PI	Propidium iodide
NOE	Normal ovarian epithelial cell line
LPAT	Lysophospholipid acyl transferase
TGF-α, -β	Transforming growth factor-α, –β
ATX/lysoPLD	Autotaxin/lysophospholipase D
LPC	Lysophosphatidylcholine
PC	Phosphatidylcholine
PLA	Phospholipase A
VEGF	Vascular endothelial growth factor
GPCR	G protein-coupled receptors
PI3K	Phosphatidylinositol-3-kinase
FBS	Fetal bovine serum
HU-K5	Human lysophospholipase homolog
LPAAT	Lysophosphatidic acid acyltransferase
FFA	Free fatty acids

2. INTRODUCTION

Ovarian cancer has the highest rate of mortality of all gynecologic cancers. In 2007, there were an estimated 23,400 new cases of ovarian cancer in the United States (Jemal et al, 2007). The majority of these women will eventually die from their disease. This depressing prognosis results from an inability to diagnose the tumor at an early, curable stage because there is no well-established screening method, so at least 75-80 % of all patients present with late stage disease for which current treatment is inadequate. Although the current therapeutic approaches, which consist of a wide variety of chemotherapeutic protocols preceded by radical debulking surgery and/or radiation therapy, result in high response rates which translate into modest increases in survival time and improvement in quality of life, there has been no notable increase in the cure rate in the last 25 years (Penson et al, 1998). Most patients rapidly develop resistance to chemotherapy and will eventually die of their disease with the overall 5-year survival rate in advanced disease remaining less than 30% (Penson et al, 1998). Multiple preventive medical therapies were evaluated and only the oral contraceptives showed preventive measure in the development for epithelial ovarian cancer but not for already established cancer (Tanyi et al, 1998). Late stage epithelial ovarian cancers also grow in an environment composed of ascitic fluid, which demonstrated to contain activities that support growth of both primary cultures of epithelial ovarian cancer cells and of epithelial ovarian cancer cell lines *in vitro* and *in vivo* (Mills et al, 1988; Eder et al 2000). Ovarian cancer genomes are remarkably variable; an advanced cancer usually harbors many genomic changes. Multiple chromosomal anomalies were published including gross chromosomal gains or losses (Tanyi et al, 1998 and 1999a); single mutations of different genes, all of which influence biologic and clinical behavior (Gray et al, 2003; Tanyi et al, 1999b, 2000 and 2001; Suzuki et al, 2000; Mills et al, 2003a). This genomic instability and tumor heterogeneity probably contribute to drug resistance and the lack of effective therapeutic approaches that can convert the high response rate into a high cure rate. Genomic analyses may improve aspects of ovarian cancer management by revealing

early events in ovarian cancer oncogenesis and progression that can be investigated as markers for early detection and by identifying specific genetic aberrations that can be targeted therapeutically. Alternatively, global genomic analysis using comparative genomic hybridization or microarray technology (Cheng et al, 2002;) may identify patterns of genetic aberrations that predict outcome or response to particular therapies, allowing tailoring of patient management to the specific genetic aberrations in the tumor: individualized molecular medicine.

Regional delivery of gene therapy constructs was heralded as an attractive alternative for the treatment of ovarian cancer because ovarian cancer tends to remain localized in the peritoneal cavity with local tumor growth contributing to morbidity and mortality. However, most gene therapy approaches currently use non-specific and non-selective prokaryotic promoters (eg, CMV and SV40) that allow a high expression of the gene in normal cells resulting in marked toxicity. Further, it has proven difficult to introduce target genes into all tumor cells and the long hoped for "bystander effect" wherein non-transfected cells would die due to interactions with their neighbors has not proven to be sufficiently effective. Additional studies are needed to clarify optimal gene therapy targets, and effective methods for tumor selective delivery such as tumor specific promoters to deliver gene therapy (Tanyi et al, 2002; Lee et al, 2004; Bao et al, 2002) will become an effective therapeutic approach.

Lysophosphatidic acid (LPA, 1-acyl-2-lyso-sn-glycero-3-phosphate) has emerged from being considered a precursor and component of lipid remodeling to clearly playing a role as an important extracellular bioactive lipid mediating cell proliferation, migration, and survival in almost every cell type, both normal and malignant (Mills and Moolenaar, 2003b). LPA, the simplest phospholipid, mediates multiple functions ranging from growth promotion and increased cell cycle progression to cell survival (Van Corven et al, 1989; Koh et al, 1989; An et al, 1998a; Chun et al, 1999; Fang et al, 2000b and Goetzl et al, 1999). LPA has also been shown to increase the production of factors involved in neovascularization, to induce protease production and action and invasiveness, suggesting the possibility that LPA may also play a role in metastatic competence (Xiao

et al, 2001, Xu et al, 1995b, Van Corven et al, 1992, Imamura et al 1993 and Moolenaar et al, 1997).

2.1. Physiologic role of LPA

LPA despite being one of the simplest of all serum phospholipids, exerts pleiomorphic activities on many cell lineages (Mills and Moolenaar, 2003b). LPA and its actions are highly conserved through ontogeny, affecting diverse organisms (Moolenaar et al, 1992). Yeast and insect cells, in which no effect has been observed, have proven powerful models to understand the mechanisms by which LPA mediates its effects (Hu et al, 2001). Although LPA induces cellular proliferation and survival in most lineages, it can induce differentiation in some cellular lineages. For example, in neurons LPA can induce necrosis and apoptosis (Nietgen and Durieux, 1998). LPA induces a number of early cellular responses, such as motility, chemotaxis, gap-junction opening, invasion, and morphologic changes that do not require new protein synthesis. Longer-term effects such as changes in cellular shape, increased cell viability, improved wound healing, and production of endothelin and proangiogenic factors may require, at least in part, new gene transcription (Mills and Moolenaar, 2003b). LPA may function as part of an autocrine signaling loop through increasing the secretion or activation of multiple peptide growth factors, including transforming growth factor-α and –β (TGFα, TGFβ), heparin-binding epidermal growth factor, insulin-like growth factor II, and endothelin 1 (Nakano et al, 1994; Pustilnik et al, 1999; Laffargue et al, 1999; Fang et al, 2000a). LPA also induces the production of a high number of paracrine growth factors active on blood vessel wall endothelial cells such as interleukin 6, interleukin 8 and vascular endothelial growth factor (VEGF), increasing neovascularization in either physiologic (wound healing) or pathologic (tumor) environments (Jalink et al, 1994; Xu et al, 1995a; Levine et al, 1997, Fang et al 2004). LPA exerts multiple other vascular effects that include alteration of monocyte attachment to blood vessel walls, plaque formation, increased endothelial permeability, and vascular smooth muscle cell contraction. These changes can

alter blood pressure in animal model systems and in humans and also play a role in the development of atherosclerosis (Shulcze et al, 1997; van Nieuw Amerongen et al, 2000). LPA is a precursor for other, more complex lipid syntheses, some of which may also function as bioactive mediators. For example, conversion of LPA to phosphatidic acid by endophilin and lysophospholipid acyl transferase (LPAT) is known to influence membrane curvature and the process of endocytosis (Schmidt, 1999). While most of the signaling effects of LPA appear to be due to binding to specific cell surface receptors, intracellular LPA has been demonstrated to activate the peroxisome proliferating activating receptor-γ, potentially contributing to atherosclerosis, adipogenesis and insulin signaling (McIntyre et al, 2003,).

2.2. LPA production and metabolism

The concentration of LPA in plasma is normally low (100-200 nM), suggesting that the production, metabolism and clearance of LPA are strictly controlled in vivo (Xu et al, 1998). LPA can be produced by multiple cell lineages such as activated platelets, adipocytes, leukocytes, fibroblasts, endothelial cells, during clotting and, of particular importance, ovarian cancer cells (**Figure 1.**) (Umezo-Goto 2004; Moolenaar et al, 1992; Goetzl et al, 1998; Fang et al, 2000b; Eder et al, 2000; Sano et al 2002; Aoki et al 2002.). The main pathway for extracellular LPA production is the activity of autotaxin/lysophospholipase D (ATX/lysoPLD) which removes a choline from lysophosphatidylcholine (LPC) (Sano et al, 2002; Aoki et al, 2002; Umezu-Goto et al; 2002, Tokumura et al, 2002). LPC is produced by the removal of a fatty acyl chain from phosphatidylcholine (PC) by phospholipase A1 or phospholipase A2 (PLA2). PLA2 has limited ability to hydrolyze lipids in intact membranes suggesting that the major source for LPA production may be vesicles or apoptotic cells where normal membrane structure is compromised. In addition, LPC is generated by the lecitin cholesterol acyl transferase pathway, in which cholesteryl esters are generated by acyl transfer from PC. PC is secreted by the liver and bound to albumin or LDLs could be hydrolyzed by the

sequential activity of PLA2 and ATX/lysoPLD producing bioactive LPA (Croset et al, 2000). LPA also can be produced by the removal of a fatty acyl chain from PA by PLA1 or PLA2 (Aoki et al, 2002; Sano et al, 2002).

ATX/LysoPLD had been previously identified as a major regulator of motility, metastases, and tumor aggressiveness (Nam et al, 2000; Nam et al, 2001; Yang et al, 2002), however, the mechanism by which it mediates these processes is unknown. The discovery that ATX and lysoPLD were encoded by the same molecule resulted in a convergence of these two major areas of research. ATX/lysoPLD is widely expressed, with the highest mRNA levels in the ovary, intestine, lung, and brain (Bachner et al, 1998). ATX/lysoPLD levels are markedly increased in multiple different cancers compatible with a role in the pathophysiology of malignant disease (Umezu-Goto et al, 2004; Tanyi et al, 2006).

LPA is efficiently metabolized maintaining LPA at low levels under physiological conditions. LPA is removed by being acylated to produce phosphatidic acid, deacylated by lysophospholipases to produce glycerol phosphate, or dephosphorylated by lipid phosphate phosphatases (LPP-s) also known as phosphatidic acid phosphohydrolases to produce monoacylglycerol which is inactive on LPA-receptors (An et al 1998a; Moolenaar et al, 1992; Tigyi et al, 1992; Erickson et al 2001 and Eicholitz et al, 1993). The most likely candidates for hydrolysis of extracellular LPA, including LPP-1 (PAP2A) LPP-2 (PAP2C), and LPP-3 (PAP2B), are six transmembrane domain containing integral membrane proteins with their catalytic site and functions on the extracellular surface of the cell (Hu et al, 2001; Brindley et al, 1998; Kai et al 1996 and 1997). LPP-like properties serve to terminate the receptor-directed signaling functions of LPA and related compounds (Waggoner et al, 1995 and 1996; Imai et al, 2000; Xu et al, 2000; Pilquil et al 2001 and Alderton et al, 2001). LPPs have been implicated in limiting LPA signaling in multiple systems (Hook et al, 2001; Pilquil et al 2001 and Alderton et al, 2001). LPPs have been proposed to degrade extracellular LPA particularly that associated with the cell membrane (Pilquil et al, 2001). Indeed, over 90% of LPA degradation by ovarian cancer cells is due to the action of LPP-like enzymes (Imai et al

2000). Alternatively, LPPs have been suggested to directly inhibit the function of G protein-coupled receptors (GPCRs) of the LPA family (Alderton et al, 2001), independent of LPA hydrolysis. Intriguingly, the effects of LPPs have under some circumstances been proposed to be independent of the known LPA receptors, implicating additional LPA receptors (Hooks et al, 2001) or LPA receptor-independent effects. Although the LPPs can hydrolyze free phosphates in phospholipids, lysophospholipids, ceramide lipids and sphingolipids, LPP-3 shows preference for LPA (Sciorra and Morris, 1999).

Other routes of LPA metabolism include acylation by lysophospholipid acyl transferase or endophilin to create phosphatidic acid and deacylation by lysophospholipases to produce glycerol phosphate (Wang and Dennis, 1999). In addition to metabolism, LPA is cleared rapidly from the circulation potentially contributing to the maintenance of LPA at appropriately low levels under physiological conditions.

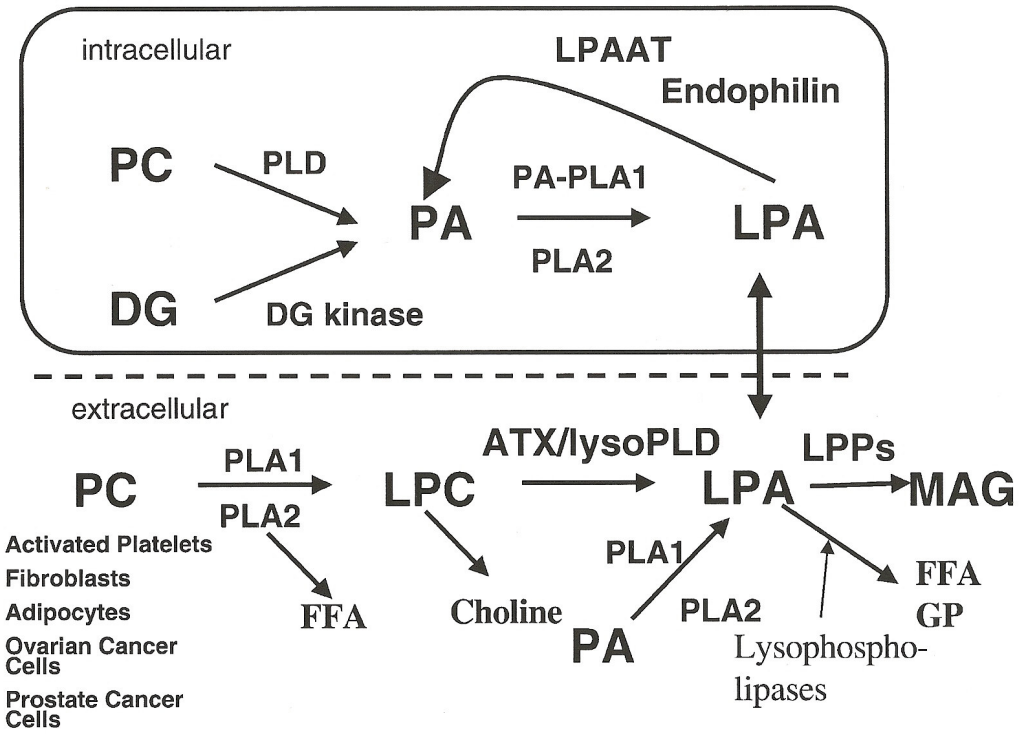


Figure 1. The major intracellular and extracellular pathways of production and degradation of LPA. Whether intracellular or extracellular LPA are independent pools or whether there is exchange between these pools are unknown. Intracellular and extracellular LPA mediate different but important functions that could contribute to the pathophysiology of cancer. Cells known to produce LPA are indicated at the lower left (Umezu-Goto et al, 2004).

2.3. LPA receptors

The outcomes of LPA signaling are determined by a broad spectrum of LPA receptors expressed on the cell surfaces. The LPA1 (Edg 2), LPA2 (Edg 4) and LPA3 (Edg 7) members of the endothelial differentiation gene (Edg) family of G protein-coupled receptors are high affinity receptors for LPA and have been proposed to mediate LPA signaling in mammalian cells (An et al, 1997 and 1998b; Bandoh et al, 1999). The extracellular LPA binds these G-protein-coupled receptors (GPCR) on the surface of mammalian cells to start a signaling cascade into the cells (An et al, 1998b; Bandoh et al, 1999). The prior Edg2, Edg4, and Edg7 LPA receptors were recently renamed LPA1, LPA2, and LPA3, correspondingly. Meanwhile, a fourth LPA receptor, LPA4, a member of the purinergic family of GPCR was recently identified (Noguchi et al, 2003). LPA1, which is widely expressed in the placenta, brain, small intestine, and colon, has a lower level of expression in pancreatic and normal ovarian tissue (Hecht et al, 1996). In some cell lineages, LPA1 is a major regulator of cellular motility by initiating Rho-dependent changes in cytoskeletal function, including cell rounding and stress fiber formation (Fukushima et al, 1998; Van Leeuwen et al, 2003). However, it is clear that LPA2 can mediate motility in at least some cellular lineages. LPA2 and LPA3 have more constrained distribution patterns that LPA1 in terms of normal tissues. LPA1 and LPA2 are aberrantly expressed in prostate and ovarian cancer (Fang et al, 2002b) as well as other cancer lineages suggesting they may be appropriate targets for therapy. LPA2 exhibits a higher affinity than the other family members for LPA suggesting that it is a major contributor to the functions of LPA, particularly at the low levels of LPA that are present in normal tissues and in plasma. LPA2 appears to be a major regulator of the production of vasculogenic factors (Fang et al, 2003; Huang et al, 2004). The lower affinity LPA receptors may function in pathologic or stress states such as those following cellular injury. These would include, for example, wound healing, ischemia reperfusion, and blood clotting (Okusa et al, 2003; Mills et al 2002). The receptors by which LPA

mediates cellular proliferation and viability remains controversial, however, LPA3 remains a strong candidate as LPA3 selective LPA homologs are potent activators of proliferation and viability (Hasegawa et al, 2003). LPA4 is expressed at a very low level in most human tissues, but a substantial level of expression has been detected in normal ovarian tissue (Noguchi et al, 2003). The role of LPA4 in physiological and pathologic states has not been elucidated.

LPA activates at least three different G proteins via the LPA receptors, which can, in turn, stimulate multiple intracellular signal transduction systems. How the integration of these signaling events leads to the functional outcome of LPA receptor ligation remains to be fully determined. Activation of G_q leads to activation of phospholipase C producing diacylglycerol and inositol trisphosphate, second messengers which in turn lead to the activation of protein kinase C and increases in cytosolic calcium respectively. Activation of G_i feeds into four different and important signaling pathways: 1) adenylate cyclase by increasing cyclic adenosine monophosphate levels and activating protein kinase A; 2) RAS and the mitogen activated protein kinase cascade; 3) activation of the phosphatidylinositol 3-kinase-; and 4) glycogen synthase kinase-3 pathway (Van Leeuwen et al, 2003; Fang et al, 2000a; Fang et al, 2000b, Fang et al, 2002b). LPA also activates $G_{12/13}$ contributing to activation of the small GTPase RhoA leading to cell rounding and cytoskeletal contraction (Etienne-Manneville and Hall, 2002).

2.4. Pathophysiology of LPA in ovarian cancer

Ascites from ovarian cancer patients frequently contain elevated concentrations of LPA (up to 80 μM) (Eder et al, 2000; Xu et al, 2003). This suggests that ovarian cancer cells in patients are exposed to a LPA rich environment. As ovarian cancer cells can produce LPA (Eder et al 2000; Sengupta et al, 2003; Shen et al, 1998; Luquain et al, 2003), the cancer cells themselves are potential sources of the elevated LPA. LPA can increase the proliferation of ovarian cancer cells (Xu et al, 1995b). LPA also contributes to the multistep process of metastasis and invasion by increasing the production of

urinary plasminogen activator (Pustilnik et al, 1999). Furthermore, LPA markedly decreases anoikis (a form of apoptosis) in ovarian cancer cell lines, suggesting that it plays a role in preventing anoikis and increasing metastasis in vivo. LPA is a potent inducer of neovascularizing factors, vascular endothelial growth factor, IL8 and IL6 production by ovarian cancer cells, potentially contributing to neovascularization and aggressiveness of ovarian cancer (Hu et al, 2001, Fang et al, 2004, Schwartz et al, 2001) as well as to the elevated levels of these factors in ovarian cancer (Zebrowski et al, 1999).

LPA is not produced in significant amounts by normal ovarian surface epithelial cells but is produced at significant levels by some ovarian cancer cell lines (Eder et al, 2000; Sengupta et al, 2003; Shen et al, 1999; Luquain et al, 2003). The levels of ATX/lysoPLD mRNA and protein levels are modestly elevated in malignant disease. At least half of ovarian cancers tissue samples exhibit at least a 2-fold increase while one quarter of these samples show up to a 3-fold increase (Umezu-Goto et al, 2004). The production of LPA by ovarian cancer cells can be increased by phorbol esters, nucleotides, laminin and surprisingly, by LPA itself (Eder et al, 2000; Sengupta et al, 2003; Shen et al, 1999, Luquain et al, 2003). The decreased LPA inactivation, the increased levels of ATX/lysoPLD protein, and LPA autocrine loop likely contributes to the increased levels of LPA in the ascites.

LPA has modest activity on normal ovarian surface epithelium that expresses predominantly LPA1. Overexpression of LPA1 in ovarian cancer cell lines decreases proliferation and increases apoptosis suggesting that LPA1 can act as a negative regulator (Furui et al, 1999). In contrast, both LPA2 and LPA3 are aberrantly overexpressed in ovarian cancer cells, suggesting that LPA plays an important role in the pathophysiology of this cancer (Furui et al, 1999; Goetzl et al, 1999; Eder 2000, Fang 2002a; Mills et al, 2002). In addition to increased LPA production and LPA receptor expression, the genetic aberrations in ovarian cancer cells may contribute to increased responsiveness to LPA receptor ligation. Both the phosphatidylinositol-3-kinase (PI3K) pathway and Ras/Erk pathways are stimulated by LPA receptors in ovarian cancer cells (Xu Y et al 1995c; Fang et al, 2000b), which contribute to LPA-induced cell proliferation and survival.

Intriguingly, the p110β catalytic subunit of phosphatidylinositol-3-kinase is selectively activated by LPA in ovarian cancer cells (Roche et al, 1998). Both the PI3K pathway and the Ras/Erk pathway are highly activated in ovarian cancer cells, potentially, at least in part due to the presence of LPA in ascites. A recent review highlights the importance of the PI3K pathway as a second lipidomics target in developing new targeted signal transduction or targeted therapeutics for the treatment of ovarian cancer (Drees et al, 2004). Fang et al, also found that next to the PI3K pathway, LPA also induces phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3), a multifactorial serine/threonine kinase (Fang et al, 2002b). The effect of LPA can be reconstituted by expression of Edg-4 and Edg-7 in cells lacking LPA responces. LPA and potentially other natural ligands utilize a PKC-dependent pathway to modulate GSK-3 (Fang et al, 2002b).

If LPA is present at an increased concentration in ascites, it could diffuse into the systemic blood circulation where it could serve as a tumor marker for early disease detection. Indeed, plasma levels of LPA from patients with ovarian cancer are lower than they are in matched ascites samples compatible with LPA in plasma representing diffusion from the peritoneal cavity (Eder et al, 2000). LPA levels have been reported to be increased in the plasma of approximately 90% of patients with ovarian cancer (Xu et al, 1998). Similarly, aberrations of particular phosphatidylcholine isoforms have been detected in the plasma of patients with ovarian cancer (Okita et al, 1997). However, there exists considerable controversy as to whether LPA levels are elevated in the plasma of ovarian cancer patients and whether the elevations are predictive of the presence of surface epithelial ovarian cancer (Xiao et al, 2000; Baker et al, 2002; Yoon et al, 2003). Similarly efforts were made to use inhibin as a tumor marker for ovarian-, mostly granulose cell tumors, but its usefulness as a tumor marker has not been proven yet (Tanyi et al. 1999c). No effort was made yet to evaluate the association of granulose cell tumors with LPA expression which can be explained with the small prevalence of granulose cell tumors compared with surface epithelial ovarian cancers.

3. AIM OF THE STUDY AND HYPOTHESIS

1. The LPA-rich environment of the ovarian cancer cell *in vivo* and the subsequent effects of cellular pathophysiology may be a consequence of both increased LPA production and decreased LPA metabolism by ovarian cancer cells. Although ovarian cancer cells can produce LPA, this may not be the major reason for altered LPA levels in ascites. We try to demonstrate that the major pathway of degradation of LPA by ovarian cancer cells is through a lipid phosphate phosphatase (LPP)-like activity.
2. At first step we evaluate the LPPs' mRNA expression to see any different between the normal epithelial cells and ovarian cancer cells.
3. We intend to introduce LPP-1 or LPP-3 into ovarian cancer cell lines in order to increase LPA hydrolysis with transient and stable transfection.
4. Our purpose is to determine the effects of decreasing LPA levels by increasing LPP activity on the pathophysiology of ovarian cancer cells. We try to demonstrate that the introduction of hLPP-1 and LPP-3 increase LPA degradation, which is associated with decreased cellular proliferation and increased death in vitro and decreased growth in vivo.
5. The specificity of the effects of LPP-3 for LPA would be demonstrated by studies with the LPP-resistant, LPA3 receptor-specific agonist, 1-Oleoyl-Sn-2-O-Methyl-Rac-Glycero-3-Phosphothionate (OMPT) and with enzymatically inactive LPP-3. Taken together, we expect that these studies will validate LPA metabolism and function as a target for therapy in ovarian cancer.
6. If it is successful, we plan to apply this method not only in vitro but also in vivo in animal models.
7. We would further manipulate the LPA production and metabolism to evaluate the relationship between the metabolizing and producing enzyme.

4. MATERIALS AND METHODS

4.1. Analysis of gene expression profiles in ovarian tissue samples from published microarray hybridization data

RNA extraction and hybridization on oligonucleotide microarrays were performed and published on 27 primary human ovarian cancer and other normal and tumors samples by previous reports (Su et al, 2001; Welsh et al, 2001a and 2001b). Affymetrix U95 array hybridization data are available (http://www.gnf.org/cancer/epican.) (Su et al, 2001; Welsh et al, 2001a and 2001b). The control samples consisted of 36 normal adult epithelial tissues (Su et al, 2001). We combined these results with our own analysis of expression arrays from an additional 26 ovarian cancers (17 from serous and 9 from endometrioid histological subtypes). All samples were assessed and demonstrated to contain at least 80% tumor with limited stromal contamination. Control samples consisted of four separate pools of normal ovarian epithelial cells scraped directly from the surface of the ovary from 13 different patients into preservative, providing an indication of mRNA levels in normal ovarian epithelial cells in vivo.

To allow comparison across multiple arrays and the two databases, the mRNA expression data of each gene was normalized to the expression detected in normal epithelial cells. The overall expression levels in the two datasets were comparable, allowing combination the data.

4.2. Tumor samples and RNA preparation

Twenty-six fresh frozen primary ovarian cancers were obtained from Duke University (9 endometrioid), the Mayo Clinic (9 stage I serous), and MD Anderson Cancer Center (8 stage III serous). 4 pools of normal ovarian surface scrapings from 13 patients were obtained from Northwestern University. The normal scrapings were

collected using a cytobrush and then the cells were resuspended and frozen in RLT buffer (Qiagen Inc., Valencia, CA). RNA was extracted using the RNeasy Kit (Qiagen Inc, Valencia, CA).

4.3. Gene Expression and data analysis

The Affymetrix GeneChip® Human Genome U95 set was used to obtain gene expression data. This series tests the expression of more than 60,000 human genes and ESTs. The cRNA preparation, hybridization, and scanning of the microarrays was performed according to manufacturer's protocols at the Mayo Clinic's microarray facility. Microarray data was analyzed using the dChip software (http://www.biostat.harvard.edu/complab/dchip/) (Li et al, 2001).

4.4. Cell cultures

OVCAR-3, SKOV3, SKOV3 IP_1, HEY and A 2780 (human epithelial ovarian carcinoma cell lines) were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). The IOSE 80 and IOSE 29 (simian virus 40 T-antigen-semi-immortalized ovarian surface epithelial cell lines) were kindly provided by Nelly Auersperg (Department of Obstetrics and Gynecology, University of British Columbia) (Lie et al, 1996). The IOSE cells were grown in medium 199/MCDB 105 supplemented with 10 % FBS. SKOV3 IP_1 is a more aggressive SKOV3 subline developed by intraperitoneal passage of SKOV3 (Yu et al, 1993).

4.5. G418 selection

All the cell lines were pre-tested to evaluate G418 (Gibco BRL, Grand Island, NY) resistance. G418 was applied in concentration from 200 μg/ml to 1000 μg/ml. OVCAR-3 and all the IOSE cell lines were cultured with 400 μg/ml, the A 2780 cell line

with 500 µg/ml and the SKOV3, SKOV3 IP_1 and HEY cell lines with 800 µg/ml G418. Medium contained the appropriate amount of G418 and was changed every third day. The lowest concentration of G418 that killed all the cells was applied later in the experiments.

4.6. First-strand cDNA synthesis and semi-quantitative RT-PCR of LPP-1

The mRNA expression of LPP-1 was determined using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Oligonucleotide primers were used for: LPP-1 primers:

5`-CGCGGATCCATGTTTGACAAGACGCG and

5`-TGGCTATGTAGTTATTCCTG, (304 bp)

and control GAPDH primers:

5`-CCCATGGCAAATTCCATGGCACCG and

5`-GTCATGGATGACCTTGGCCAGGGG (344 bp).

The RNA samples were treated with DNAse before first strand cDNA synthesis or RT-PCR reaction following the manufacturer's instructions (DNA-free, DNAse Treatment and Removal Kit) (Ambion, Austin, Texas, USA). In some cases, RT-PCR was performed as a single step with the RT-PCR reaction mixture consists 1µg of total RNA, 0.4 µM of sense and antisense primers, 0.2 mM of dNTPs, 0.5 U of reverse transcriptase and Taq-DNA polymerase enzymes (in enzyme mix), 5 mM DTT-solution, 5 U RNAse inhibitor, 1.5 mM $MgCl_2$ in final volume of 50 µl (Titan One Tube RT-PCR System, Roche Molecular Biochemicals, Mannheim, Germany). The reverse transcriptase reaction was performed at 60 C° for 30 minutes followed by 35 cycles of PCR in a Thermal Cycler (Perkin-Elmer 480, Atlanta, GA). Each cycle of PCR included 30 s of denaturation at 94 C°, 2 min of annealing at 56 C° and 1 min of extension at 72 C°.

In the cases of the primary tumor samples and the ovarian surface epithelial samples, the RT-PCR was performed as a two step approach using first strand cDNA synthesis was with SuperScript™ First-Strand Synthesis System (Invitrogen Corporation,

Carlsbad, CA) according to the manufacturer's instruction using 5 µg total RNA from each primary ovarian tumor. PCR was then performed as described above using 1/5 of the RT product. RT-PCR with GAPDH (29 cycles) was used to demonstrate equal amounts of RNA in each sample and used to normalize results. PCR products were visualized by electrophoresis in a 2 % agarose gel with ethidium bromide.

4.7. Analysis of total LPP and LPP-1 activity in ovarian cancer cell lines

Cells were grown in 35 mm diameter dishes to ~80% confluence, washed once in PBS and lysed by scraping in buffer containing 20 mM Tris, pH 7.4, 2 mM EDTA and protease inhibitors (benzamidine and PMSF). The cells were disrupted by brief probe sonication. LPP activity in the lysates was determined using [^{32}P]-LPA presented in mixed micelles of Triton X-100 as described previously (Sciorra et al, 1999; Roberts et al, 2000). For the data shown in Figure 3 assays contained 1-5 µgs of protein. Immunoprecipitations were performed as described previously (Sciorra et al, 1999; Roberts et al, 1998 and 2000). LPP activity in washed immune complexes was determined as above using [^{32}P]-LPA.

4.8. Preparation of Affinity-purified LPP Anti-Peptide Antibodies

A peptide corresponding to residues 2-17 (QNYKYDKA-IVPESKNG) of the sequence of human LPP-3 (PAP2b) was synthesized. Rabbits were immunized, and antibody titers were determined by enzyme-linked immunosorbent assay using the individual peptide antigen as the solid phase. Antibodies were purified by affinity chromatography using the individual immobilized peptide antigen as described (Harlow and Lane, 1988). Selectivity of the antibodies was determined by detection of recombinantly expressed PAP enzymes by Western blotting. Quality Controlled Biochemicals prepared the peptide antisera.

4.9. Indirect immunofluorescence

Swiss 3T3 cells or COS7 cells were grown directly on sterilized glass coverslips. HEK 293 cells were grown on poly-L-lysine-coated glass coverslips and transfected with pcDNA-PAP2b before processing. Cells were washed three times with ice-cold PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, fixed by incubation in 4% paraformaldehyde (in PBS) for 20 min at 4°C, and permeabilized with PBS containing 0.1% TX100 (Pierce) for 10 min at 4°C. Fixed cells were incubated for 30 min with blocking buffer (4% nonfat dry milk in TBS + TX100) followed by a 1-h incubation with rabbit polyclonal antibody against PAP2b (1:100) in blocking buffer (or overnight at 4°C). Coverslips were washed followed by a 30-min incubation with rhodamine-conjugated goat anti-rabbit IgG (1:200). Coverslips were rinsed extensively and mounted in antifade reagent (VectaShield, Burlingame, CA). For double-labeling experiments, fixed cells were incubated with rabbit anti-PAP2b at 1:200 and mouse anti-BiP (1:200) for 1 h, washed, and stained with rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG. For Golgi labeling, Swiss 3T3 cells were incubated with 2 μM (1-caproyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-2n-glycerol-3) NBD-ceramide (Molecular Probes, Eugene, OR) in serum-free media for 10 min at 37°C. The medium was then removed, and cells were further incubated in serum-free medium containing 0.68 mg/ml fatty acid-free BSA for 30 min. NBD-ceramide-labeled cells were fixed (omitting TX100) and mounted as described above.

Stained cells were viewed by confocal laser scanning epifluorescence microscopy using a Nikon Diaphot microscope equipped with a 40× (NA 1.4) PlanApo objective oil immersion lens. Epifluorescent images were captured with an Optronics color camera and processed using Image I software and Adobe Photoshop 3.0. Control images were acquired and processed in exactly the same way as their corresponding image.

4.10. Cell preparation for analysis of LPP-1 and LPP-3 activity

For studies using SKOV3 cell membranes or detergent-extracted membrane proteins, the monolayer of infected cells was washed gently with PBS and lysed by the addition of 4 ml of ice-cold lysis buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride) and scraping (Roberts et al, 1998). The cell suspension was transferred to a 15-ml conical tube and the cells were disrupted by sonication (Vertis Systems Sonifier), 10-s pulses on ice. The disrupted cells were centrifuged at 20,000xg at 4 C° for 20 min. The cytosolic fraction was removed, and the membrane fraction was resuspended in ice-cold lysis buffer. Detergent extracts were prepared from the membranes by the addition of Triton X-100 to final concentration of 1% followed by incubation at 4 C° with constant rocking for 1 h. The solubilized material was centrifuged at 26,000xg at 4 C° for 30 min, and the supernatant was removed. To assess LPP-3 activity, stable LPP-3 expressing SKOV3 were washed gently with phosphate-buffered saline and collected by the addition of 4 ml of ice-cold lysis buffer followed by scraping. The cell suspension was processed as above.

4.11. Preparation of (^{32}P) LPA as substrate

The (^{32}P)LPA was prepared by phosphorylation of Oleoyl monoacylglycerol (monoolein) (Avanti Polar Lipids, Alabaster, AL) using E. Coli diacylglycerol kinase (Calbiochem, San Diego, CA) and (γ^{32}P)ATP(ICN Pharmaceuticals, Costa Mesa, CA). The reaction was terminated by extraction with acidified CHCL3 and MeOH, and the dried organic phase obtained was resuspended in 0.4 ml of 20:9:1 CHCL3/MeOH/H2O (solvent A) and neutralized by the addition of a small volume of 20 % NH_4OH in MeOH. This material was applied to an Econosil NH_2 5U high-pressure liquid chromatography column (250X4.2 mm) (Alltech Associates, Baulkham Hills, NSW, Australia). The column was washed with 20 ml of solvent A and then eluted with a 40-ml linear gradient of 0-1 M ammonium acetate in solvent A. 0.5-ml fractions of the eluant were collected,

and associated radioactivity was determined by liquid scintillation counting. ^{32}P-labeled products were pooled and extracted from the eluant by the addition of 3 M HCL and CHCL3 to give two phases.

4.12. LPP-1 and LPP-3 enzyme assays and immunoprecipitation

The assay procedures used were adapted from those described previously (Bandoh et al, 2000). In brief, assays were performed in medium containing 20 mM Tris, pH 7.5, 1 mM EGTA, and 2 mM EDTA. ^{32}P-labeled LPA was dried under vacuum and resuspended in 6.4 mM Triton X-100. The assay volume was 100 µl, and each assay contained a final concentration of 3.2 mM Triton X-100 and 100 µM ^{32}P-labeled lipid substrate. Detergent-extracted membrane proteins (generally 0.1-5 µg of protein) were added directly. Assays were performed at 37 C° and were terminated by addition of ice-cold 10 mg/ml bovine serum albumin and 10 % trichloroacetic acid. The samples were centrifuged for 5 minutes in a microcentrifuge, and $(^{32}P)PO_4^{2-}$ released into the supernatant was quantitated by liquid scintillation counting. This assay was validated by demonstrating that the water-soluble radioactivity released from the substrate was $(^{32}P)PO_4^{2-}$ by quantitative extraction with ammonium molybdate. Immunoprecipitation, SDS-polyacrylamide gel electrophoresis and protein determinations were performed as described previously (Hammond et al, 1997; Roberts et al, 1998). The only modification for the LPP-1 enzyme assay was that we used 12.5 % SDS-polyacrylamid gel instead of the previously published 7.5 % (Hammond et al, 1997; Roberts et al, 1998).

To assess LPP-3 activity on cell supernatants both parental and stably SKOV3 transfected cells were resuspended in OPTI-MEM medium containing 3 % bovine serum albumin. ^{32}P LPA was added to a final concentration of 20 µM by bath sonication. Assays were initiated by adding 1 ml of this substrate preparation to 1 ml of cells ($4x10^5$ cells) followed by incubation at 37 C° with constant shaking. Aliquots of the suspension were removed at various times for determination of LPP-3 activity by measurement of $[^{32}P]PO^{-2}_4$ release as described above.

4.13. Cytoplasmic $[Ca^{2+}]_i$ assay

After starvation in serum-free medium for 12-24 h, C3H10T1/2 cells were harvested and loaded with 1 μM Indo-1 AM in phosphate-buffered saline (PBS) for 30 min at 37 °C. Cells were washed in PBS and resuspended at 2×10^6 cells/ml in a $[Ca^{2+}]_i$ assay buffer (140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Hepes, and 10 mM glucose, pH 7.4). Cytoplasmic $[Ca^{2+}]_i$ was determined at an excitation wavelength of 331 nm and an emission wavelength of 410 nm using a fluorescence spectrophotometer (F-4000, Hitachi). Approximately 3×10^6 cells were used for $[Ca^{2+}]_i$ determination in a stirred quartz cuvette kept at 37 °C. PC-3M cells were plated onto opaque, 96-well clear bottom plates at about 80% of confluence. The next day, cells were washed with Hanks' Basal Salts Solution containing 0.1% fatty acid-free BSA and loaded in the same solution with the calcium-sensing dye, Fluo-4AM. Calcium signals were recorded using a fast kinetic fluorometer (FLEXStation, Molecular Devices, Inc.). Excitation was at 485 nm, and records were collected at 525 nm. For the cytoplasmic $[Ca^{2+}]_i$ assay in Sf9, ~2×10^6 cells were infected with recombinant baculovirus engineered to express the LPA_2, LPA_3, or LPA_1/LPA_2 chimeric receptor (Bandoh et al, 1999). The preparations of the virus and infection of Sf9 cells have been described previously (Bandoh et al, 1999). Two days after virus infection, cells were starved and harvested for cytoplasmic $[Ca^{2+}]_i$ assays. After cells were loaded with 1 μM Indo-1 AM in PBS for 30 min at 27 °C, cytoplasmic $[Ca^{2+}]_i$ was determined at 27 °C in the same $[Ca^{2+}]_i$ assay buffer as described above for C3H10T1/2 cells.

4.14. Evaluation of the ERK activation in LPP-3 transfected cells

After 24 hrs starvation of 90 % confluent cell cultures of LPP-3 and neo stably transfected SKOV3 cells, LPA (1μM) was added and cell lysates prepared at the indicated time points. Western blotting was performed with anti-phospho-ERK1/2 (Cell

Signaling Technology, Inc. Beverly, MA) and anti-ERK2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Relative density of specific bands was determined using NIH Image 1.62.

4.15. Western Blot analysis

SDS-polyacrylamide gel electrophoresis (12.5% SDS), western blotting, and protein determinations were performed as described previously (Hammond et al, 1997; Sciorra et al, 1999) with slight modification using the indicated antibodies. Protein concentration was determined by the method of Bradford et al, 1976, using BSA as a standard. Samples were prepared for SDS-PAGE by boiling in sample buffer. Proteins were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) for 2 hours at 200 mA at 4°C. Blots were blocked in 5% nonfat dry milk in TBS with 0.1% Tween-20 (vol/vol) (TBST) for 2 hours at room temperature. Primary antibody incubations were performed in PBS containing 4% BSA for 2 hours at room temperature or overnight at 4°C. Secondary antibody incubations were performed in TBST containing 5% nonfat dry milk at 1:10,000 for 1 hour at room temperature. Protein bands were visualized by enhanced chemiluminescence using Super Signal (Pierce, Rockford, IL). Films were scanned with a (Bio-Rad) (Hercules, CA) scanning densitometer.

4.16. LPA determination in cell supernatants

LPA 18:1 (1μM) was added to SKOV3 cells, LPP-1 transfected SKOV3, LPP-3 transfected SKOV3 cells in separated experiments and cell supernatants collected at the indicated times. An unnatural LPA, 17:0, was added to the supernatants after collection to monitor efficiency of isolation and detection of LPA. LPA was extracted from 1ml of cell supernatant using Waters Oasis HLB 1 cc, 30 mg solid phase extraction cartridges (Milliford, CT) preconditioned with 1 ml methanol and 1 ml water. Cartridges were washed twice with 1 ml water and dried under vacuum for 5 min. LPA was eluted from

the cartridges using 1ml 95:5:5 methanol:chloroform:1M NH_4OH. Twenty-five µl of eluent was injected into the LC/MS/MS using a Water's XTerra 3.5 um C18 2 x 100 mm micropore column in a Agilent 1100 binary HPLC. The column was run in the isocratic mode using a mobile phase of 90:5:5 methanol:chloroform:1M ammonium hydroxide. LPA isoforms were detected using a MicroMass QuattroUltima triple quadrapole mass spectrometer (Beverly, MA) using electrospray negative ionization with the instrument operating in a multiple reaction-monitoring mode. Specific transitions for each LPA are as follows: LPA 18:1 is 435.24 > 152.8 and LPA 17:0 423>152.8. Instrument settings are as follows: cone voltage, 50 V; capillary voltage 3.00 KV; collision energy 22.

4.17. The LPP-1 and LPP-3 cDNA containing vectors driven by CMV and hTERT promoters

The complete cDNA of LPP-1 (Roberts et al, 2000) was amplified using primers with the sequences:
5-CGCGGATCCATGTTTGACAAGACGCG -3 (forward) and
5-GCTCTAGAAGGCTGGTGATTGCTCG -3 (reverse) using PCR, cut with Bam HI and Xba I and inserted into Bam HI and Xba I restriction sites of the pcDNA3.1-HisA epitope tagged eukaryotic expression vector (Invitrogen, Carlsbad Ca). The CMV promoter was replaced in the pcDNA3.1-HisA vector with the hTERT promoter (Tanyi et al, 2002) using Bgl II and Kpn I. The validity of all PCR products was confirmed by sequencing and of the vectors by restriction endonuclease analyses.

To create the LPP-3 expressing eukaryotic expression vectors, four different constructs were assessed. The CMV promoter driven pcDNA3.1-HisA vector, which adds an in frame NH_2-terminal HisA and V5 epitope tag, and pcDNA3.1-HisA-LacZ constructs were from Invitrogen (Carlsbad, CA). The human LPP-3 complete cDNA expression vector and an inactive mutant have been previously described (Roberts et al, 1998, Sciorra et al, 1999). To inactivate hLPP3, the critical histidine in the catalytic domain was mutated to proline (from CAC to CCA at position 561-563 of the cDNA).

This has been shown to inactivate LPP-1 (Roberts et al, 2000) and to block the activity of LPP-3 expressed in HEK 293 (not presented). The complete cDNA of LPP-3 was amplified using primers with the sequences:
5-CGCGGATCCATGCAAAACTACAAGTA -3 (forward) and
5-GCTCTAGACATCATGTTGTGGTGAT-3 (reverse) using polymerase chain reaction (PCR). Bam HI (site-921 in pcDNA3.1-HisA vector) and Xba I (-983) restriction sides were built into the primer pairs allowing the cDNA to be ligated into the pcDNA3.1-HisA epitope tagged eukaryotic expression vectors. The CMV promoter was deleted, and the hTERT378 promoter was inserted into the pcDNA3.1-HisA expression vector with Bgl II (-13) and Kpn I (-913). The validity of the expression constructs was confirmed by sequencing and restriction endonuclease analyses.

4.18. Transient and stable expression of LPP-1 and LPP-3

SKOV3 or OVCAR-3 cells were plated in 60-mm plastic dishes at a density of $0.5x10^6$ cells/dish. After 48 hours, when they were approximately 60-80 % confluent, cells were washed twice in PBS (phosphate-buffered saline - pH 7.4)(Sigma, St. Louis, MO). Cell transfection was carried out using FuGene 6 carrier (Roche Molecular Biochemicals, Indianapolis, USA) according to the manufacturer's instructions with minor modifications. Briefly, 10 µl FuGene 6 was added into 100 µl serum free medium and incubated 10 minutes at room temperature. 1.5 µg of expression vector was mixed and incubated for an additional 30 minutes at room temperature. Co-transfection with 0.5 µg of green fluorescent protein expressing vector (pGFP) was performed as a control for transfection efficiency. This mixture was applied into the 60-mm plate containing 3 ml of medium. Transiently transfected cells were used 48 hours later. Stably transfected SKOV3 and SKOV3 IP_1 cell lines were developed by continuous culture in G418. Despite multiple attempts with OVCAR-3, HEY and A 2780 as well as IOSE 29 and 80, the ability to generate LPP-3 expressing stable lines was not successful.

Quantitation of transfection efficiency was done by flow cytometry using a FACScan with CELLQUEST 3.3 software package (Becton-Dickinson, San Jose, California). Green fluorescent protein (GFP) was identified using a single parameter histogram display of log Green Fluorescent. Cells transfected with an empty vector were used as a negative control to develop a gate to determine the percent of GFP positive transfected cells. The transfection efficiency varied from 4-21 %. To create stable lines, 48 hours after transient transfection, medium containing the appropriate amount of G418 (selection medium) was changed every third day for 6-8 weeks.

4.19. Colony Formation Assay

The colony formation assay was performed as described previously with slight modifications with both transiently and stably transfected cell lines (Gerwin et al, 1992; Yu et al, 1999). Two days after transient transfection of the SKOV3 or OVCAR-3 ovarian carcinoma cell lines or the MCF-7 breast carcinoma line, cells were trypsinized, washed in PBS twice, and counted. $3x10^4$ cells were seeded in 30-mm 6 well plates. $2x10^3$ stably transfected SKOV3 cells were seeded into 30-mm 6-well plates. Two weeks later, colonies were stained with 0.1 % Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA) in 30 % methanol and 10 % acetic acid. Colonies (greater than 500 cells or 1mm in diameter) were counted by two independent investigators and the variation was under 10 %. Where indicated, 100 nM OMPT was added at the initiation of culture.

4.20. GFP targeted cell cycle progression assay (CCPA) with transient transfection.

LPP-1 expressing vectors: Cells were transiently transfected using FuGene 6 when they were 60-80 % confluent. The parental lines were transfected with either pcDNA3.1-LacZ-HisA, pc-hTERT-DNA3.1-HisA, pcDNA3.1-LPP-1-His A or pchTERT-DNA3.1-LPP-1-HisA, constructs separately, and co-transfected with pGFP. 48 hours later, the medium was removed and the cells were washed twice in PBS and

trypsinized. Both floating and adherent cells were harvested and subjected to flow-cytometry. Cells were fixed with 0.25% paraformaldehyde (Poly-Scientific, Bay Shore, NY) in PBS solution followed by DNA staining with Propidium Iodide staining buffer (10 ug/ml PI, 0.1 % Tween20 and 100 μg/ml RNAse A in PBS). To assess cell cycle progression, a two-color cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CELLQUEST 3.3 software for acquisition and analysis. GFP positive cells were gated and analyzed for cellular DNA content. LPP-3 expressing vectors: OVCAR-3 and SKOV3 parental lines were transfected with either pc-DNA3.1-LacZ-HisA, pc-hTERT-DNA3.1-HisA, pcDNA3.1-LPP-3-His A or pc-hTERT-DNA3.1-LPP-3-HisA constructs and co-transfected with pGFP, as described above. Where indicated, pcDNA3-LPP-3 mutant vectors were assessed to determine the requirement for an intact catalytic activity in LPP-3. 48 hours later, the medium was removed and the cells were washed twice in PBS and trypsinized. Both floating and adherent cells were harvested and subjected to flow-cytometry as prescribed above. Where indicated, OMPT was added 24 hours before assessing cell cycle progression.

4.21. Analysis of bystander effects of LPP-1 and LPP-3 using transient and stable transfection

Two days after transient transfection (see above), OVCAR-3 and SKOV3 cells were trypsinized and counted. Transfected cells ($3x10^4$) were mixed at different ratios with their respective non-transfected parental cell line and were plated in 6 well plates. Stably transfected SKOV3 cells ($2x10^3$) were mixed with an equal number of respective non-transfected parental SKOV3 cells and plated into 30-mm dishes in the absence of G418. Two weeks later, colonies were stained and counted as described above.

4.22. Migration assays of LPP-1 over-expressing SKOV3 cells

Migration assays were performed using a transwell chamber membrane (8µm pore size; Biocoat, Becton Dickinson Labware, Franklin Lakes, NJ). LPA, 10µM, was added to the lower chambers. Cells were initially starved of serum. Cells ($5x10^4$) were added to the upper chamber and allowed to migrate for 24 h at 37 ºC. Cells that had not migrated were removed from the upper chamber with a cotton swab. The remaining cells were fixed and stained with crystal violet.

4.23. Semi-quantitative RT-PCR of LPP-3

The mRNA expression of LPP-3 was determined using semi-quantitative RT-PCR. Oligonucleotide primers were used for:
LPP-3 primers:
5`-CGCGGATCCATGCAAAACTACAAGTA and
5`-CGTGATGATCGCGAGGATGG (306 bp)
GAPDH primers:
5`-CCCATGGCAAATTCCATGGCACCG and
5`-GTCATGGATGACCTTGGCCAGGGG (344 bp).
RNA samples were treated with DNAse before the RT-PCR reaction following the manufacturer's instructions (DNA-free, DNAse Treatment and Removal Kit)(Ambion, Austin, Texas, USA). The RT-PCR reaction mixtures consisted of cDNA derived from 1 µg of total RNA, 0.4 µM of sense and antisense primers, 0.2 mM of dNTPs 0.5 U of either reverse transcriptase or Taq-DNA polymerase enzymes, 5 mM DTT-solution, 5 U RNAse inhibitor, 1.5 mM $MgCl_2$ in a final volume of 50 µl (Titan One Tube RT-PCR System)(Roche Molecular Biochemicals, Mannheim, Germany). The reverse transcriptase reaction was performed at 60 C° for 30 minutes followed by 35 cycles of PCR reaction carried out in a Thermal Cycler (Perkin-Elmer 480, Atlanta, GA). Each cycle of PCR consisted of 30 s of denaturation at 94 C°, 2 min of annealing at 54 C° and

1 min of extension at 72 C°. The PCR products were visualized by electrophoresis in a 2 % agarose gel with ethidium bromide.

4.24. Tumor Growth in Nude Mice

SKOV3 parental and SKOV3 hLPP-3 or SKOV3 IP_1 parental and SKOV3 IP_1-hLPP-3 stably transfected ovarian cancer cells ($4x10^6$ cells) were injected subcutaneously into the left and right thigh of 6-8 week old female nude mice (Harlan Laboratories, LTD, Indianapolis, Indiana). Tumor sizes (in width and length) were measured and tumor volumes (mm^3) were estimated according to the formula: Tumor volume = (long dimension) x (short dimension)^2/2 (Pang 2000). On day 50 (SKOV3) or day 26 (SKOV3 IP_1), the mice were euthanized and autopsy was carried out.

Intraperitoneal growth was assessed by injection of $1x10^7$ cells of SKOV 3 parental and SKOV3 hLPP-3 or SKOV3 IP_1 parental and SKOV3 IP_1-hLPP-3. Abdominal circumference and body weight were assessed every second day after day 9 (SKOV3 IP_1) or day 21 (SKOV3). The mice were euthanized on day 41 (SKOV3) and day 30 (SKOV3 IP_1) after injection. At the time of euthanasia, autopsy was performed. Animals with any detectable tumor were counted. Animals free of detectable tumor were excluded from the analysis.

4.25. Evaluation of Autotaxin/LysoPLD effect on ovarian cancer cells

The autotoxin (ATX)/Lysophopholipase D (LysoPLD) cDNA containing eukaryotic expression vector was provided by Junken Aoki and Makiko Umezu-Goto (Umezo Goto 2002). The effect of ATX/lysoPLD was evaluated on LPP-3 overexpressing cells after transient transfection with ATX/lysoPLD expressing vector. Two days after transfection, the cells were trypsinized, washed twice in PBS, and counted. Cells ($3X10^4$) were seeded into a 30-mm, six well plates. Selection medium supplemented with G418 was changed every third day. Two weeks later, colonies were

stained with 0.1 % Coomassie blue (Serva, Heidelberg, Germany) in 30 % methanol and 10 % acetic acid. The effect of ATX/lysoPLD on the rate of cellular apoptosis induced by LPP-3 was also evaluated. Forty-eight hours after transient transfection with LPP-3 and a green fluorescent protein containing vector, cells were harvested and fixed with 0.25 % paraformaldehyde in PBS solution followed by propidium iodide (10 μg/ml) DNA staining. Two-color cytometric analysis was performed, and the percentages of hypodiploid cells as an indication of apoptosis were determined (CellQuest software; Becton Dickinson, Franklin Lakes, NJ).

4.26. Statistical analyses

Unpaired continuous outcomes were compared using Wilcoxon rank sum tests and exact permutation tests. Paired continuous outcomes were compared using Wilcoxon sign-rank tests. Proportions were compared using chi-square analyses. Longitudinal data were compared using repeated measures of analysis of variance. Significance was set at $P<0.05$.

5. RESULTS

5.1. The LPP-1 mRNA expression is decreased in ovarian cancer samples by Affymetrix microRNA database.

LPP-1 and human lysophospholipase homolog mRNA expression is decreased in ovarian carcinomas. Using Affymetrix array data, we were able to analyze levels of expression of a number of enzymes involved in hydrolysis of LPA in multiple tumor types (**Table I.**). LPP-1 mRNA levels in ovarian cancer samples were decreased approximately 5 fold as compared to normal epithelium. Further, LPP-1 RNA levels were lower in ovarian cancers as compared to other tumor lineages. For example, the levels of LPP-1 RNA in prostate and kidney samples were at least 10 fold higher than the levels in ovarian cancer cells. In contrast, LPP-2 and LPP-3 RNA levels were similar between ovarian cancer samples and normal epithelium. The overall total of LPP-1, -2 and –3 RNA levels were lower in ovarian cancer than in any other tumor type analyzed, compatible with decreased LPP and particularly LPP-1 levels contributing to the elevated LPA levels in ovarian cancer patients. The human lysophospholipase homolog (HU-K5) mRNA expression was decreased in ovarian cancers approximately 2.5 fold when compared with normal epithelium. LPAAT-β mRNA expression was decreased approximately 30% in ovarian cancer cells, whereas no significant changes were detectable in LPAAT-α or LPL1. We evaluated changes in LPPs and HU-K5 mRNA levels in individual patients by combining data from the published database with our own data using the same U95 Affymetrix arrays (**Fig. 2.**). This combined analysis of 53 ovarian cancer samples compared to 4 separate pools of normal epithelial cells scraped directly from the ovaries of 13 patients demonstrated that LPP-1 mRNA levels were markedly decreased in all samples, and in many cases, LPP-1 levels were decreased more than 10 fold. LPP-1 expression was decreased at least 2 fold in 89 % of the tumors as compared to normal epithelium. When LPP-2 and LPP-3 mRNA levels were considered, 2/3 of patients showed some decrease in levels (>30%).

Table I. Analysis of the DNA array data

[a]Values at the medians (range) of expressed DNA array results in each tumor type in Su et al, 2001, and Welsh et al, 2001a-b. (http://www.gnf.org/cancer/epican)

Enzyme	Gene Bank Number	Averages									Ratios (%)		
		Normal epithelium	Ovarian[a] (n=27)	Breast[a] (n=25)	Lung[a] (n=27)	Colon[a] (n=23)	Prostate[a] (n=26)	Kidney[a] (n=11)	Pancreas[a] (n=6)	Bladder[a] (=8)	Ovary/ Normal	Breast/ Normal	Lung/ Normal
LPP-1	AF0 14402	388	91	163	199	119	2310	980	284	614	0.2	0.4	0.5
LPP-3	AFO 17786	634	663	404	686	311	1767	3009	612	782	1.0	0.6	1.1
LPP-2	AFO 35959	230	264	642	509	718	230	103	856	457	1.1	2.8	2.2
LPAATα	U 56417	490	812	773	632	757	595	725	653	623	1.7	1.6	1.3
LPAATβ	U 56418	544	359	481	411	953	399	640	473	347	0.7	0.9	0.8
HU-K5	U 67963	339	151	109	296	292	106	815	494	88	0.4	0.3	0.9
LPL1	AFO 81281	512	458	665	417	561	576	248	355	941	0.9	1.3	0.8

5.2. Semi-quantitative RT-PCR analysis of human ovarian carcinoma cell lines and primary ovarian tumor samples

Based on the microarray data, we decided to further evaluate LPP-1 mRNA expression levels in ovarian cancer cells as compared to normal epithelium. We characterized the expression of LPP-1 in 20 primary ovarian tumor samples, all with greater than 80% tumor cell content, 6 normal surface epithelium samples, one normal surface epithelial short term culture (NOE), and 5 ovarian carcinoma cell lines, including OVCAR-3, SKOV3, SKOV3-IP_1, HEY, and A 2780. As indicated in **Figure 3.**, LPP-1 levels in normal ovarian epithelium from multiple individuals were tightly clustered. All ovarian tumors samples and ovarian cancer cell lines were lower than the average of the NOE. Using the lowest normal ovarian epithelial sample as a cut-off, 16/20 ovarian cancer samples and 4/5 ovarian cancer cell lines demonstrated decreased LPP-1 RNA levels, with some of the levels being markedly decreased. These data suggested the possibility that decreased LPP-1 levels contribute to the increased LPA levels in ascites of ovarian cancer patients and to the increased levels of LPA in ovarian cancer cell supernatants.

5.3. LPP-1 activity is decreased in ovarian cancer cell lines

As indicated in **Figure 4.**, HEY, OVCAR-3 and SKOV3 demonstrated lower total LPP activity, representing the activity of all of the LPP isoforms, than the non-tumorigenic ovarian surface epithelial cell line (IOSE). When LPP-1 was immunoprecipitated with LPP-1 specific antibodies, approximately 10% of the LPP-1 activity present in IOSE was accounted for by LPP-1. Strikingly, HEY, OVCAR-3 and SKOV3 demonstrated markedly lower levels of LPP-1 activity than did IOSE. Indeed, the difference in LPP-1 activity between IOSE and the tumor cells was much greater than that for total LPP activity, compatible with the contention that LPP-1 activity is electively

decreased in ovarian cancer cells. The LPP-1 activity correlated with that of the mRNA levels with SKOV3 having the lowest levels in both assays.

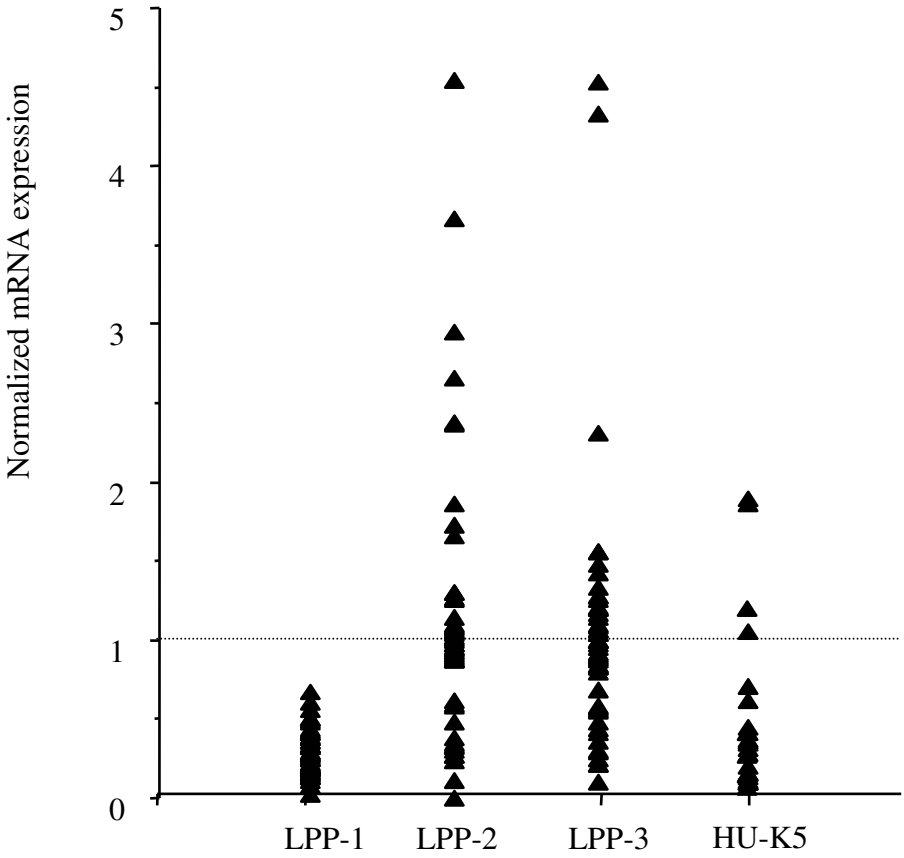


Figure 2. Human LPP-1, LPP-2, LPP-3 and HU-K5 gene expression in ovarian cancer. The normalized values of hybridization signal of LPP-1, -2, -3 and HU-K5 transcripts from 53 ovarian carcinoma samples (see Materials and Methods). The average of the expression of the transcripts in the normal epithelial tissues normalized to 1 is shown for comparison as a horizontal dashed line.

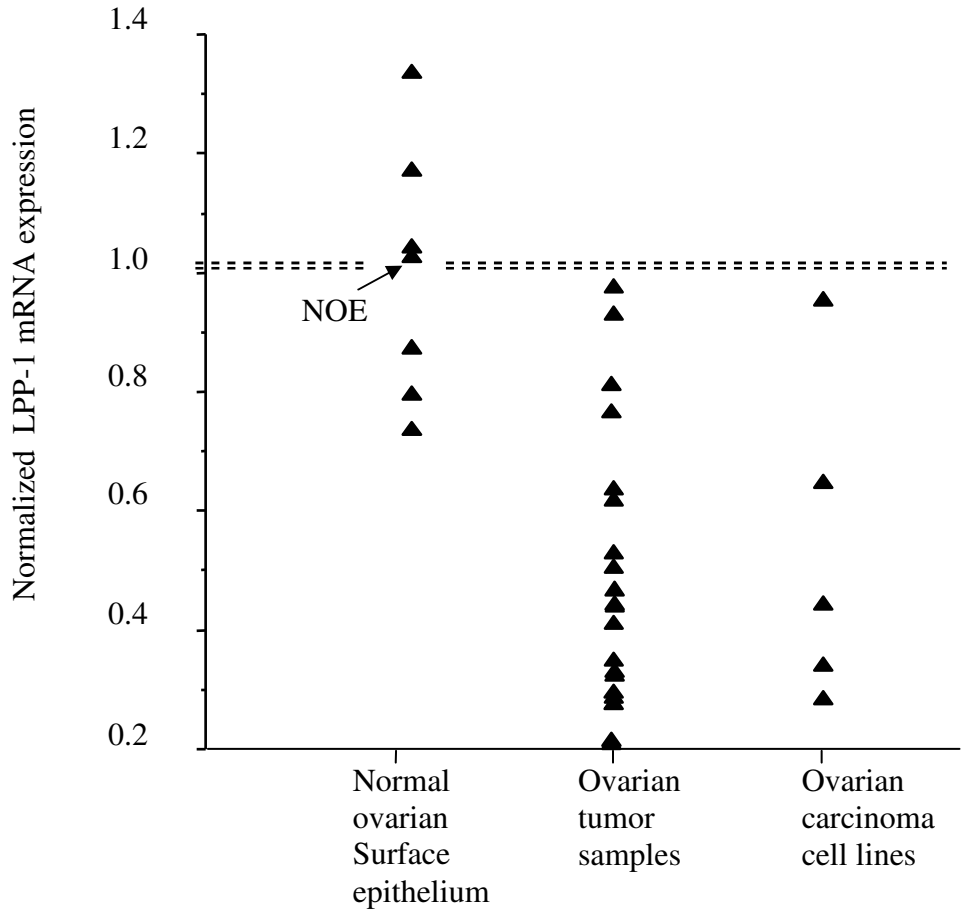


Figure 3. Decreased LPP-1 mRNA expression in ovarian carcinoma cell lines and tissue samples. The mRNA expression data of normal epithelium and malignant ovarian tissue samples and cell lines after normalization. (NOE: normal epithelial cell line.) The average of the expression of LPP-1 in normal epithelial tissues normalized to 1 is shown for comparison as a horizontal double line. RT-PCR reaction and normalization are described in Methods.

Ratio LPP-1 IP/ Total LPP activity
IOSE 0.082143

HEY 0.048837

OVCAR 0.033333

SKOV3 0.037838

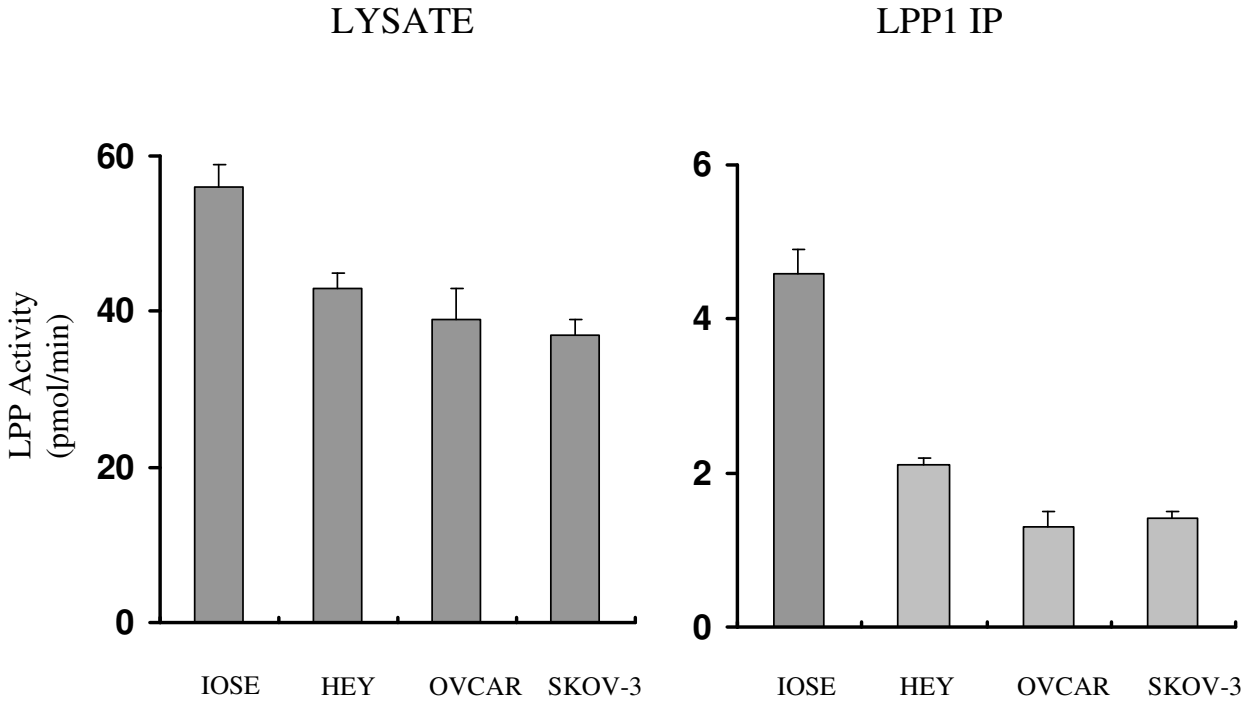


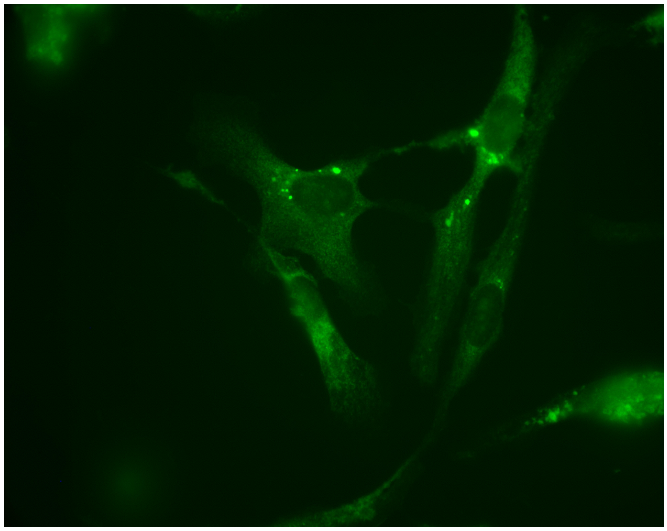
Figure 4. Determination of LPP activity in Ovarian Cancer Cell Lysates and LPP-1 Immune precipitates. Data normalized to 1 μg protein in lysates. The data shown are means +/- SD of triplicate determinations from a single representative experiment.

5.4. Functionally competent expression of LPP-3 in SKOV-3 cancer cell line

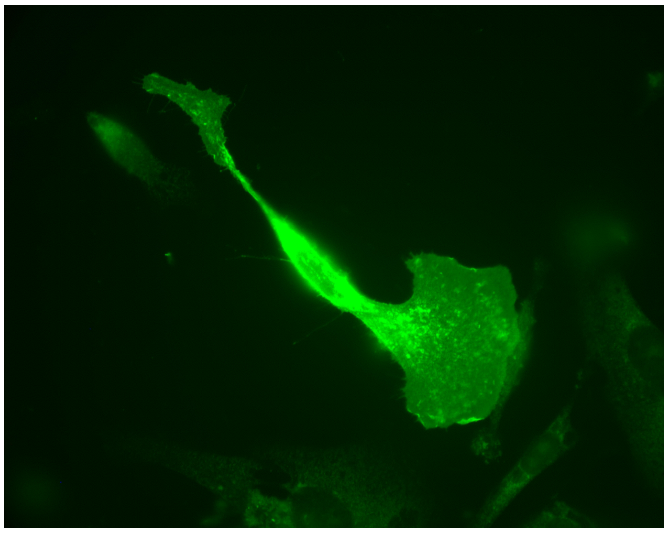
To characterize the effect of LPP-s on the growth of ovarian cancer cells, we attempted to establish stable cell lines. We were able to establish stable LPP-3 expressing SKOV3 and SKOV3 IP_1 cell lines. Ovarian cancer cell lines and normal control epithelial cells were transiently and stably transfected with LPP-1- and LPP-3-expressing vectors, and enzyme overexpression was confirmed by semiquantitative reverse transcriptionase polymerase chain reaction, immunoprecipitation, Western blotting and immunofluorescence (**Picture 1.**).

The cell lines contained modestly increased amounts of LPP-3 as assessed by RT-PCR and western blotting (**Fig. 5.A and 5.B**). There is selective over expression of the higher molecular weight species of LPP-3 in the stable cell line, which corresponds to the mature plasma membrane localized form of the enzyme (Sciorra et al, 1999). The expression of LPP-3 on the cell surface was confirmed by analysis of the ability of transfected cells to hydrolyze radiolabeled LPA added to the media. As indicated in **Figure 5.C**, transfected cells demonstrate increased ability to hydrolyze radiolabeled LPA in the media compared to the parental line (P=0.03). This was reflected in an increased rate and magnitude of LPA hydrolysis. As indicated above, expression of LPP3 results in increased rates of LPA hydrolysis and LPA concentrations in media.

Parental SKOV3 ovarian cancer cells



LPP-3 enzyme-overexpressing stably transfected SKOV-3 cells



Picture 1. LPP-3 overexpression in the stably transfected SKOV3 ovarian carcinoma cell lines. The parental SKOV3 cells and the LPP-3 enzyme-overexpressing stably transfected SKOV3 cells were fixed and stained with antibodies against LPP-3. Immunoreactive proteins were visualized using a fluorescein-conjugated secondary antibody as described be Smyth et al, 2003. and Tanyi et al, 2006.

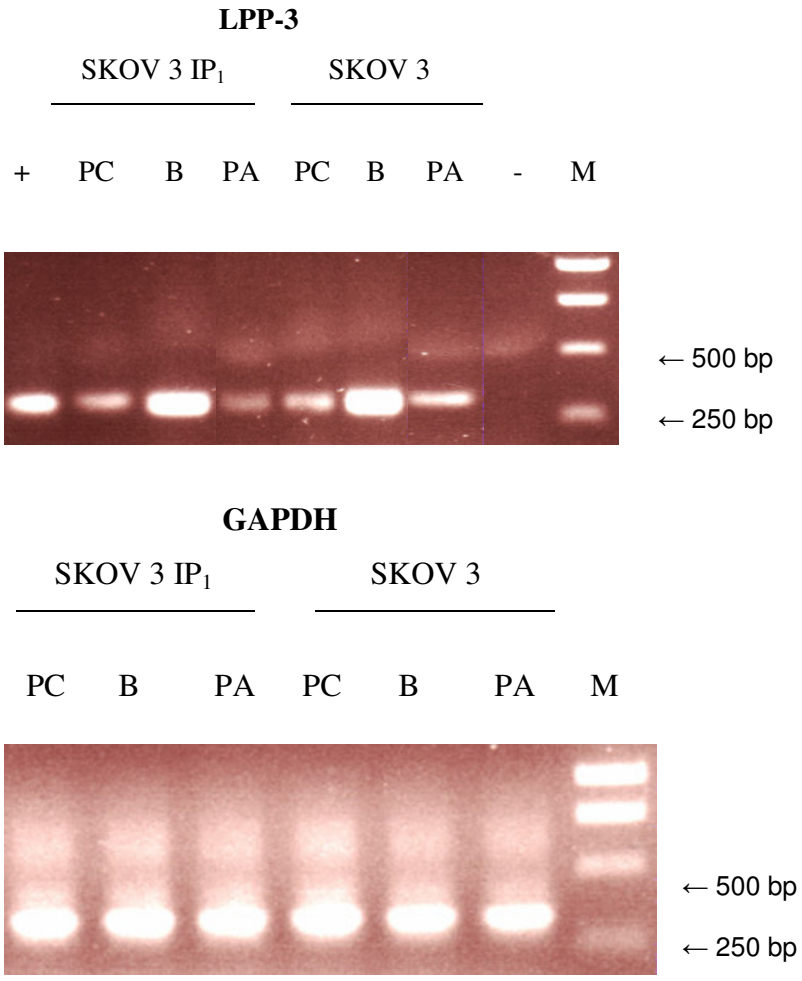


Figure 5.A. LPP-3 expressed in SKOV3 in functionally competent: Expression of LPP-3 assessed by RT-PCR is shown in parental and stably transfected cell lines. GAPDH expression as a control was checked in the parental and stably transfected cell lines. #B# represents cells stably transfected with LPP-3. #PC# represents the cell lines stably transfected with the effectorless pcDNA3.1-His-A vector. #PA# represents the untransfected original parental line. PCR products created with the same primer pairs and LPP-3 cDNA was used as positive control. The lanes have been reorganized to remove extraneous data. The positions of the size markers (base pairs) are shown in the lane labeled "M". Results are from one of three repeats.

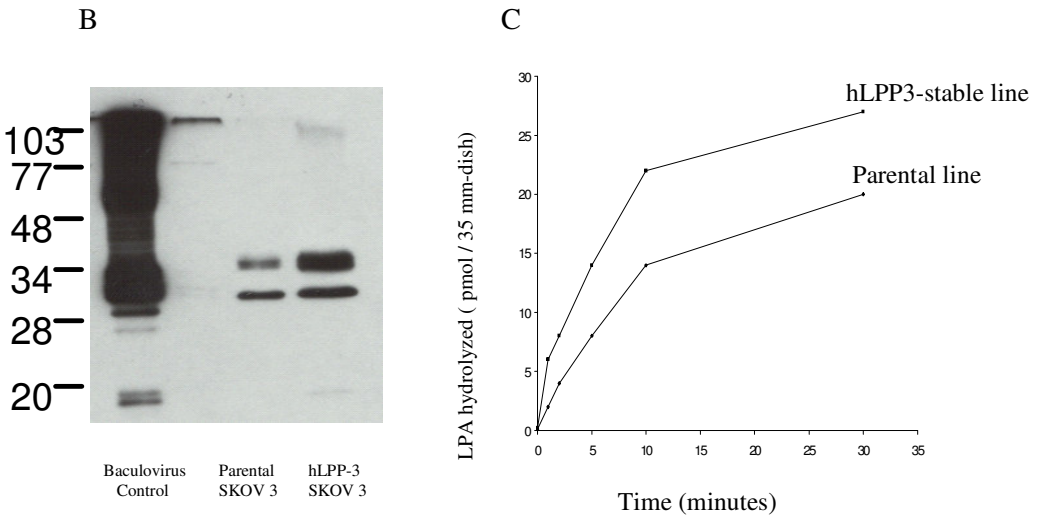


Figure. 5.B.: LPP-3 expressed in SKOV3 in functionally competent : Proteins from cell transfected with pcDNA3.1-LPP-3 were separated by SDS-polyacrylamide gel electrophoresis on a 10 % gel and analyzed by Western blotting as described in Methods. Sizes of prestained markers are shown. **C:** SKOV3 parental and LPP-3 stably transfected cells were harvested, washed, and resuspended in OPTI-MEM medium as described in Methods. LPP activity was determined using LPA as substrate for hydrolysis in samples of cells that had been disrupted by sonication. The data shown are means $\pm$ S.D. of triplicate determinations and are representative of three separate experiments

5.5. Evaluation of the ERK activation and intracellular calcium mobilization in LPP-3-transfected cells compared to non-transfected cells.

To determine whether this resulted in functional consequences, we assessed the effect of expression of LPP3 on LPA-induced phosphorylation of erk kinases, a sensitive indicator of LPA signaling. As indicated in **Figure 5. D-E**, expression of LPP3 resulted in a decrease in maximal levels of erk phosphorylation, which was associated with a rapid decrease in erk phosphorylation levels. At later time points (2-3 hrs), erk phosphorylation returned to baseline in both parental and transfected SKOV3 cells, compatible with the decrease in LPA levels to undetectable levels at late times as described above in both cell lines. Erk activation is based on LPA receptor activation, but we wanted to know whether LPP-3 overexpression had any effect on LPA receptor binding. Once LPA binds the receptor, it may be inactivated and internalized within a few minutes. The intracellular calcium mobilization of the parental line was compared with that of the LPP-3-overexpressing ovarian tumor cell line, and no difference was observed (**Fig. 5.F**.). This indicates that the ability of LPA to bind to the receptor is independent from the presence of the LPP-3 enzyme on the surface, and the activity is due to LPA hydrolysis rather than an effect on receptor function.

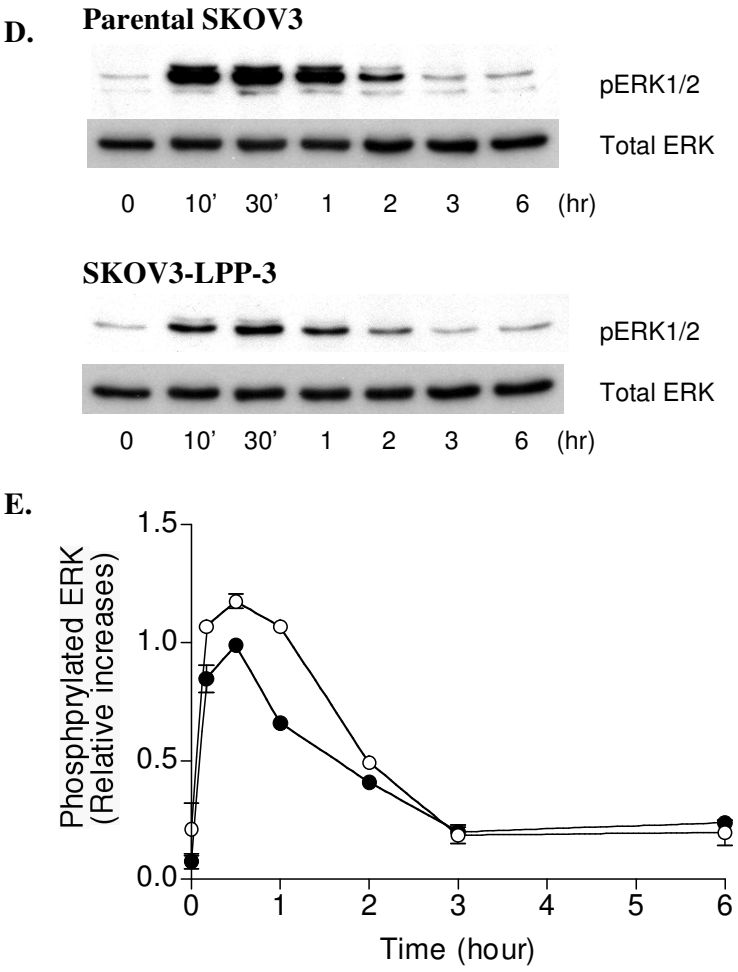


Figure 5. D-E. LPP-3 expressed in SKOV3 in functionally competent. **D:** After over-night starvation, SKOV3 and LPP3 transfected SKOV3 were incubated with LPA (1μM). Cell lysates were prepared in each time point and Western blotting was performed with anti-phospho-ERK1/2 and anti-ERK2 antibodies. **E:** Relative increases were determined using NIH Image 1.62. The solid circles represent LPP-3 transfected SKOV3 and empty circles the parental SKOV3 cell line. The data represents the mean ± S.E.M. from three independent experiments.

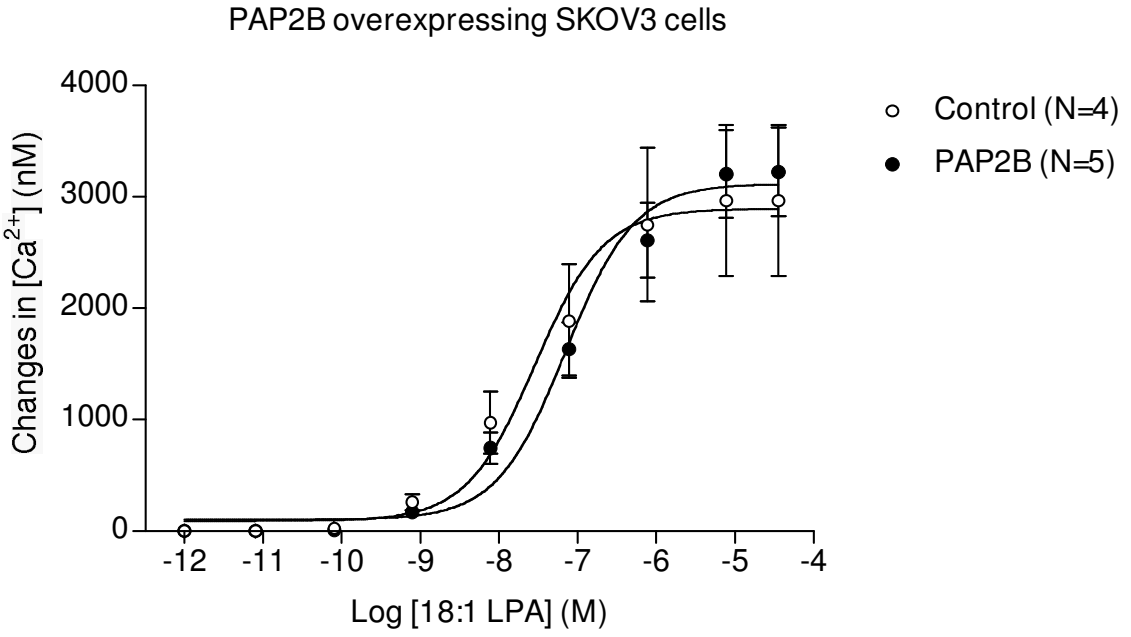


Figure 5.F. LPA induces intracellular calcium mobilization independently of LPP-3 (PAP2B) expression. Parental and LPP-3-overexpressing SKOV3 cell lines were grown to subconfluence, starved in serum-free medium, and harvested for cytoplasmic $[Ca^{2+}]_I$ assay. Cytoplasmic $[Ca^{2+}]_I$ was determined at an excitation wavelength of 331 nm and an emission wavelength of 410 nm using a fluorescence spectrophotometer (F-4000, Hitachi, Tokyo, Japan). Approximately 3 x 10^6 cells were used for $[Ca^{2+}]_I$ determination in a stirred quartz cuvette and kept at 37 ºC as detailed by Hasegawa et al, 2003 and Tanyi et al, 2006.

5.6. LPP-1 and LPP-3 decreases colony-forming ability of carcinoma cells

LPA is a potent regulator of multiple activities in ovarian cancer cells. To evaluate the potential role of decreased LPP-1 levels in the pathophysiology of ovarian cancer, we assessed the effect of increasing LPP-1 levels on the ability of ovarian cancer cells to proliferate as indicated by colony forming cell activity, a sensitive method to determine effects of mediators on cellular function (Gerwin et al, 1992; Yu et al 1999).

We attempted to establish stable cell lines expressing LPP-1 in IOSE 29, IOSE 80, OVCAR-3, HEY, and A2780. Despite multiple attempts and a ready ability to develop neomycin resistant lines, we were unable to develop stable LPP-1 expressing cell lines based on these lines. This suggests that LPP-1 decreases the growth or survival of ovarian epithelial and ovarian cancer cell lines. It was published by Eder et al., that the SKOV3 cell line releases high levels of LPA into cell supernatants (Eder et al, 2000). Consistent with this observation, we were able to develop stable LPP-1 expressing cell lines in SKOV3 and in SKOV3 IP_1, a variant of SKOV3 selected for the ability to grow intraperitoneally. These cells demonstrated an approximately 4.9 fold increase in LPP-1 RNA levels as assessed by RT-PCR analysis (not presented). However, even on the SKOV3 background, LPP-1 mRNA levels and activity deceased during cell passage. Thus, for analysis of LPP-1 activity early passage transfected cells were used.

We examined the ability of stably transfected and parental SKOV3 cell lines to hydrolyze LPA presented in the medium. This assay reflects the activity of all of the LPP isoforms on the cell surface. The LPA hydrolysis activity of LPP-1 stably transfected SKOV3 was 7.7 times higher (32 ± 2.3 pmol LPA hydrolysed/30min/mg protein vs. 246 ± 4.3 pmol LPA hydrolysed/30min/mg protein) than in the non-transfected parental line. As indicated by immunoprecipitation of cell lysates with LPP-1 specific antibodies (Roberts et al, 1998), SKOV3 cell lysates demonstrated decreased LPP-1 enzyme activity (10 ± 1.1 vs. 23.1 ± 1.9 pmo;/30min/mg protein immunprecipitated) as compared to NOE. Stably transfected SKOV3 (late passage) demonstrated 27.0 ± 0.9 pmol/30 min/mg protein immunoprecipitated LPP-1. This activity data was compatible with the 4.9-fold increase in LPP-1 mRNA expression in the stable lines. Thus ectopic expression of LPP-1 in ovarian cancer cells results in a marked increase in the ability of cells to hydrolyze extracellular LPA.

The increased ability of LPP-1 transfected SKOV3 to hydrolyze radiolabeled LPA, suggested that LPP-1 transfection would decrease the concentration of LPA in the culture media. When 1μM of 18:1 LPA was added to media alone, there was no detectable change in LPA concentration over time (Time 0, 780 nM: 10 min, 740 nM: 1

hour, 845 nM: and 8 hours, 819 nM). SKOV3 induced hydrolysis of extracellular LPA cells, (Time 0, 727 nM: 10 min, 580 nM: 1 hour, 555 nM: and 8 hours, <100 nM [levels above 100nM were readily detectable in calibration curves]). The expression of LPP-1 in SKOV3 cells resulted in a marked increase in the hydrolysis of LPA (Time 0, 701 nM: 10 min, 633 nM: 1 hour, 364 nM: and 8 hours <100 nM), compatible with increased LPP-1 activity. Thus the extracellular concentration of LPA is markedly decreased by transfected of LPP-1.

As indicated by the ability to form colonies, stable expression of LPP-1 decreased the growth of the SKOV3 ovarian cancer cells approximately 1.9 fold (49$\pm$8.4 vs. 26$\pm$4.3 p=0.0002) (**Fig. 6.B.**). Due to the difficulty in obtaining high level LPP-1 expression in stable cells lines, we assessed the effect of transient transfection of LPP-1 on colony forming cell activity of SKOV3 and OVCAR-3 ovarian cancer cell lines. As indicated in **Figure 6.A**., transient expression of LPP-1 caused a marked growth inhibition in both OVCAR-3 and SKOV3. The inhibitory effected ranged from 2.3 fold to 5.8 fold when LPP-1 was driven by the CMV and hTERT promoters in the OVCAR-3 and SKOV3 cell lines (P= 0.0022, P= 0.0022, P= 0.0416, P=0.0022), respectively. Thus ectopic expression of LPP-1 strongly inhibits the ability of ovarian cancer cell lines to proliferate as indicated by colony forming cell activity in vitro.

We determined the effects of introduction of LPP-3, which degrades LPA, on the ability of ovarian cancer cells to form colonies (greater than 500 cells and greater than 1 mm in diameter). Following transient transfection of hLPP-3 driven by either the CMV or human telomerase promoter (both of which are highly active in ovarian cancer cells, (Tanyi et al, 2002), hLPP-3 markedly decreased the ability of both SKOV3 (7.3 fold (86%decrease) with hTERT-hLPP-3 and 7.1 fold (86% decrease) with CMV-hLPP-3) and OVCAR-3 (6.8 fold with (85% decrease) hTERT-hLPP-3 and 6.7 fold (85% decrease) with CMV-hLPP-3) ovarian cancer cells to form colonies (P=0.002) (**Fig. 7.A.**). As shown in **Figure 7.B.**, the ability of hLPP-3 to decrease colony forming activity of ovarian cancer cells was dependent on an intact catalytic activity, compatible with the

degradation of LPA, as transfection of mutant hLPP-3 lacking catalytic activity (Sciorra et al, 1999; Roberts et al, 2000) had no effect on colony forming ability.

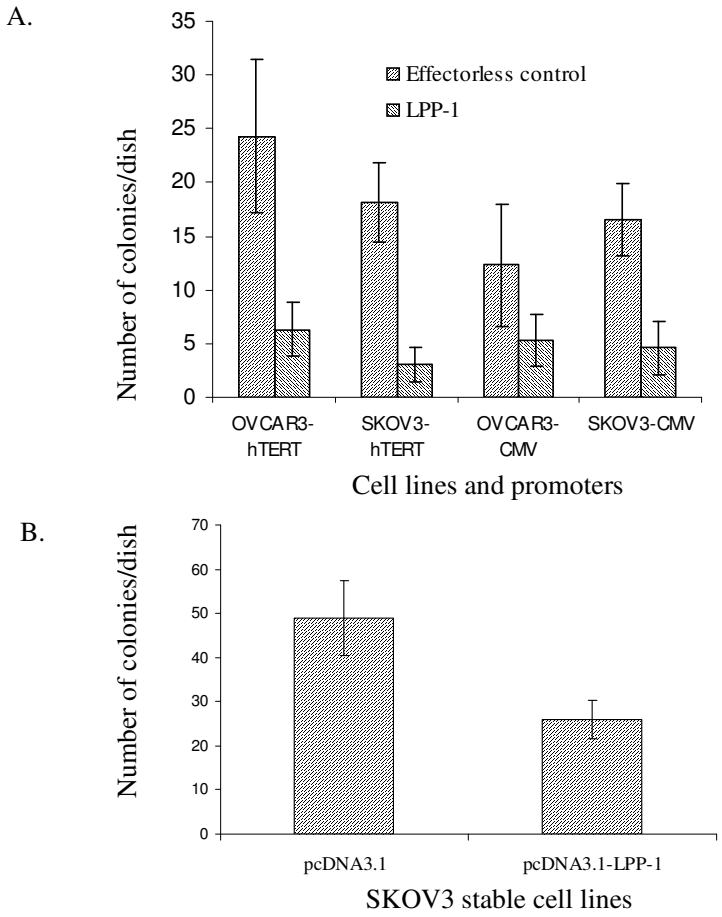


Figure 6. Growth inhibitory effect of LPP-1 overexpression assessed by colony forming cell activity. **A**, Two days after transient transfection, cells were trypsinized, washed in PBS twice and counted. 3x104 cells were seeded into 30-mm six well plates. The selection medium supplemented with G418 was changed every third day. Two weeks later, colonies were stained by 0.1 % Coomassie blue in 30 % methanol and 10 % acetic acid. Average number of colonies/dish is presented. **B**, Growth inhibition effect of LPP-1 in stably transfected SKOV3 cell line. 2x103 of cells from the stable SKOV3 line were seeded into 30-mm six well plates. Complete culture medium supplemented with G418 was changed every third day. Two weeks later, colonies were stained by 0.1 % Coomassie blue in 30 % methanol and 10 % acetic acid. The data are the mean of six separate experiments $\pm$ standard deviation.

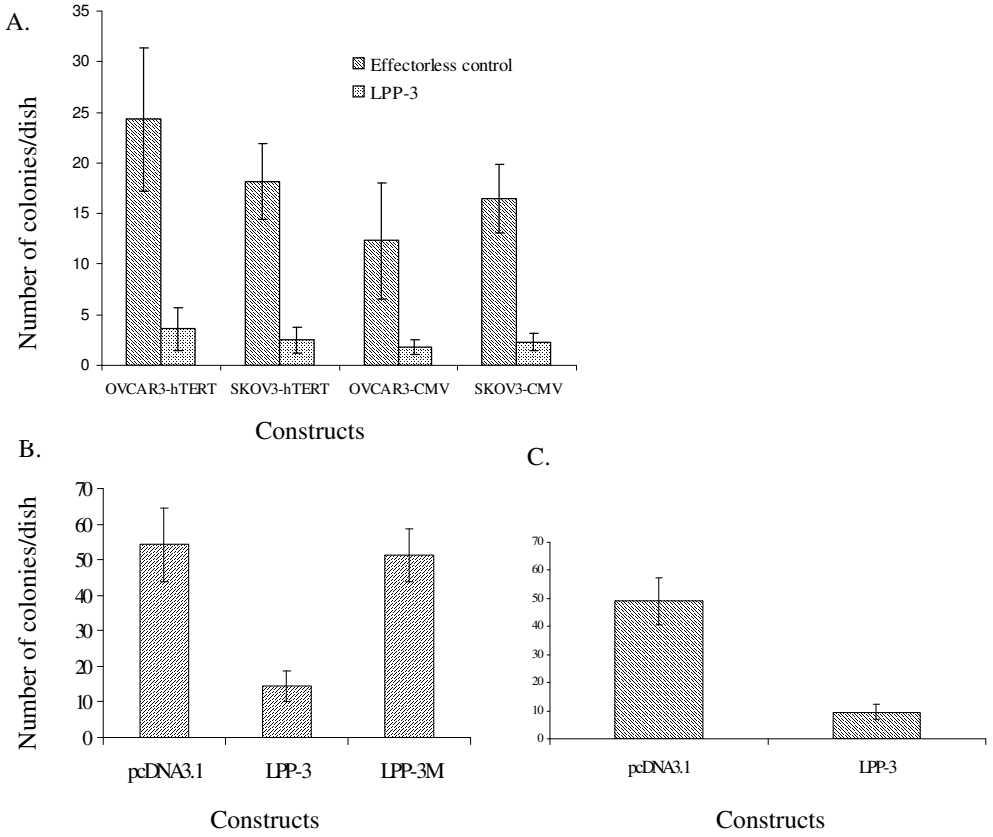


Figure 7. Growth inhibition following transfection of LPP-3. **A,** Two days after transient transfection, the cells were trypsinized, washed in PBS twice and counted. 3x104 cells were seeded into 30-mm six well plates. Selection medium supplemented with G418 was changed every third day. Two weeks later, colonies were stained with 0.1 % Coomassie blue in 30 % methanol and 10 % acetic acid. Average number of colonies/dish is presented. **B,** The experiment was repeated with a LPP-3 mutant (LPP-3m) construct. **C,** Growth inhibitor effect of the LPP-3 enzyme in stably transfected SKOV3 cell lines. 2x103 cells from stable LPP-3 transfected SKOV3 cells were seeded into 30-mm six well plates. The selection medium supplemented with G418, was changed every third day. Two weeks later, colonies were stained with 0.1 % Coomassie blue in 30 % methanol and 10 % acetic acid. The data represented three separate experiments, mean $\pm$ SE of six samples.

5.7. LPP-1 and LPP-3 markedly increases the apoptosis rate in ovarian carcinoma cell lines

The decreased ability of ovarian cancer cells to form colonies could either be due to decreased cell cycle progression or due to increased rates of apoptosis. There were no obvious differences in cell cycle progression as indicated by number of cells in G1, S or G2M in control or LPP-1 expressing SKOV3 or OVCAR-3 cells (**Fig. 8.**). However, as indicated by an accumulation of a hypodiploid peak on cell cycle analysis, LPP-1 expression increased the rate of apoptosis in both SKOV3 and OVCAR-3 (**Fig. 8.**). Thus the decreased ability of LPP-1 expressing ovarian cancer cells to form colonies is associated with an increased rate of cellular apoptosis. SKOV3 and OVCAR-3 cells were co-transfected with GFP (to mark transfected cells) and LPP-3 under either the CMV or hTERT promoters and assessed for cell cycle progression and apoptosis (hypodiploid peak) by staining with propidium iodide. There were no obvious differences in cell cycle progression as indicated by number of cells in G1, S or G2M (**Fig. 9. B.)** in control or LPP-3 expressing SKOV3 or OVCAR-3 cells. However, there was a significant and consistent increase in the hypodiploid peak in hLPP-3 expressing cells ($P<0.0001$) compatible with an increased rate of apoptosis (**Fig. 9. A.**). Thus the decreased ability of LPP-3 expressing ovarian cancer cells to form colonies is associated with an increased rate of cellular apoptosis. This experiment was repeated with the mutant, inactive LPP-3 using the same condition and as indicated in **Figure 10.** a mutant LPP-3, which is unable to hydrolyze LPA (Sciorra et al, 1999; Roberts et al, 2000), did not alter the apoptosis rate, confirming a need for intact enzyme activity in the effect of LPP-3 on cell death.

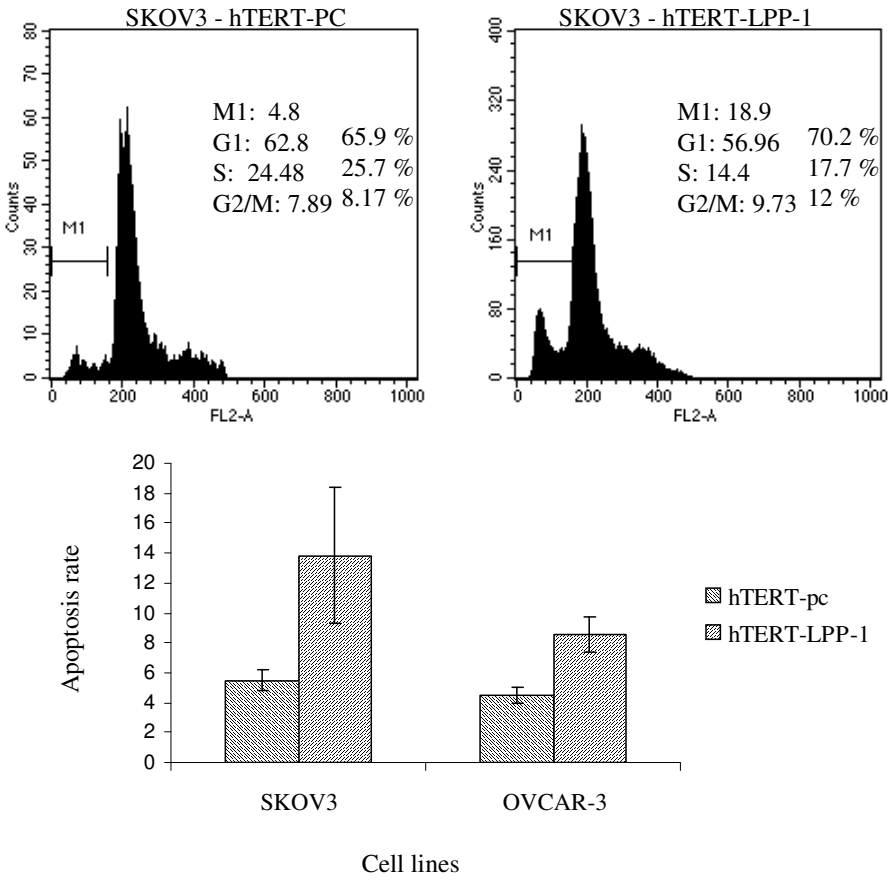


Figure 8. LPP-1 increases apoptosis in ovarian cancer cell lines. Forty-eight hours after transient transfection, cells were harvested and fixed by 0.25% paraformaldehyde in PBS solution followed by Propidium Iodide (10 ug/ml) for DNA staining. Two-color cytometric analysis was performed and the percentages of hypodiploid cells as an indication of apoptosis determined using CellQuest softwere. Primary flow cytometric graphs from SKOV3 cell line transfected with hTERT driven constructs are presented. M1 represents hypodiploid cells. Percentage of cells in G1, S and G2/M is presented with (right) and without (left) correction for hypodiploid cells. The data shown in the bar graph are the mean of three separate experiments $\pm$ standard deviation.

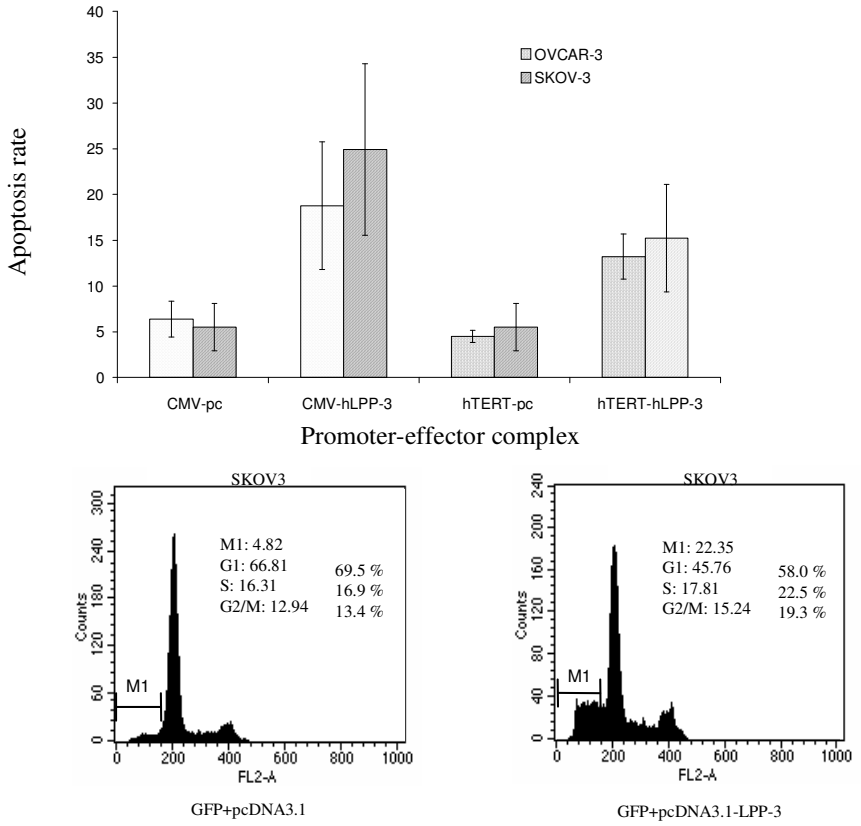


Figure 9. Transient transfection of hLPP-3 increases apoptosis. 48 hours after transient transfection with LPP and a GFP containing vector, cells were harvested and fixed by 0.25% paraformaldehyde in PBS solution followed by Propidium Iodide (10 μg/ml) DNA staining. Two-color cytometric analysis was performed and the percentages of hypodiploid cells as an indication of apoptosis determined using CellQuest software. Primary flow cytometric graphs from SKOV3 cell line transfected with CMV promoter driven constructs are presented. M1 represents hypodiploid cells. Percentage of cells in G1, S and G2/M is presented with (right) and without (left) correction for hypodiploid cells. The data shown are the mean $\pm$ SE of three separate experiments.

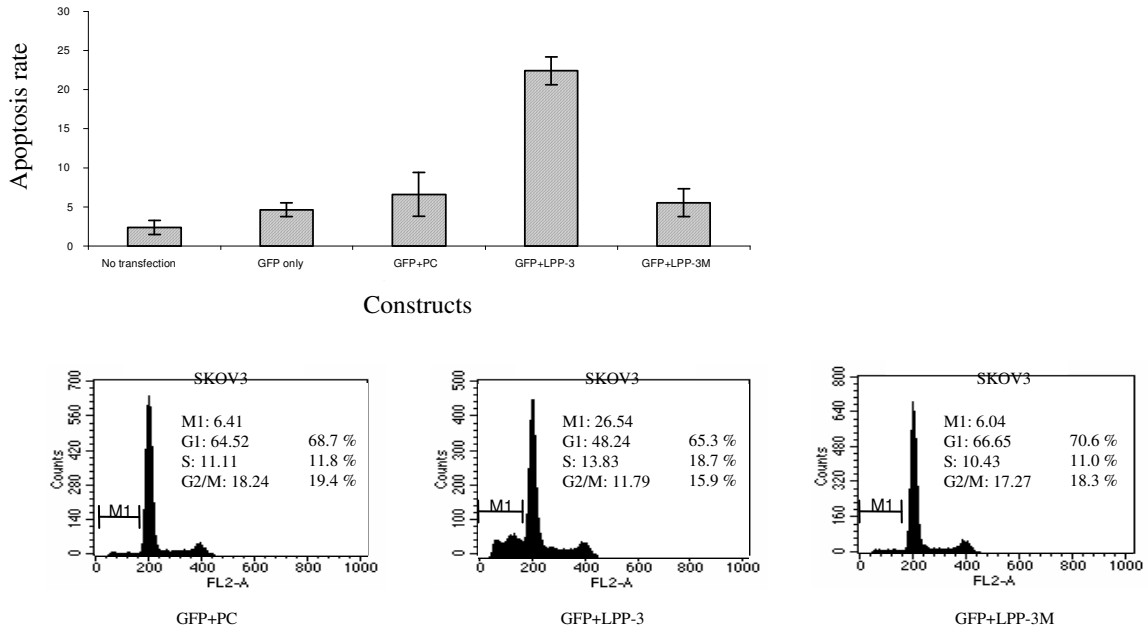


Figure 10. Transient transfection of LPP-3 increases apoptosis. Primary flow cytometric graphs from SKOV3 cell line transfected with CMV promoter driven constructs are presented. M1 represents hypodiploid cells. Percentage of cells in G1, S and G2/M is presented with (right) and without (left) correction for hypodiploid cells. The data shown are the mean $\pm$ SE of three separate experiments. The experiment was repeated with a mutated LPP-3 (LPP-3M, catalytically inactive) expression vector.

5.8. LPP-3 decreases growth of ovarian cancer cells through LPA hydrolysis

As indicated above, the catalytic activity of LPP3 is required for the ability to decrease cell growth and further the expression of LPP3 results in a decrease in extracellular LPA levels. To determine whether the effects of LPP-3 on the growth of ovarian cancer cells was due to hydrolysis of extracellular LPA, we assessed the ability of addition of exogenous LPA or a non-hydrolysable LPA analog, OMPT (Hasegawa et al, 2001), to reverse the effects of LPP-3 expression. As indicated earlier, LPA phosphatase activity was increased 4.4-fold by stable expression of LPP-3 resulting in considerable LPA hydrolytic activity. However, as indicated in **Figure 11.A.** and **11.B**. OMPT was able to substantially reverse the effects of LPP-3 on both colony forming activity and on apoptosis with an OMPT concentration of 100nM proving optimal in both assays. The ability of exogenous OMPT to reverse the effects of LPP-3 suggests that the major effect of LPP-3 on the growth of ovarian cancer cells was due to hydrolysis of extracellular LPA.

5.9. LPP-1 decreases the growth of non-transfected bystander cells

The ability of LPP-1 expression to increase the hydrolysis of extracellular LPA suggests that LPP-1 may function to decrease extracellular LPA concentrations. If this were the case, expression of LPP-1 in one population of ovarian cancer cells could decrease the colony forming cell activity of bystander non-transfected cells. To assess this possibility, we performed a series of cell mixing assays with LPP-1 transfected cells and control parental cells.

Our preliminary data indicated that the number of colonies formed demonstrated essentially a linear relationship related to the number of cells plated, i.e. there were no effects related to cell crowding at higher concentrations (not presented). To assess the

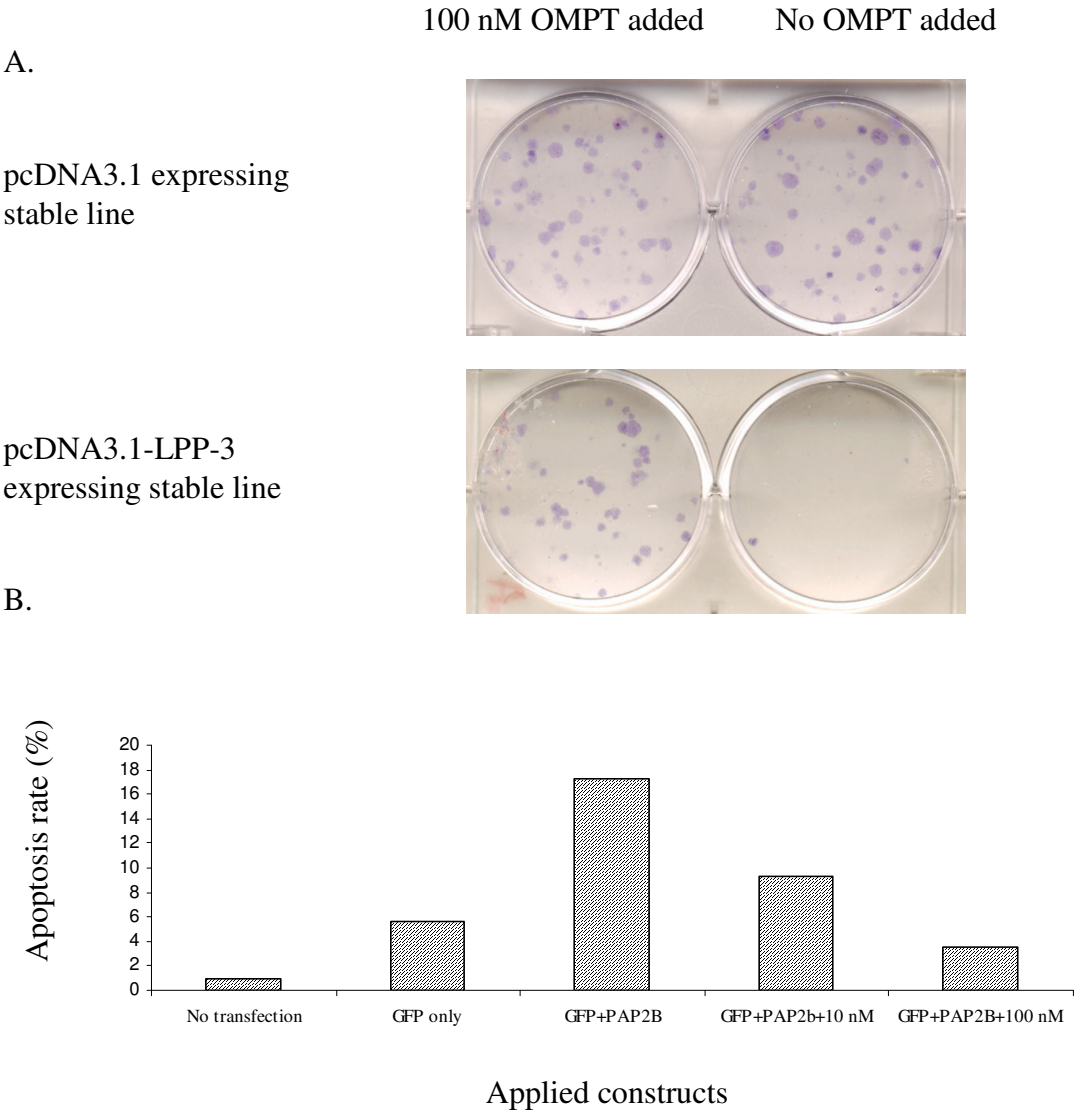


Figure 11. OMPT reverses the effects of LPP-3 (PAP2B). **A,** A representative picture of the effect of OMPT administration on colony forming activity of stably transfected SKOV3 cell lines. In the right 2 wells, the stable transfected lines were cultured without OMPT. In the left two wells, 100 nM OMPT was added to the selection medium every third day. **B,** Apoptosis rates were determined as described in Figure 8. with and without addition of the indicated concentration of OMPT.

effects of LPP-1 transfected cells on the growth of non-transfected cells, we combined equal numbers of stably or transiently LPP-1 transfected cells and parental cells. The predicted number of colonies in the combination experiment would be the sum of the number of colonies produced by parental cells and the number of colonies produced by the transfected cells cultured separately. As indicated in **Figure 12.A.** and **12.B.** with either transient transfection or stable cell lines, there was a marked decrease in the number of colonies observed compared to the expected number of colonies (P=0.01, P=0.01). Therefore, LPP-1 transfected cells were able to decrease the proliferation of parental cells compatible with the effect of LPP-1 being related to the effects of an extracellular mediator, likely LPA.

We evaluated the bystander effect with LPP-3 also. As indicated earlier, overexpression of LPP-3 results in decreased LPA levels in cellular supernatants. Thus by decreasing extracellular LPA, expression of LPP-3 in one population of ovarian cancer cells could decrease the colony forming cell activity of bystander non-transfected cells. This would be particularly important if LPP-3 were used in a "gene therapy" type of approach where it is difficult to transfect all cells. To assess this possibility, we performed a series of cell mixing assays with LPP-3 transfected cells and control parental cells.

We initially determined that the number of colonies formed demonstrated essentially a linear relationship related to the number of cells plated, i.e. there were no effects related to cell crowding at higher concentrations (not presented). As described previously (**Fig. 7.A. to 7.C.**), expression of LPP-3 either transiently or stably resulted in a marked decrease in colony forming cell activity. To assess the effects of LPP-3 transfected cells on the growth of non-transfected cells, we combined equal amounts of hLPP-3 expressing cells and parental cells. Thus the expected number of colonies in the combination experiment would be the number of colonies produced by parental cells plus the number of colonies produced by the transfected cells. As indicated in **Figure 13.A.** and **13.B.** with both transient transfection and with stable cell lines, there was a marked decrease in the number of colonies observed compared to the expected number of the

colonies (P=0.028, P=0.012). Therefore, LPP-3 transfected cells were able to decrease the proliferation of parental cells compatible with the effect of LPP-3 being related to the effects on an extracellular mediator, likely LPA.

5.10. LPP-1 overexpression decreases the ability of the tumor cells to migrate

LPA has been demonstrated to markedly increase cellular migration. We thus assessed whether overexpression of LPP-1 in ovarian cancer cells would also decrease LPA induced cellular migration. As indicated by the ability of cells to migrate towards LPA in a transwell assay (**Fig. 14.**), LPA (10μM) stimulated the migration of empty vector pcDNA3.1 transfected SKOV3 cells. In contrast, LPA failed to induce migration in LPP-1 over-expressing SKOV3 cells. Thus the increased hydrolysis of LPA of LPP-1 is translated into alterations in cellular motility as well as in proliferation, colony formation and survival, compatible with the decreases in LPP-1 in ovarian cancer cells contribution to these important components of the transformation cascade.

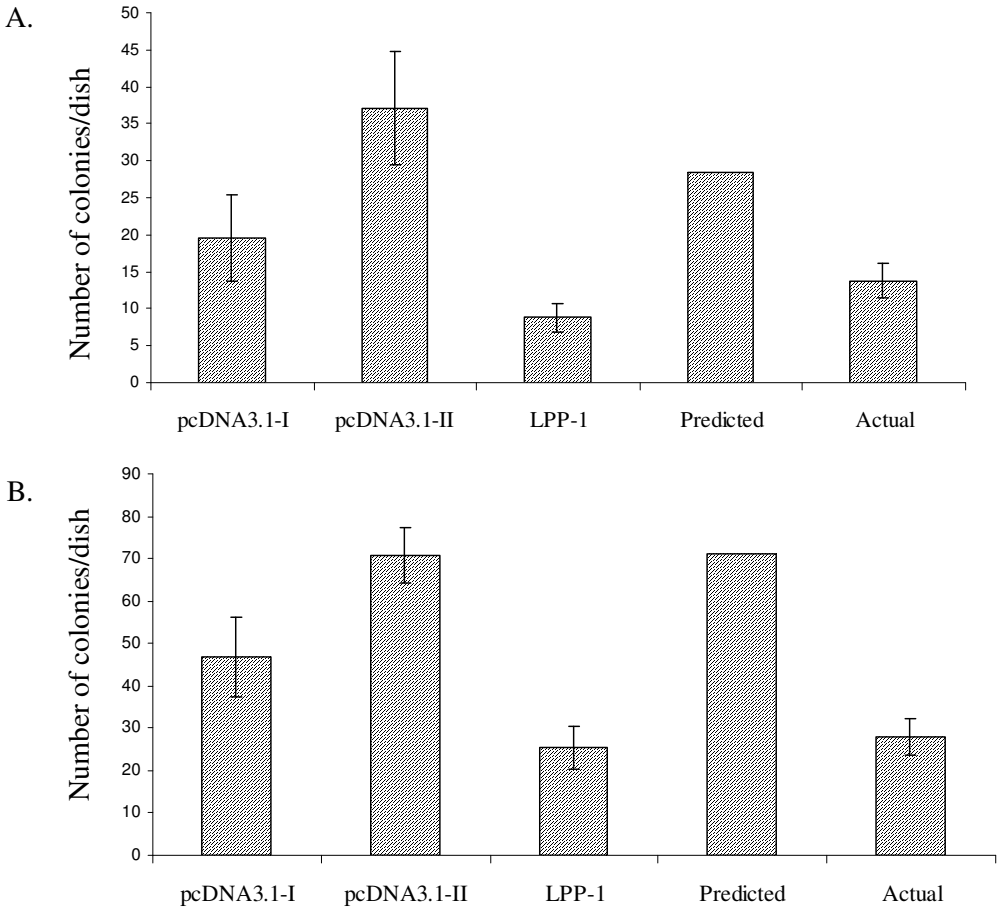


Figure 12. LPP-1 decreases the colony forming ability in bystander cells. PcDNA3.1 transfected control cells (I), or twice as many cells (II) or LPP-1 expressing cells (3x104 cells with transient, or 2x103 cells with stable transfection) were plated separately. To determine the bystander effect one unit of control cells (3x104 cells with transient or 2x103 cells with stable transfection) were mixed with equal amounts of LPP-1 expressing cells and two weeks later the number of colonies were counted. The predicted value presents the number of the colonies, which is the sum of the colonies when the control and LPP-1 expressing line were seeded separately. The actual value presents the end number of the colonies in the experiment when the control cells and LPP-1 expressing cells were plated together. **A:** The bystander effect of LPP-1 using transient transfection. (See Methods). **B:** Bystander effect of the LPP-1 enzyme on stably transfected SKOV 3 cells. The data are the mean $\pm$ SD of six independent experiments.

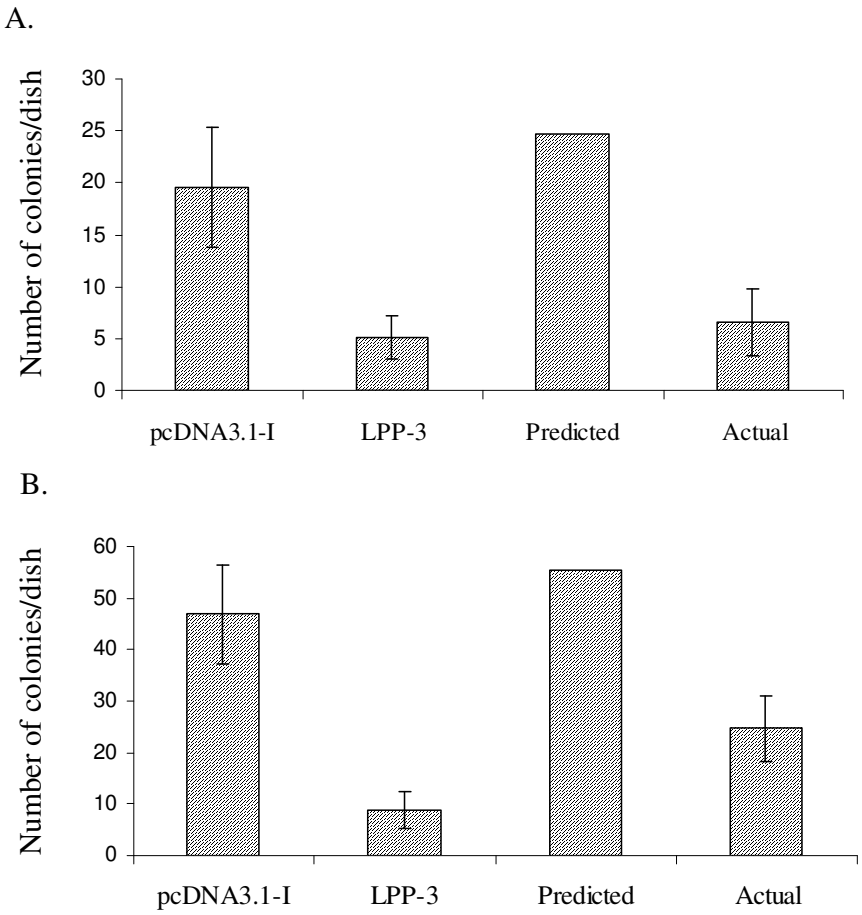


Figure 13. Bystander effect of LPP-3 on SKOV3 cells. The inhibition of bystander cell proliferation was assessed by combining transfected and non-transfected cells. As controls, pcDNA3.1 and LPP-3 transfected cells were plated separately. To determine bystander effects control cells were mixed with equal numbers of LPP-3 expressing cells and two weeks later the number of colonies was counted. The predicted value presents the sum of the colonies when the control and LPP-3 expressing line were seeded separately. The actual value presents the number of the colonies where the control cells and the LPP-3 expressing cells were plated together. **A:** The bystander effect of LPP-3 using transient transfection. Two days after transient transfection, cells where harvested and counted. Cells where plated as described in Material and methods. **B:** Bystander effect of LPP-3 on stably transfected SKOV3 cells. The studies were repeated with stably transfected cells. The results show the mean $\pm$ SD of six independent experiments.

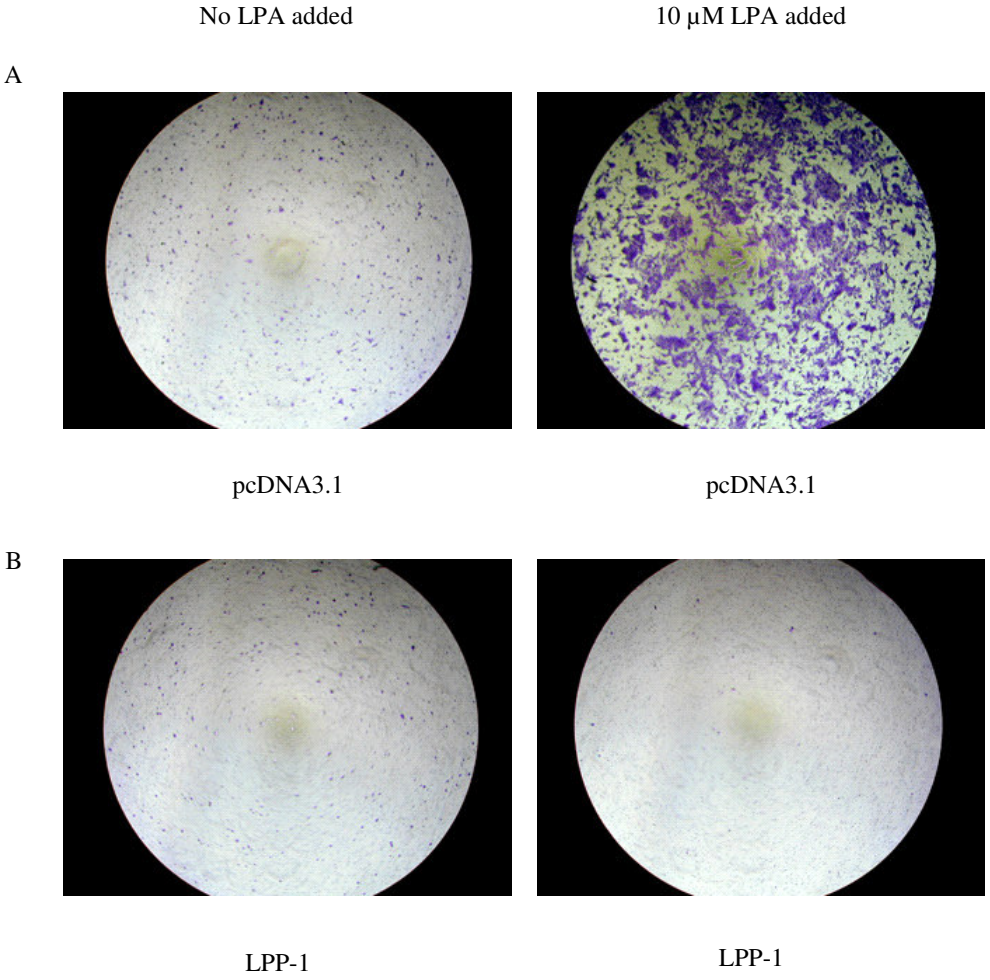


Figure 14. LPA-induced migration of ovarian cancer cell lines. **A:** represents the migration after LPA stimulation on the empty vector (pCDNA3.1) transfected cells, and **B:** the LPP-1 over-expressing cells. Left pictures show migrated cells without, while right pictures show with 10µM of LPA, respectively.

5.11. LPP-3 decreases tumor growth of ovarian cancer cells *in vivo*

Following subcutaneous injection of SKOV3 cells, tumors developed in 9/10 mice as compared to 4/10 mice injected with LPP-3 expressing SKOV3 cells. Following subcutaneous injection of SKOV3-IP_1 cells, a more aggressive SKOV3 subline developed by intraperitoneal passage of SKOV3 (Yu et al, 1993), tumors developed in 10/10 mice as compared to 5/10 mice injected with LPP-3 expressing SKOV3 IP_1 cells. Thus the take rate of the tumors expressing LPP-3 was markedly less than that of the parental lines. In addition to a decreased take rate, as indicated in **Figure 15.**, the growth rate of both the SKOV3 and SKOV3-IP_1 LPP-3 expressing tumors was markedly decreased as compared to the parental cell lines (note: only mice that developed tumors are used to derive the growth curves) (P value for both SKOV3 and SKOV3-IP_1<0.0001, **Fig. 15.A. and 15.B.).** At the termination of the study (mandated by tumor size and AALAC guidelines), the average SKOV3 parental tumor was 0.48$\pm$0.15 g in weight while the hLPP-3 expressing SKOV3 tumors averaged 0.13$\pm$0.04 g in weight. Similarly, with the SKOV3 IP_1 parental tumor the average weight was 0.44$\pm$0.11 g while LPP-3 expressing tumors had an average weight of 0.12$\pm$0.02 g. Thus with both tumor lines, for the animals that developed tumors the average tumor weight was 4 times greater in the parental than in the LPP-3 expressing tumors.

When SKOV3 or SKOV3-IP_1 cells were injected into the orthotopic site in the peritoneal cavity, LPP-3 expression also decreased the take rate (1 of 5 from SKOV3 LPP-3 injected and 3 of 5 from SKOV3 IP_1-LPP-3 injected mice with all parental cell injections (10/10) resulting in tumor growth). With both SKOV3 and SKOV3 IP_1 cells, LPP-3 expression decreased intraperitoneal growth as assessed by both animal weight (P=0.005, P<0.0001) and abdominal circumference (P=0.042, P=0.001) (**Fig. 15. C-F.**).
As indicated above, LPP-3 resulted in a marked decrease in take rates and in those cases where tumors formed, a decrease in growth rates. However, after a delay in growth, the LPP-3 expressing tumors appeared to enter a more rapid growth phase. As the LPP-3 construct was not under selective pressure *in vivo*, it was possible that the eventual

increase in growth rate was due to loss of LPP-3. As indicated in **Figure 16.** this was indeed the case. Following *in vivo* growth, LPP-3 levels in the transfected lines were markedly decreased. Even more striking, however, following *in vivo* growth, LPP-3 levels were markedly decreased in the parental cell lines. It thus appears that *in vivo* growth of SKOV3 cells is associated with a down regulation of expression of both endogenous LPP-3 and transfected LPP-3. This suggests that a very strong negative selection exists against hLPP3 expression in ovarian cancer cells *in vivo*.

5.12. ATX/LysoPLD oppose the effct of LPPs

The level of LPA in the ascites of ovarian cancer patient depends on the rates of production and catabolism. As LPPs decreases and ATX/lysoPLD increase the LPA level these two enzyme groups appear to be the major contributors to this process. This suggests that overexpression of ATX/lysoPLD could potentially increase LPA production and overcome the effect of LPP-3 activity. We designed an experiment in which ovarian cancer cells were transiently transfected with ATX/lysoPLD and/or LPP-3 expressing eukaryotic expression vectors. Colony forming cell assay was done and as seen on **Figure 17.** and **18.** ATX/LysoPLD reversed the growth inhibitory and apoptosis-inducing effect of LPP-3 overexpression in ovarian carcinoma cell lines. This clearly suggests that high level of LPA is able to overrule the effect of even increased LPP-3 expression.

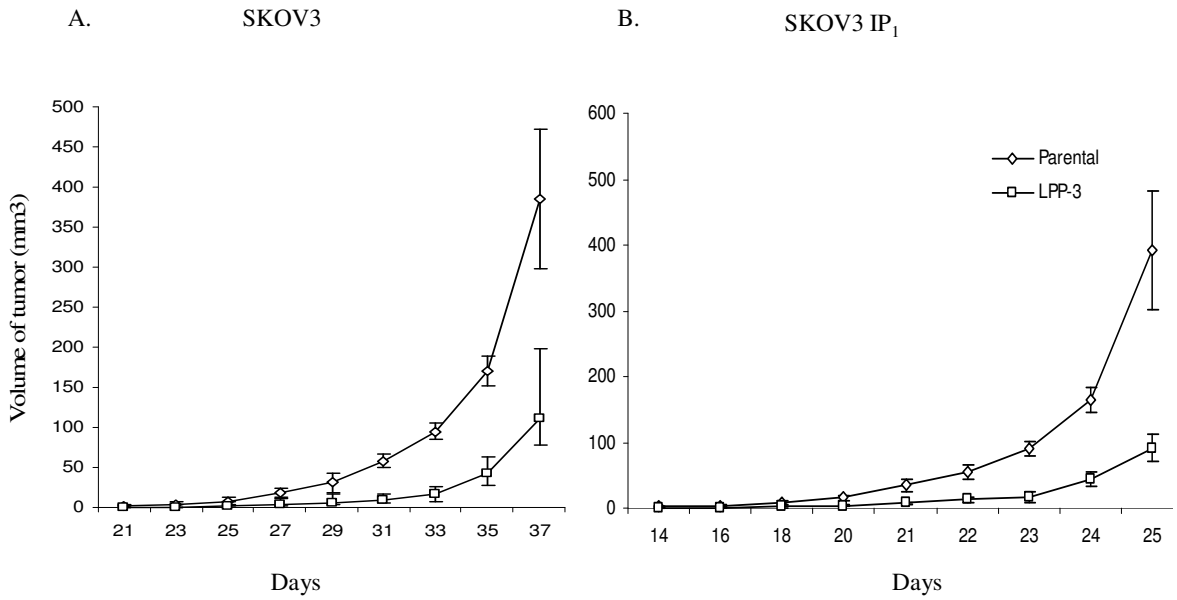


Figure 15 A-B. Effect of LPP-3 on tumor growth. The tumor growth curves in athymic nude mice injected subcutaneously with parental and hLPP-3 expressing stable lines. **A:** 4 million SKOV3 parental ovarian carcinoma cells were injected subcutaneously above the left thigh while 4 million hLPP-3 stably expressing cells were injected on the right side at the same time. Tumor growth was apparent after 21 days. Tumors were measured every second day. **B:** 4 million SKOV3 IP1 parental ovarian carcinoma cells were injected subcutaneously above the left thigh while 4 million hLPP-3 stably expressing cells were injected on the right side at the same time. Tumor growth was apparent on day 14. Tumors were measured every 1-2 days.

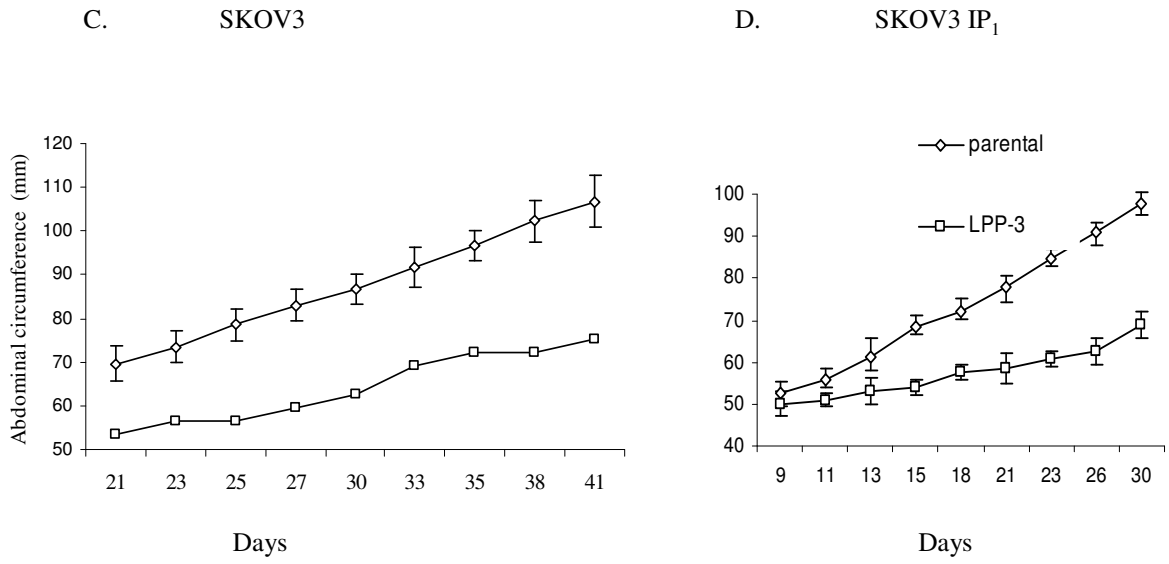


Figure 15 C-D. Effect of LPP-3 on tumor growth. The tumor growth curves in athymic nude mice injected intraperitoneally with parental and LPP-3 expressing stable lines. 10 million SKOV3 parental ovarian carcinoma cells and the same number of LPP-3 expressing stable cells were injected separately, intraperitoneally into 5 athymic nude mice. The abdominal circumference **C:** was measured starting 21 days after injection. 10 million SKOV3 IP1 parental ovarian carcinoma cells and the same number of LPP-3 expressing stable cells were injected, intraperitoneally into 5 athymic nude mice. The abdominal circumference **D:** was measured starting 9 days after injection.

E. SKOV3 F. SKOV3 IP_1

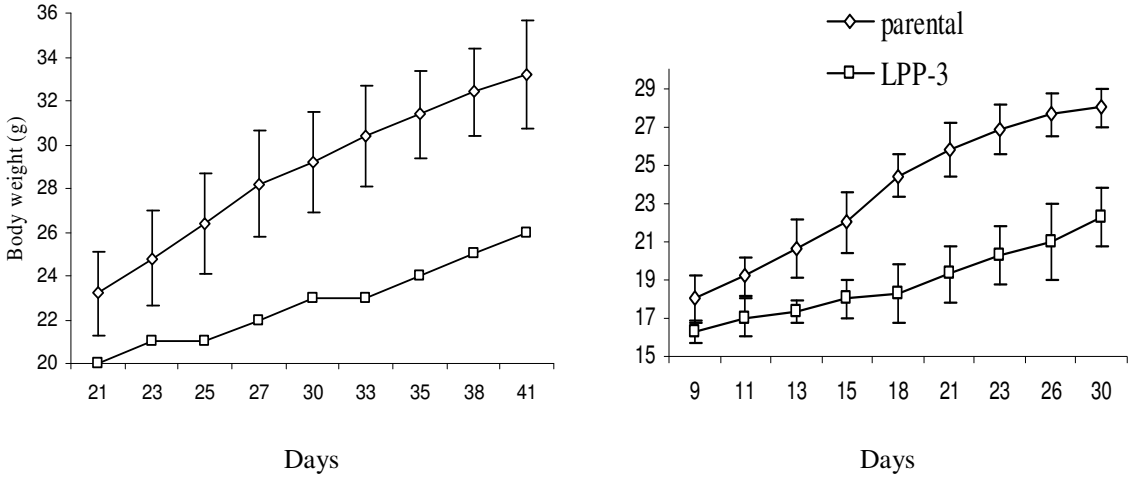


Figure 15 E-F. Effect of LPP-3 on tumor growth. The tumor growth curves in athymic nude mice injected intraperitoneally with parental and LPP-3 expressing stable lines. 10 million SKOV3 parental ovarian carcinoma cells and the same number of LPP-3 expressing stable cells were injected separately, intraperitoneally into 5 athymic nude mice. The body weight **E:** was measured starting 21 days after injection. 10 million SKOV3 IP1 parental ovarian carcinoma cells and the same number of LPP-3 expressing stable cells were injected, intraperitoneally into 5 athymic nude mice. The body weight **F:** was measured starting 9 days after injection.

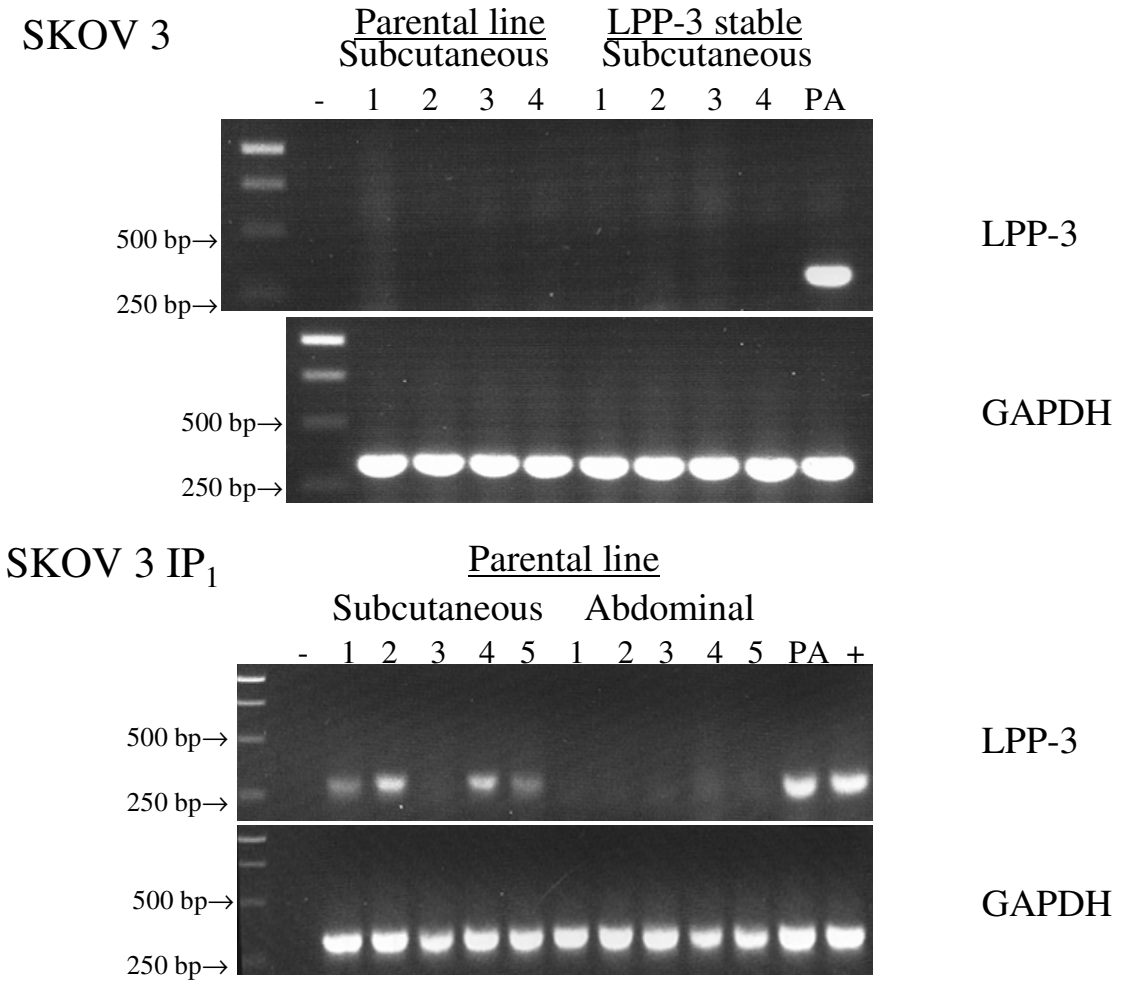


Figure 16. Semi-quantitative RT-PCR analysis for LPP-3 and GAPDH on tumor samples recollected from the mice. Expression of LPP-3 is shown in the subcutaneous and abdominal tumor samples 41 (SKOV3) and 30 (SKOV3 IP1) days after the injection with SKOV3 parental, LPP-3 stably transfected , and SKOV3 IP1 parental cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a RNA control. #PA# represents the RNA sample from the untransfected original parental lines. Empty water sample as negative control and PCR products created with the same primer pairs and the cDNA were used as positive control. Tumors contained at least 70 % malignant cells as assessed by histology.

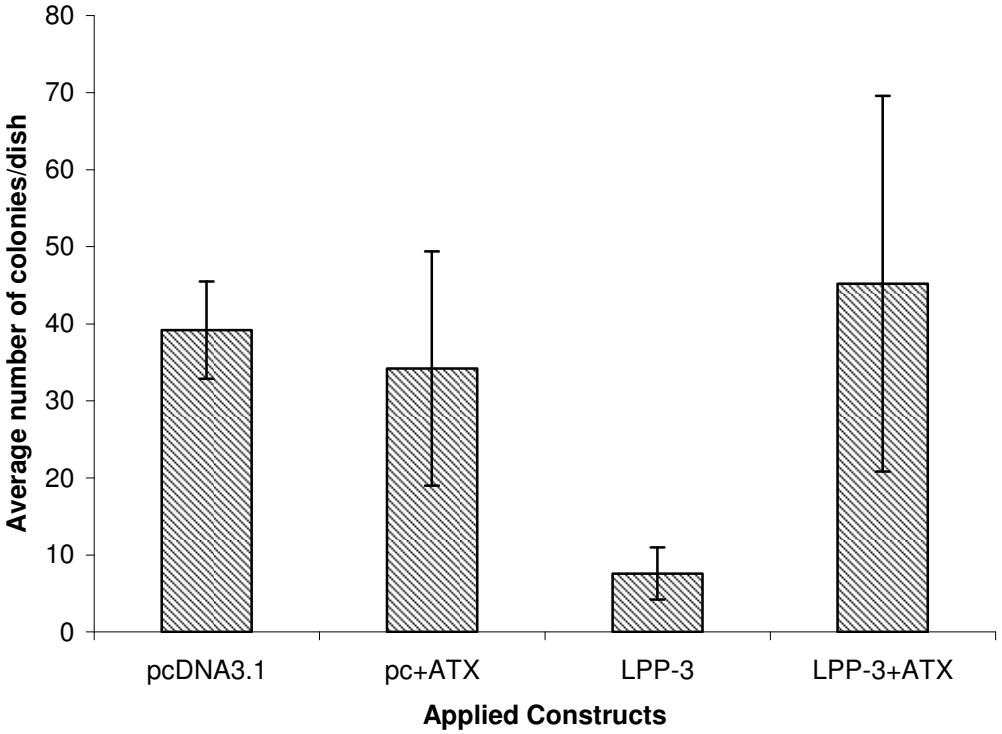


Figure 17. Growth-inhibitory effects of LPP-3 overexpression after transfection of LPP-3 are opposed by ATX/lysoPLD transient transfection. *pcDNA3.1*: cells transfected with empty vector as control, *pc+ATX*: cell were transfected with autotoxin expression vector alone, *LPP-3:* cells were transfected with LPP-3 expression vectors only, *LPP-3+ATX*: cells were transfected with both vector. The mean number of colonies per dish is shown. The data represent the means $\pm$ standard error in three separate experiments.

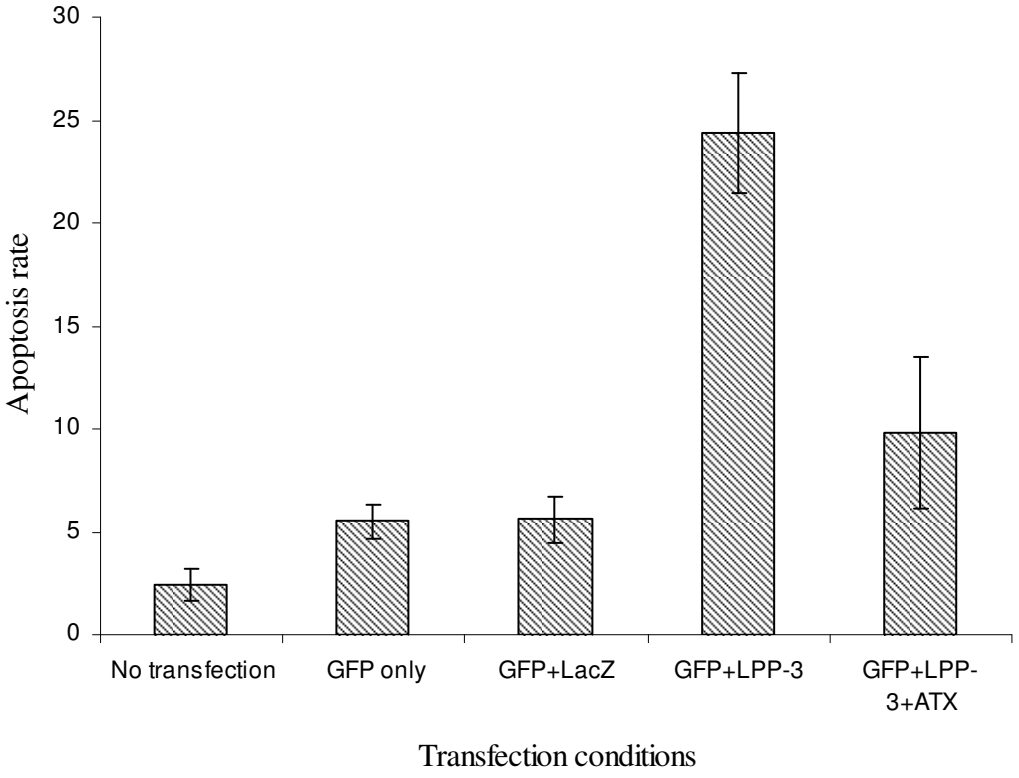


Figure 18. Transient transfection of LPP-3 increases apoptosis, but transient transfection with ATX/lysoPLD opposes this effect. The percentage of apoptotic cells are seen in the figure transfected with the vector written on the particular column.Two-color cytometric analysis was performed, and the percentages of hypodiploid cells as an indication of apoptosis were determined (CellQuest software; Becton Dickinson, Franklin Lakes, NJ). The data shown are the means + standard error of three separate experiments.

6. DISCUSSION

Under physiologic circumstances, LPA levels in bodily fluids, cell membranes, or cells are low (submicromolar), likely reflecting rapid clearance or degradation of LPA (Goetzl et al, 1998). Elevated LPA concentrations in ascites of ovarian cancer patients could be a consequence of altered levels or altered activity of enzymes involved in LPA production, an increase in the number of LPA producing cells (i.e. tumor cells), or altered clearance. These changes in LPA levels and its possible effects on the tumor cells seemed to be an attractive field for evaluation. Using public transcriptional profiling databases as well as the CGAP SAGE database and the transcriptional profiling databases of M.D. Anderson Cancer Center, we analyzed the transcriptional profiles of genes involved in LPA production and metabolism in ovarian cancer tissue samples and cell lines as compared to normal ovarian surface epithelium and normal ovarian cell lines. There were obvious decreases in the levels of LPPs (Tanyi et al 2003a), which degrade LPA in ovarian cancer, concurrent with increases in levels of LysoPLD/autotaxin, which is the key enzyme regulating LPA production (Umezu-Goto et al; 2002, Tokumura et al, 2002). ATX/LysoPLD mRNA levels are elevated up to 200 fold in a proportion of ovarian cancer cell preparations obtained directly from the patient (Umezu-Goto et al 2004). The combined expected effects of these enzymes would be to increase LPA levels in the local tumor environment. In contrast to ATX/lysoPLD and compatible with the increased LPA levels in ascites, the overall total levels of all three LPP mRNA was lower in ovarian cancer tissue samples and cell lines than it was in normal epithelial tissues and cell lines derived from normal ovarian epithelium (Tanyi et al, 2003a). The biggest part of the difference in total LPP mRNA levels could be attributed to decreased LPP-1, which was, on average, five times lower in ovarian cancers than in normal epithelial tissues. It is important to note again that only some patient samples demonstrated marked aberrations in LPP-1 mRNA levels. In contrast to LPP-1, LPP-2 and LPP-3 expression was similar in ovarian cancer and normal epithelium. The same pattern of enzyme expression was

observed in the HEY, OVCAR-3, and SKOV3 ovarian cancer cell lines (Tanyi et al, 2003a).

Taken together, data suggest that ovarian cancer cells have an increased ability to produce LPA compared to normal ovarian epithelium. This is indeed the case with ovarian cancer cells producing high levels of LPA constitutively or in response to stimuli such as LPA itself, phorbol esters, laminin, and nucleotides (Eder et al, 2000; Sengupta et al, 2003; Shen et al, 1999; Luquain et al, 2003). The ability of ovarian cancer cell lines to produce LPA constitutively or inducibly is dependent on the action of phospholipase A2 isozymes and phospholipase D (Eder et al, 2000; Sengupta et al, 2003; Shen et al, 1999; Luquain et al, 2003).

In addition to alterations in the production or action of enzymes involved in LPA production, even a modest ability of ovarian cancer cells to produce LPA could result in marked aberrations in levels of LPA in ovarian cancer patients. It is not unusual for ovarian cancer patients to present with over a kilogram of tumor in the peritoneal cavity and several liters of ascites, which can contain up to 10^8 tumor cells per ml. Thus the number of ovarian cancer cells in a patient at presentation can reach very high levels. Taken together, these studies suggest that the ovarian cancer environment is LPA rich due to aberrations in both LPA production and metabolism.

Combined with the observations described above that the expression of LPA receptors is aberrant in ovarian cancer, the evidence that LPA levels and metabolizing enzymes are aberrant in ovarian cancer suggests that LPA production or action could provide novel effective targets for therapy of ovarian cancer. We have validated LPA production, metabolism and action as potential therapeutic targets in ovarian cancer both in vitro and in vivo by evaluating the effects of decreasing LPA production by ovarian cancer cells by transfecting LPP into ovarian cancer cells. Ovarian cancer cell lines and control normal epithelial cells were transiently and stably transfected with LPP-1- and LPP-3-expressing vectors, with enzyme overexpression being confirmed by semiquantitative reverse transcription polymerase chain reaction and immunoprecipitation (Tanyi et al, 2003a; 2003b; 2006). Overexpression of either the

LPP-1 or LPP-3 isozymes decreased proliferation and survival as indicated by colony-forming activity of ovarian cancer cells as well as by MTT dye reduction. The ability of LPP-3 and LPP-1 to decrease the colony-forming activity of ovarian cancer cells was dependent on LPA degradation as mutant, biologically inactive LPP-3 had no effect on colony-forming activity. To further determine whether the effects of LPP-3 on the growth of ovarian cancer cells were due to hydrolysis of extracellular LPA, we assessed the ability of a nonhydrolyzable LPA3 receptor-selective agonist (OMPT) (Hasegawa et al, 2003), to reverse the effects of LPP-3 expression. OMPT was able to substantially reverse the inhibition both colony-forming activity and apoptosis by LPP-3 (Tanyi et al, 2003b and 2006; Mills et al, 2002). To determine whether OMPT was acting as an agonist for LPA receptors or by inhibiting LPP activity, we assessed the ability to OMPT to inhibit LPP-mediated LPA hydrolysis. While high concentrations of OMPT (>10 μM) could inhibit LPPs, the concentrations used in these studies (100 nM) failed to alter LPP activity. The ability of exogenous OMPT to reverse the effects of LPP-3 suggests that the major effect of hLPP-3 on the growth of ovarian cancer cells is due to hydrolysis of extracellular LPA. LPP-1 and LPP-3 overexpression markedly increased the apoptosis rate in ovarian carcinoma cell lines with lesser effects on cell cycle progression suggesting that the decreased growth rate was a consequence of both decreased proliferation and increased cell death.

The amount of LPA produced by ovarian cancer cells is dependent on both the rate of production and catabolism. ATX/LysoPLD and LPPs appear to be the major contributors to this process (Umezo-Goto et al, 2002 and 2004; Imai et al, 2000). This would suggest that overexpression of ATX/LysoPLD could potentially increase LPA production to sufficient levels to overcome the effects of LPP transfection. Indeed, ATX/LysoPLD was able to reverse the growth inhibitory and apoptosis-inducing effects of LPP-3 overexpression in ovarian carcinoma cell lines. This suggests that high levels of LPA production can over-ride the effects of LPP expression. Taken together, these studies suggest that autocrine loops involving LPA play a critical role in the proliferation and survival of ovarian cancer cells.

LPA has been shown to markedly increase cellular migration which could contribute to tumor aggressiveness or to metastases (Van Leeuwen et al, 2003b; Stahle et al, 2003). We thus assessed whether overexpression of LPP-1 in ovarian cancer cells would alter LPA-induced migration. LPA (10 μM) stimulated migration of control transfected SKOV3 cells in a transwell assay. Strikingly, LPA failed to induce migration in LPP-1-overexpressing SKOV3 cells. These results indicate that the increased extracellular hydrolysis of LPA by LPP-1 is translated into alterations of cellular motility as well as proliferation, colony formation, and survival. These findings are compatible with the hypothesis that decreases in LPP-1 levels and activity in ovarian cancer cells contribute to the pathophysiology of ovarian cancer through both increased proliferation and survival as well as the metastatic cascade. This is supported by our and other previous studies indicating that LPA increased the activity and amount of a number of proteases involved in invasion and metastases (Pustilnik et al, 1999; Fishman et al, 2001; Mills et al, 2002; Tanyi et al, 2006).

LPP-1 and LPP-3 are transmembrane enzymes with their catalytic surface on the external side of the cell membrane. Thus overexpression of these enzymes should decrease in LPA concentrations in cell culture media. We demonstrated that the transfected LPPs had an increased ability to hydrolyze radiolabelled LPA, compatible with this hypothesis (Tanyi et al, 2003a). To confirm an ability to hydrolyze extracellular LPA, we thus determined the concentration of LPA isoforms in cell supernatants of transfected and non-transfected cells using a MicroMass QuattroUltima triple quadrapole mass spectrometer (Waters, Milford, MA). Non-transfected SKOV3 ovarian cancer cells induced a moderate hydrolysis of extracellular LPA with LPA levels decreasing to 76% of the original concentration at the end of the first hour. In contrast, transfection of LPP-1 and LPP-3 into SKOV3 resulted in a decrease in the extracellular concentration of LPA to 52% and to 28%, respectively, of the original concentrations after 1 hour.

If expression of LPP-1 and LPP-3 decreases extracellular LPA, then the overexpression of it in one population of ovarian cancer cells should also influence the colony-forming ability of nontransfected tumor cells. This could prove very important in

gene therapy approaches as LPP-1 or LPP-3 could then induce death of non-transfected bystander cancer cells. To evaluate this theory, LPP-1- and LPP-3-transfected cells were mixed with an equal number of parental cells (Tanyi et al, 2003a,b). Both LPP-1-andLPP-3-transfected cells decreased the proliferation of non-transfected tumor, which is compatible with the hypothesis that the effect of LPP-1 or LPP-3 is related to degradation of an extracellular mediator, likely LPA.

The mechanisms by which overexpression of LPP isoenzymes alter cellular function appears to be through degradation of LPA and limiting LPA-induced signaling. LPA induces changes within seconds of addition to the media. In contrast, other signaling events such as phosphorylation of Erk kinases are delayed and prolonged. If the effects of LPPs are primarily due to degradation of LPA, then LPP overexpression should not alter changes in cytosolic calcium which occur prior to LPA degradation but in contrast LPP overexpression would be expected to decrease Erk phosphorylation (Tanyi et al, 2006). As already showed in this study (Figure 5.F.) there was no difference in the ability of LPA to induce increases in intracellular calcium mobilization in parental or LPP-transfected cells. In contrast, transfection of LPP-3 resulted in a decrease in maximal levels of Erk phosphorylation, and further in a more rapid decline in Erk phosphorylation levels to baseline. The differential rate of decline in erk phosphorylation was compatible with the rate of degradation of LPA by LPP transfected and parental cells (Tanyi et al, 2003a).

The ability of LPPs to decrease cellular proliferation and apoptosis in vitro was reflected by a marked decrease in tumor take rates and growth rates in vivo. After subcutaneous injection of SKOV3 cells, tumors developed in 90% of the mice, compared with only 40% of the mice injected with LPP-3-expressing SKOV3 cells. Thus, the take rate of the tumors expressing LPP-3 was markedly less than that of the parental line. In addition, the growth rate of the hLPP-3-expressing SKOV3 tumor cells was markedly decreased compared with that of the parental cell lines (Tanyi et al, 2003b). At the termination of the study (mandated by tumor size and Association for Assessment of Laboratory Animal Care guidelines), the average weight of SKOV3 parental tumors was

4 fold higher than that of hLPP-3-expressing SKOV3 tumors. When SKOV3 cells were injected into the orthotopic site for ovarian cancer, ie. the peritoneal cavity, LPP-3 expression decreased the take rate to 20% in the injected mice, whereas all parental cell injections resulted in tumor growth. LPP-3 expression decreased intraperitoneal growth as assessed by both animal weight and abdominal circumference (Tanyi et al, 2003b).

Thus, LPP-3 resulted in a marked decrease in tumor take rates, and, in those cases in which tumors formed a decrease in growth rates. However, after a delay in growth, the LPP-3-expressing tumors appeared to enter a more rapid growth phase. Because the hLPP-3 construct was not under selective pressure in vivo, it was possible that the eventual increase in the growth rate was due to loss of LPP-3. Strikingly, when the rapidly growing tumors were excised and assessed for the presence of the LPP-3 transgene, LPP-3 levels in the transfected lines were markedly decreased. Furthermore, LPP-3 levels were decreased in the parent lines in vivo (Tanyi et al, 2003b). It therefore appears that in vivo growth of SKOV3 cells is associated with downregulation of expression of transfected LPP-3. This suggests that a very strong negative selection exists against LPP-3 expression in ovarian cancer cells in vivo.

The changes of the LPA signaling cascade in ovarian cancer offers multiple further field for investigations. ATX/LysoPLD is a particularly attractive target for therapy. Its enzyme face is external and should be accessible to small molecule drugs or to inhibitory antibodies. Its role in cancer, both prior to its demonstration to be a LysoPLD, and through studies of LPA in cancer is well validated. Indeed, application of RNAi to ATX/LysoPLD potently downregulates cellular proliferation and survival (Umezu-Goto et al 2004; Tanyi et al 2006). A series of adenoviral vectors have been developed with broadly active promoters and also with ovarian cancer specific promoters such as telomerase, survivin and ceruloplasmin (Tanyi et al, 2002; Bao et al, 2002; Lee et al, 2004) and are also exploring liposomal delivery approaches.

Although a wide variety of LPA receptor-selective agonists and antagonists have been investigated (Fischer et al 1998; Bandoh et al, 1999; Fischer et al, 2001; Okusa et al, 2003; Lynch et al, 2002; Hasegawa et al, 2003; Feng et al, 2003), a highly effective

therapeutic agent with drug like characteristics that could be used in treatment of ovarian cancer is not yet available. The effects of altering LPA receptor or LPP expression on the onset of tumorigenesis remain to be determined. The structural basis of LPA receptor selectivity is beginning to be determined through structure activity relationships and molecular modeling to suggest that rational drug design could contribute to create new and more selective receptor agonists and antagonists to serve as therapeutic mediators (Feng et al, 2003; Tigyi et al, 2003; Virag et al, 2003; Wang et al, 2001).

Taken together, these studies suggest that an autocrine LPA loop is critical for the proliferation, survival, motility and in vivo growth of ovarian cancer cells. A similar autocrine loop appears to be present in prostate cancer (Xie et al, 2002) and in renal cell carcinoma (Umezu-Goto et al, 2004). Further, the results suggest that this autocrine loop is a potential target for therapy of ovarian and likely other cancers.

7. CONCLUSIONS

1. LPP-1 expression is decreased in ovarian cancer cells. The mechanisms leading to the elevated levels of LPA in ovarian cancer ascites are not currently clear. Our data suggest that loss of LPP-1 expression could result in increased extracellular LPA levels.

2. Introduction of LPP-1 and LPP-3 into ovarian cancer cells significantly decreased the ability of ovarian cancer cells to form colonies and to grow both *in vitro* and *in vivo*.

3. The decreased colony forming cell activity caused by increased expression of LLP-1 and LPP-3 was associated with a marked increase in apoptosis with no obvious differences in cell cycle progression as assessed by flow cytometric analysis.

4. The inability to stably express LPP-1 or LPP-3 in other ovarian cell lines is compatible with a strong negative selection for high-level expression of LPP-1, LPP-3 both in *in vivo* and *in vitro.*

5. The LPP-1 and LPP-3 exerts its effects on ovarian cancer cells through decreasing extracellular LPA. It was supported by the observation that addition of a non-hydrolysable LPA analog, OMPT, to the medium substantially reversed the effects of LPP-3 on both colony forming activity and cellular apoptosis.

6. Both the ability to decrease colony forming activity and to induce apoptosis was dependent on an intact catalytic activity of LPP-3.

7. Point 6. is further proven by the decreased phophorylation of erk kinases. The signaling downstream of LPA as indicated by phosphorylation of erk kinases was curtailed in LPP-3 expressing cells.

8. Both LPP-1 and LPP-3 demonstrated a clear bystander effect also consistent with the effects of LPP-1 and LPP-3 being due to hydrolysis of an extracellular mediator, likely LPA.

9. Overexpression of LPP-1 in SKOV3 cells markedly decreased cellular motility. This data is compatible with the fact that decrease in LPP-1 level in ovarian cancer cells increase their motility and ability to form metastases.

10. The high level of LPA production driven by overexpression of ATX/lysoPLD can override the effect of LPP expression in ovarian cancer cell lines.

11. Taken together these data suggest that LPA production, metabolism, receptor binding and downstream signaling pathways are attractive targets for therapy in ovarian cancer.

8. SUMMARY

Ovarian carcinoma has the highest mortality rate of all gynecologic malignancies owing to late diagnosis and a lack of effective tumor-specific therapeutics. Ovarian carcinogenesis and metastasis is accompanied by a complicated cascade of genetic, molecular, and biochemical events. Indeed over the last several years an ever expanding collection of aberrations has been identified in this tumor. Abnormal lysophosphatidic acid (LPA) production, receptor expression, and signaling are frequently found in ovarian cancers suggesting that LPA plays a role in the pathophysiology of the disease. Moreover, the LPA pathway may provide novel molecular targets, illustrating how the development of new therapeutic and diagnostic strategies can contribute to disease management. The recent identification of the metabolizing enzymes that mediate the degradation and production of LPA and the development of receptor selective-analogues open a potential new approach to the treatment of this deadly disease.

Understanding the physiologic aberrations that originate from genetic alterations of ovarian cancer will lead to the development of new therapeutic approaches for treating ovarian cancer. In this study it is clearly proven that aberrations of LPP production contribute to the progression of ovarian cancer, just us overexpression of these metabolizing enzymes return the physiologic situation and inhibit the growth of the cancer cells. Further manipulating the metabolism, increasing the expression of the enzyme involved in LPA production, can override the effect of the metabolizing enzymes. LPPs exert their effect through metabolizing extracellular LPA. This also clearly explains the excellent “bystander effect” seen in this study. LPA through its production, metabolism, and receptors may provide an excellent target for the development of molecular therapeutics, and the early detection of molecular forms of LPA, other lysolipids, and the activities of LPA pathway receptors and enzymes may facilitate both diagnosis and monitoring the response of a given patient to therapy. The impressive development of knowledge about the pathway regulating LPA production and

the identification of selective LPA-receptor agonists suggest that targeting the LPA cascade could be a real addition to the management and treatment of this still-deadly disease. Additional studies of the LPA cascade and other phospholipids in ovarian cancer are essential to further elucidate their critical roles.

8. ÖSSZEFOGLALÁS

Az ovárium tumorok mortalitása a legmagasabb a nőgyógyászati tumorok között. Ez egyrészt a késői diagnózisnak másrészt a hatásos terápia hiányának köszönhető. Az ovárium tumorok karcinogenezise és metasztázis képzése egy komplikált genetikai, molekuláris és biokémiai folyamatsor eredménye. A lizofoszfát sav (LPA) termelésének, receptor státuszának és szignál transzdukciós útvonalának abnormalitása gyakran megtalálható az ovárium tumorokban, ami azt sejteti hogy az LPA nagyon fontos szerepet játszik ennek a betegségnek a kialakulásában és patofiziológiájában. Igy jogosan feltételezhetjük, hogy a LPA szignál kaszkád számos célpontot szolgáltat a molekuláris kezelési módok kialakítására és jó példát mutat arra, hogyan lehet új diagnosztikus és terápiás módszereket kialakítani egyes betegsegek ellen. A LPA-t lebontó és termelő enzim családoknak csak a közelmultban tőrtént felfedezése és a receptor specifikus molekulák kifejlesztése egy új fejezetet nyithat ennek a potenciálisan halálos betegségnek a kezelésében.

Egyértelműen bizonyítottuk ebben a tanulmányban azt, hogy a tumorsejtekben lévő LPA-t lebontó enzimek aktivitása csökkent, és ez hozzájárul a tumor progressziójához. Ugyanezen enzimek mesterségesen létrehozott, fokozott aktivitása csökkenti a tumorsejtek növekedését és elősegíti a fiziológiás viszonyok helyreállását. Ha tovább manipuláljuk az LPA metabolikus kaszkádot és növeljük a az LPA termelésében résztvevő enzimek aktivitását akkor meglepve tapasztaljuk hogy ezek hatása erősebb a lebontó enzimek aktivitásánal. Egyértelmüen bizonyítottuk ebben a tanulmányban továbbá azt is, hogy a lipid foszfát foszfatáz enzimek hatásukat a sejten kivüli LPA lebontásával érik el. Minthogy ez a lebontás extracellulárisan történik, ez megmagyarázza a “bystander” effect előfordulását, amit szintén leírtunk. Az LPA lebontás, termelés enzimei illetve az LPA-t kötő receptorok kitünő célpontok, új molekuláris terápia kidolgozására. A különböző LPA izoformák és más lizofoszfolipidek szint változásainak korai detektálása segíthet a tumor korai diagnosztizálásában illetve később a kezelés hatékonyságának követésében. A közelmúlt jelentős kutatási eredményei a LPA szignál

kaszkádról azt sejtetik hogy azok jelentős szerepet fognak játszani ennek a még mindig halálos betegségnek a kezelésében, habár még további kutatások lesznek szükségesek a részletek pontos megértéséhez.

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