Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

Lysophosphatidic acid stimulates cell migration, invasion, and colony formation as well as tumorigenesis/metastasis of mouse ovarian cancer in immunocompetent mice

Hui Li,[1] Dongmei Wang,[1] Hong Zhang,[1,2] Kashif Kirmani,[1] Zhenwen Zhao,[1] Rosemary Steinmetz,[1] and Yan Xu[1]

[1]Department of Obstetrics and Gynecology, Indiana University, Indianapolis, Indiana and [2]2nd Hospital of Jilin University, Jilin University, Changchun, Jilin, People's Republic of China

Abstract

We have already established human xenographic models for the effect of lysophosphatidic acid (LPA) on tumor metastasis *in vivo*. The purpose of this work is to establish a preclinical LPA effect model in immunocompetent mice. We first characterized the mouse epithelial ovarian cancer (EOC) cell line ID8 for its responsiveness to LPA in cell proliferation, migration, and invasion and compared these properties with those of human EOC. The signaling pathways related to cell migration were further investigated using pharmacologic and genetic approaches. The effects of LPA on the tumorigenesis of ID8 cells and mouse survival were then examined using two different mouse models (i.p. and orthotopic injections). LPA stimulated cell proliferation, migration, and invasion of mouse EOC ID8 cells in a manner closely resembling its activity in human EOC cells. The signaling pathways involved in LPA-induced cell migration in ID8 cells were also similar to those identified in human EOC cells. We have identified cyclooxygenase-1 and 15-lipoxygenase as two new signaling molecules involved in LPA-induced cell migration in both human and mouse EOC cells. In addition, LPA enhanced the tumorigenesis/metastasis of ID8 cell *in vivo* as assessed by increased tumor size, early onset of ascites formation, and reduced animal survival. We have established the first LPA-EOC preclinical model in immunocompetent mice. Because ID8 cells respond to LPA similar to human EOC cells, this model is very valuable in developing and testing therapeutic reagents targeting LPA in EOC. [Mol Cancer Ther 2009;8(6):1692–701]

Received 11/20/08; revised 2/19/09; accepted 3/13/09; published OnlineFirst 6/9/09.

Grant support: RO1 CA095042 and CA-89228 (Y. Xu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Yan Xu, Indiana University-Purdue University Indianapolis, 975 West Walnut Street, IB355A, Indianapolis, IN 46202. Phone: 317-274-3972; Fax: 317-278-4828. E-mail: xu2@iupui.edu

doi:10.1158/1535-7163.MCT-08-1106

Introduction

Epithelial ovarian cancer (EOC) is the most deadly gynecologic disease mainly due to the lack of highly sensitive and specific methods for early detection and effective treatments for late-stage diseases. Since our first report of elevated lysophosphatidic acid (LPA) levels in human ovarian cancer ascites and the identification of the stimulatory role of LPA in EOC more than 13 years ago (1), extensive information has accumulated showing that LPA plays an important role in EOC development. LPA regulates almost every aspect of EOC cell biology, including cell proliferation, apoptosis, morphology, drug resistance, adhesion, migration, and invasion (2–4). Most of the studies on the mechanisms of LPA in ovarian cancer have been conducted *in vitro*. Only recently, animal studies showing a role for LPA and/or its receptors in the development of cancers *in vivo* have been published (5–9). We have recently provided the first direct evidence that LPA stimulates tumor metastasis *in vivo*, which is inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor, and by 17-dimethylaminoethylamino-17-demethoxygeldanamycin, an inhibitor of the heat shock protein 90, which stabilizes hypoxia-inducible factor-1α (10, 11). However, these experiments were conducted using nude or severe combined immunodeficient mice, which are immunocompromised. It will be important to test the same concepts in immunocompetent mice.

The two most common and critical processes in metastasis are cell migration and invasion. We and others have shown that LPA stimulates the migration and invasion of ovarian cancer cells (10–22). Although most, if not all, of these studies were conducted in established ovarian cancer cell lines, we have recently conducted LPA- and S1P-induced cell migration and invasion experiments in primary EOC and nonmalignant human ovarian surface epithelial cells (11, 23).

All three LPA receptors have been implicated in the migration of different cell types and under different assay conditions (24, 25). Whereas LPA_1 plays a very important role in migration of breast, pancreatic, and prostate cancer cells (25, 26), our data suggest that LPA_2 and LPA_3 are more important in cell adhesion, migration, and invasion of EOC cells, which are consistent with reports showing that LPA_1 is involved in the negative growth regulation in ovarian cancer cells and that LPA_2 and LPA_3, but not LPA_1, expression is up-regulated in last-stage EOC (27). In addition, we have shown that either inhibitors and/or dominant-negative forms of G_i protein, phosphatidylinositol 3-kinase, or cytosolic phospholipase A_2 ($cPLA_2$) completely or nearly completely block LPA-induced cell migration in human EOC cells, suggesting that these three molecules play a pivotal role in this process (2, 10, 11, 24). The PLA_2 family of

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

Translational Relevance

Lysophosphatic acid (LPA) has been shown to be an oncolipid in epithelial ovarian cancer (EOC). We have recently shown that LPA stimulates tumor metastasis of human EOC in orthotopic ovarian cancer models in immunocompromised mice. However, it is critically important to establish a model to test LPA's effect in immuno-competent mice, since the immune system plays an important role in tumor development in human. In the current work, we have characterized LPA responsiveness in a mouse EOC cell line, ID8, and found that these cells closely resemble the properties of human EOC in response to LPA. In addition, signaling studies in these studies have identified enzymes that are potential novel targets for EOC. Using ID8 cells, we have shown for the first time that in immuno-competent mice, LPA stimulated tumorigenesis/metastasis of EOC. This preclinical mouse model will be valuable in testing novel therapeutic reagents targeting LPA *in vivo*.

enzymes catalyzes the hydrolysis of the sn-2 position of phospholipids to generate free fatty acids (arachidonic acid in particular) and lysophosholipids. We have recently shown that LPA regulates both $cPLA_2$ expression and activation (assessed by its phosphorylation) in human EOC cells (11, 24). The role of $cPLA_2$ in cell migration is likely related to its ability to produce arachidonic acid (15, 17, 24). Arachidonic acid is converted to a group of bioactive compounds called eicosanoids (including prostagladins and leukotrienes) by cyclooxygenase (COX) and by lipoxygenase (LOX) enzymes. These enzymes and their lipid products play an important role in inflammation and cancer (28). The role of COX and LOX isoforms, particularly COX-2, in inflammation and cancer has been well established (28). However, COX-1, but not COX-2, is overexpressed in human EOC tissues, which has been confirmed by several independent studies (29, 30). In addition, COX-1, but not COX-2, is also overexpressed in all of the major current EOC mouse models, including the TVA mouse models and the models with deletions of both p53 and Rb, with induction of the transforming region of SV40 under the control of Mullerian inhibitory substance type II receptor, or with local activation of K-Ras in the absence of Pten in the ovarian surface epithelium (29). Thus, COX-1 has been proposed as a potential marker and a possible target for the prevention and/or treatment of EOC (29). There are three major groups of LOX enzymes: 5-LOX, 12-LOX, and 15-LOX. Expression of LOX pathway receptors have been found in human EOC tissues (31). 15-LOX-2, but not 15-LOX-1 or COX-2, is overexpressed in human EOC and EOC metastases (32).

Roby et al. have established mouse EOC cell lines using mouse ovarian surface epithelial cells isolated from normal mouse ovaries through repetitive passaging and spontaneous transformation *in vitro* (33). Ten clonal cell lines were established with distinguishable chromosomal changes and tumorigenic potential. Among these cell lines, ID8 cells form tumors in syngeneic C57/BL6 mice with the highest incidence and shortest lag time when i.p. injected (33). Mouse ovarian surface epithelial cells can be used in immunocompetent mice.

In this work, we characterized the LPA responsiveness in ID8 cells and examined the signaling pathways by which LPA induced cell migration. The *in vivo* effects of LPA on tumorigenesis, metastasis, and mouse survival were determined in syngeneic mice. Our results suggest that the ID8 cells represent a valuable system to develop and evaluate therapeutic reagents targeting LPA in immunocompetent mice.

Materials and Methods

Reagents

18:1 LPA was from Avanti Polar Lipids. Human fibronectin and other extracellular matrix proteins were purchased from Chemicon International. AACOCF3, Akt inhibitor, LY294002, and U0126 were from Calbiochem. Ki16425, thiazolyl blue tetrazolium bromide, AA-861, PD146176, baicalein, and poly(2-hydroxyethyl methacrylate) were from Sigma. SC-560 was from Cayman Chemical. Nordihydroguaiaretic acid and NS398 were from Biomol. Antibodies against phosphorylated $cPLA_2$ and total $cPLA_2$ were from Cell Signaling Technology. We have used nontoxic doses of the inhibitors. The effects on cell survival were checked for all of the inhibitors used.

Cell Culture and Transfection

ID8 cells were obtained from Dr. Paul F. Terranova (University of Kansas Medical Center). Cells were cultured in DMEM supplemented with 4% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite. To establish ID8 cells that stably expressing luciferase, cells were transfected with LUCIE (a kind gift from Dr. Mark Kelley, Indiana University School of Medicine) and selection for resistance to zeocin (100 μg/mL) was initiated 48 h after transfection. Cells were transfected with a dominant-negative $cPLA_2^{514-733}$ (a kind gift from Dr. Sabine Adam, Christian-Albrechts-Universität Kiel) using the Nucleofector Solution V and program T-020 (Amaxa). 15-LOX or COX-1 small interfering RNA (siRNA; Ambion; s62269 and s72391) were transfected into cells using the same method as above and harvested for experiments 36 h post-transfection.

Proliferation Assays

Cells (10^4) in 100 μL serum-free DMEM were added in 96-well plates and incubated at 37°C overnight. LPA (in 100 μL serum-free DMEM) at a 2-fold concentration was then added to the wells and cells were incubated for an additional 24 or 48 h. Cells were trypsinized and counted in the presence of trypan blue using a hemocytometer. For colony assays, CytoSelect 96-well cell transformation assay

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

(Soft Agar Colony Formation; Cell Biolabs) was done according to the company's manual with some modifications. In brief, the cell agar layer was prepared by mixing equal volumes of the 1.2% agar in 2× DMEM and cell suspension (4×10^5 cells/mL in DMEM with various stimulators). DMEM (100 μL) containing the stimulus was layered on the top of the cell agar layer. The assay was done for 8 days. For anoikis assays, 96-well plates were coated twice with poly(2-hydroxyethyl methacrylate) (10 mg/mL in 95% ethanol) and rinsed thoroughly with PBS. ID8 cells in 100 μL serum-free medium with various concentrations of LPA were plated on the coated plates. At the indicated times, the cells were subjected to a brief trypsin treatment to dissociate spheroids and counted in a hemocytometer.

Migration and Invasion Assays

Cell migrations were done in 24-well modified Boyden chambers (Corning Life Sciences) with 8 μm pore size polycarbonate polyvinylpyrrolidone-free Nucleopore filters. Briefly, the bottom phase of the top chamber was coated with 10 μL laminin or other extracellular matrix at a concentration of 10 μg/mL. ID8 cells were serum starved overnight and 5×10^4 cells in 300 μL DMEM were added to the top chamber. DMEM (300 μL) containing the indicated concentrations of LPA was added to the bottom chambers as the chemoattractant. The chambers were then incubated at 37°C for 4 h. After incubation, the top chambers were rinsed with PBS and swabbed with a cotton swab to remove nonmigrated cells. The migrated cells were then fixed in methanol for 30 min and stained with crystal violet (Fisher Scientific) for 30 min followed by a wash with water. After the chambers were dried, pictures were taken with a Nikon SMZ 1000 microscope and migrated cells were counted in three different fields. Invasion assays were done using modified Boyden's chamber (Becton Dickinson Labware). The Matrigel in the chamber was allowed to swell for 2 h in PBS before the experiment. Cells were starved for 24 h and 5×10^4 cells were resuspended in 300 μL DMEM. The cells were added to the top chamber and 300 μL serum-free medium with or without LPA was introduced to the bottom chamber. The invasion assays were conducted for 16 h. The same method for fixing and counting cells described above for cell migration was used.

Reverse Transcription-PCR

The expression levels of $LPA_{1\text{-}3}$ receptors were evaluated by reverse transcription-PCR. In brief, total RNA was extracted from cells using RNeasy mini kit (Qiagen) and reverse transcribed by MMLV (Invitrogen). Derived cDNAs were amplified using PCR master mix (Promega). Primer sequences for $LPA_{1\text{-}3}$ are as follows: LPA_1, 5′-AATCGAGAGGCACATTACGG-3′ and 5′-GTTGAAAATGGCCCAGAAGA-3′; LPA_2, 5′-TTGTCTTCCTGCTCATGGTG-3′ and 5′-TCAGCATCTCGGCAAGAGTA-3′; and LPA_3, 5′-TGCTCATTTTGCTTGTCTGG-3′ and 5′-GCCATACATGTCCTCGTCCT-3′. β-Actin was amplified as a housekeeping gene with primers 5′-ACCGCTCGTTGCCATTAGTGATGA-3′ and 5′-AAGGCCAACCGTGAAAAGATGACC-3′. PCR was also done using 15-LOX primers: 5′-TACCTGTGGTTGATCGGACA-3′ and 5′-AGTTCCTCCTCCCTGTGGTT-3′ and COX-1 primers: 5′-CATTGCACATCCATCCACTC-3′ and 5′-CCCAGAGCCAGTATCCATGT-3′.

Western Blot Analyses

Cells in 6-well plate were rinsed with PBS and lysed in 100 μL Laemmli sample buffer (Bio-Rad). Proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in 5% skim milk for 2 h and then with the indicated primary antibodies overnight followed by incubation with appropriate secondary antibodies for 1 h. Specific proteins were detected using Enhanced Chemiluminescence Plus Western blotting detection system (GE Healthcare). Protein loading was verified by stripping and reprobing blots with antibodies against β-actin or total $cPLA_2$.

Animal Models

Four- to 6-week-old female C57/BL6 mice were purchased from Charles River Labs and housed at the Laboratory Animal Resource Center at the Indiana University School of Medicine. All surgeries were done under approved animal protocols and conducted as described previously (10, 11). LPA was delivered using Alzet microosmotic pumps (model 1002) as described previously (10, 11). Briefly, the pumps were first siliconized with Sigmacote (Sigma) to prevent LPA from adhering to the wall of the pumps. Pumps containing 100 μL concentrated LPA (4 mmol/L in PBS) or PBS were implanted into the peritoneal cavities. Following surgery, ID8-Luc cells (5×10^6) in 200 μL PBS were i.p. injected into the peritoneal cavity. Pumps were replaced after 14 days. In a separate model, LPA was also delivered by daily i.p. injection. Mice were i.p. injected with 5×10^6 ID8 cells in 1 mL PBS and then administered daily with LPA (100 μmol/L) in 200 μL PBS. The LPA injection lasted for 4 to 8 weeks. In the orthotopic model, 5×10^5 ID8 cells in 5 μL PBS were injected into the bursa of a mouse ovary, and LPA was administered by daily i.p. injection for 4 weeks. Animal's health was monitored daily. Animals were euthanized when they developed large volumes of ascites (evidenced by abdominal detention). Tumorigenesis was recorded by counting the numbers and sizes of tumor foci on each organ.

Statistical Analysis

The results were presented as mean ± SE of at least three individual experiments unless otherwise stated. Statistical significance was assessed by Kaplan-Meier for animal average survival and Student's *t* test for the other experiments. $P < 0.05$ was considered to be significant.

Results

LPA Did Not Stimulate Proliferation in Two-Dimensional Cell Culture but Stimulated Colony Formation of ID8 Cells

We conducted a series of experiments to characterize ID8 mouse ovarian cancer cells for their responsiveness to LPA to test the effect of LPA *in vivo* in immunocompetent mice. The effect of LPA on cell proliferation was first tested in two-dimensional tissue culture dishes in the presence or

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

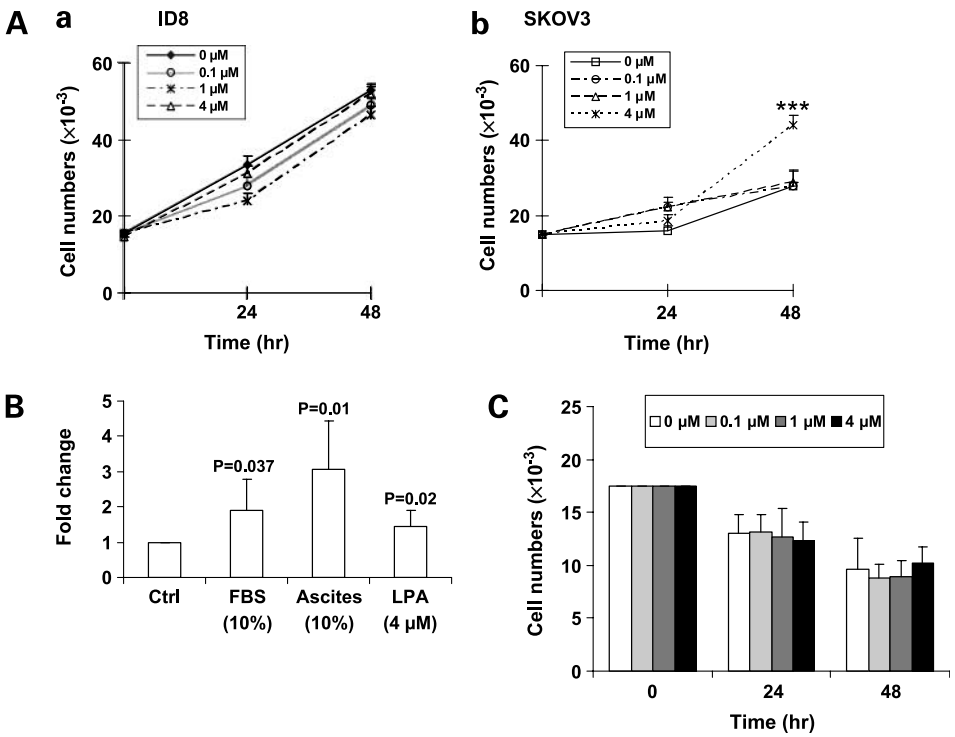

Figure 1. LPA stimulated colony formation but not cell proliferation in two-dimensional cell culture. **A,** cell proliferation in two-dimensional cell culture. Cells were treated with or without indicated doses of LPA for 24 or 48 h. *a,* ID8 cell proliferation. *b,* SKOV3 cell proliferation. **B,** colony formation assays were conducted as described in Materials and Methods. **C,** anoikis assays of ID8 cells. ***, $P < 0.001$. Representative of at least three independent experiments.

absence of LPA (0.1-4 μmol/L) and no significant effect was seen after 24 or 48 h, suggesting that LPA did not affect survival or proliferation of ID8 cells. In comparison, LPA (4 μmol/L) significantly (but rather weakly) stimulated proliferation of human ovarian cancer SKOV3 cells at 48 h (Fig. 1A). Using a 96-well soft agar colony formation assay, we found that although it was not as potent as fetal bovine serum (10%) or human ovarian cancer ascites (10%), LPA (4 μmol/L) significantly stimulated colony formation of ID8 cells (Fig. 1B), which is similar to the effect of LPA in human ovarian cancer cells (34). We showed further that the promoting role of LPA in colony formation was not likely through protecting cells from anoikis, because LPA did not increase cell survival when ID8 cell attachment was prevented by poly(2-hydroxyethyl methacrylate) (Fig. 1C).

LPA Stimulated Migration of ID8 and SKOV3 Cells via $cPLA_2$-, COX-1-, and 15-LOX-Dependent Pathways

Similar to human EOC cells (24, 35–40), LPA (1 μmol/L) induced migration of ID8 cells toward four different extracellular matrix proteins: collagen I, fibronectin, laminin, and vitronectin (Supplementary Fig. S1A).[3] The basal levels of activities (the haptotactic activity toward extracellular matrix proteins) were different, with vitronectin having the strongest effect. LPA induced the highest fold changes of migration to laminin. Because laminin is important for EOC (41), we conducted the remainder of the migration assays using laminin. LPA dose-dependently stimulated migration of ID8 cells to laminin (Supplementary Fig. S1B).[3] LPA (1 μmol/L) was used for the most of the experiments unless otherwise specified.

We tested whether $cPLA_2$ and/or $iPLA_2$ were similarly involved in LPA-induced migration of ID8 cells as those human EOC cells (2, 10, 11, 13, 24). A general inhibitor (AACOCF3) for both $cPLA_2$ and $iPLA_2$ almost completely blocked LPA (1 μmol/L)-induced ID8 cell migration, suggesting that one or both of these enzymes are involved in the process (Fig. 2A). To distinguish the effect between $iPLA_2$ and $cPLA_2$, we tested a more specific inhibitor for $iPLA_2$, HELSS. As shown in Fig. 2B, HELSS significantly reduced basal levels of cell migration as well as migration induced by LPA. To investigate the effect of $cPLA_2$, ID8 cells were transiently transfected with a dominant-negative form of $cPLA_2$ (42). We found that the LPA-induced cell migration were significantly (~70%) inhibited (Fig. 2C), suggesting that the $cPLA_2$ signaling pathway is also involved in the effect of LPA on mouse ovarian cancer cell migration.

Activation of $cPLA_2$ involves phosphorylation by either extracellular signal-regulated kinase (ERK) or p38 mitogen-activated protein kinase (43). LPA (1 μmol/L) time-dependently induced phosphorylation of $cPLA_2$ in ID8 cells, which was inhibited by the MEK-ERK inhibitor U0126 (10 μmol/L; Supplementary Fig. S2A[3]; Fig. 3A, a), suggesting that ERK is the upstream kinase involved in $cPLA_2$ phosphorylation induced by LPA in ID8 cells. When tested in cell migration, the MEK-ERK inhibitor U0126 (10 μmol/L), but not an inhibitor for Akt (10 μmol/L), inhibited LPA-induced cell migration, suggesting that the MEK-ERK-$cPLA_2$ signaling pathway is involved in LPA-induced ID8 cell migration. Similar to human cells (2, 10, 11, 24), LY294002, a

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

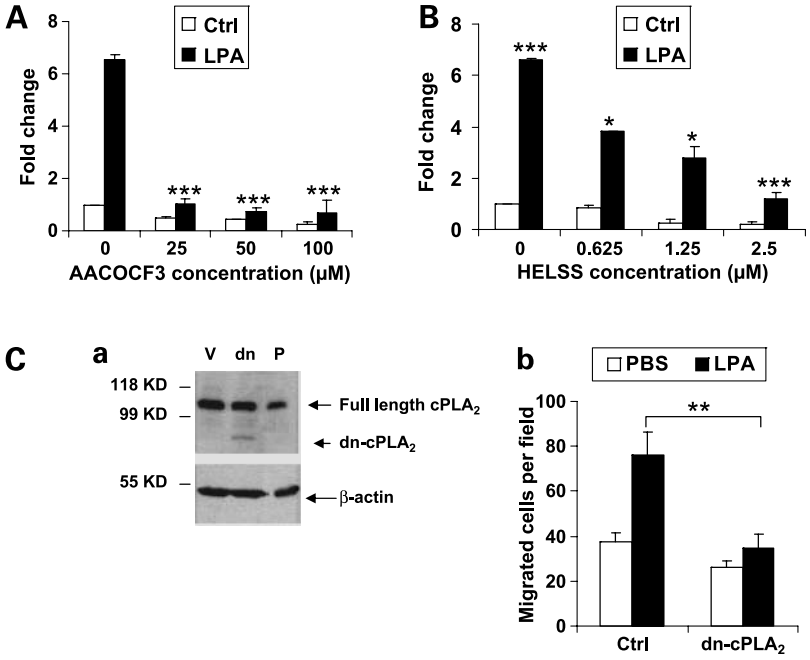

Figure 2. $cPLA_2$ was involved in ID8 cells migration stimulated by LPA. **A,** starved ID8 cells were pretreated with AACOCF3 for 30 min before introducing to the top chamber. The bottom chamber contains LPA (1 μmol/L). **B,** ID8 cells were pretreated with HELSS for 30 min before the migration assays. LPA (1 μmol/L) was used as the attractant. **C,** *a,* 10^6 dominant-negative $cPLA_2$- and vector-transfected ID8 cells were lysed and analyzed by Western blot analyses for $cPLA_2$. *V,* vector transfected clone; *dn,* a dominant-negative $cPLA_2$-transfected clone; *P,* parental cells. *b,* cell migration induced by LPA (1 μmol/L) in transfected cells. *, $P < 0.05$; ***, $P < 0.001$. Representative of at least three independent experiments.

phosphatidylinositol 3-kinase inhibitor, also inhibited LPA-induced migration in ID8 cells (Fig. 3A, b).

To explore downstream effectors of $cPLA_2$, we first used some inhibitors. The general LOX inhibitor nordihydroguaiaretic acid (5 μmol/L), but not the COX-2 inhibitor NS398 (5 μmol/L), significantly inhibited LPA-induced migration of ID8 cells, suggesting that one or more LOX enzymes are involved in LPA-induced cell migration. Interestingly,

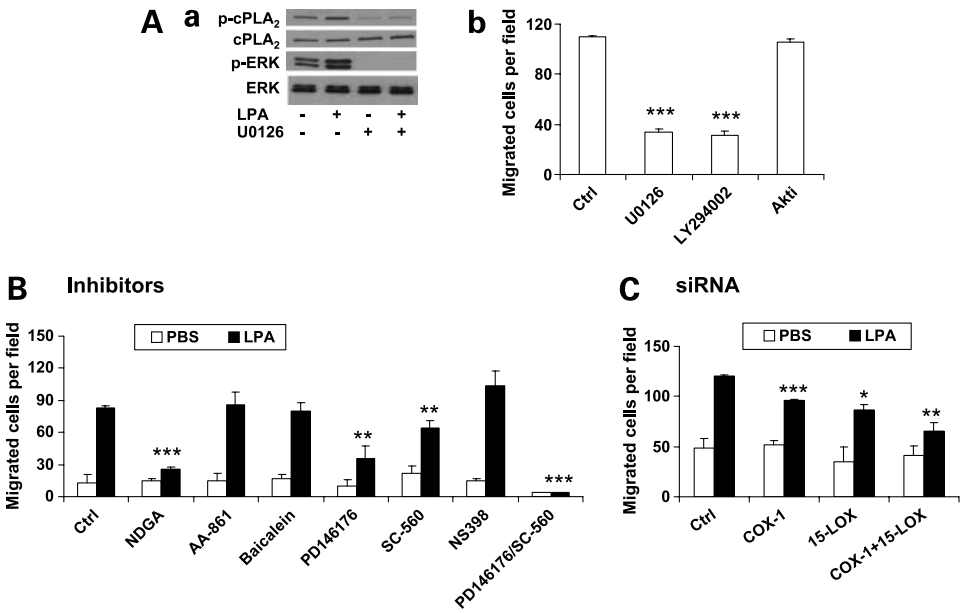

Figure 3. ERK was the upstream kinase for $cPLA_2$ phosphorylation and COX-1 and 15-LOX were involved in LPA-induced migration of ID8 and SKOV3 cells. **A,** *a,* MEK-ERK inhibitor U0126 inhibited LPA-induced $cPLA_2$ phosphorylation. Starved ID8 cells were pretreated with U0126 (10 μmol/L) for 30 min followed by LPA (1 μmol/L) stimulation for 20 min. *b,* U0126 (10 μmol/L) and LY294002 (10 μmol/L), but not an inhibitor for Akt (10 μmol/L), inhibited LPA (1 μmol/L)-induced migration of starved ID8 cells. Cells were treated with inhibitors for 30 min before the assay. **B,** starved ID8 cells were treated with nordihydroguaiaretic acid (5 μmol/L), NS398 (5 μmol/L), AA-861 (2 μmol/L), baicalein (2 μmol/L), PD146176 (2 μmol/L), and/or SC-560 (2 μmol/L) for 30 min before the migration assays. **C,** ID8 cells were transfected with siRNAs for COX-1 and/or 15-LOX and harvested at 36 h post-transfection for the migration assays. Laminin was used to coat the chambers and LPA (1 μmol/L) was used to stimulate cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Experiments were repeated at least three times.

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

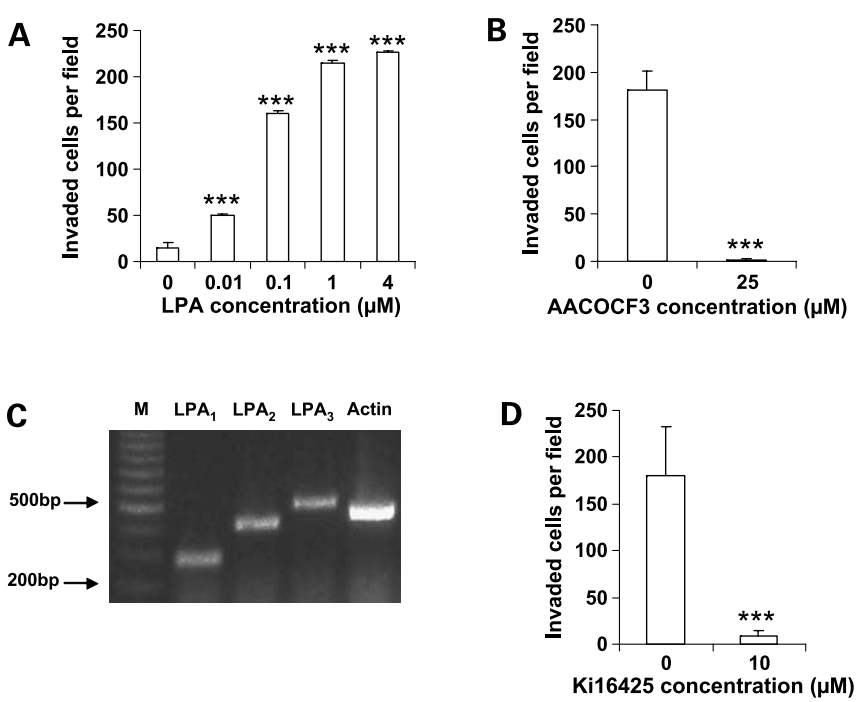

Figure 4. LPA stimulated invasion of ID8 cells and $cPLA_2$ and one or both LPA_1 and LPA_3 receptors were involved. **A,** starved ID8 cells were chemoattracted by various doses of LPA. **B,** starved ID8 cells were pretreated with AACOCF3 (25 μmol/L for 30 min) before introduction to the top chamber. LPA (1 μmol/L) was used for chemoattraction. **C,** total RNA was extracted from ID8 cells and specific primers for LPA_{1-3} were used in reverse transcription-PCR. **D,** starved ID8 cells were pretreated with Ki16425 (10 μmol/L for 30 min). LPA (1 μmol/L) was used. ***, $P < 0.001$. Experiments were repeated at least three times.

COX-1, but not COX-2, is overexpressed in human ovarian cancer tissues and all major mouse ovarian cancer models (29–32). Therefore, we tested whether COX-1 was likely to be involved in LPA-induced cell migration. The COX-1 inhibitor SC-560 (2 μmol/L) partially inhibited LPA-induced cell migration, suggesting that COX-1 is involved in this activity. Next, specific LOX enzyme inhibitors were used to determine which LOX enzymes are involved in LPA-induced cell migration, and 2 μmol/L PD146176, AA-861, and baicalein were used to inhibit 15-LOX, 5-LOX, and 12-LOX, respectively. Our results suggested that 15-LOX, but not 5-LOX or 12-LOX, was involved in the effect of LPA. A combination of PD146176 and COX-1 specific inhibitor SC-560 (2 μmol/L for each inhibitor) almost completely blocked both basal level and LPA-induced cell migration (Fig. 3B), suggesting that each of the signaling pathways contribute partially to the activity and together they mediate the major effects of LPA on cell migration. To confirm these results, ID8 cells were transfected with siRNAs for COX-1 or 15-LOX. The reverse transcription-PCR results showed that the mRNA level of 15-LOX was diminished and the level for COX-1 was only partially reduced (Supplementary Fig. S2B).[3] However, each of these siRNAs resulted in reduced cell migration (Fig. 3C). Combined siRNAs did not further reduce LPA-induced cells migration, which was more likely to be related to the transfection and down-regulation efficiency of these siRNAs. To examine whether human EOC cells also use the same or similar signaling pathways, the same set of inhibitors were used to pretreat SKOV3 cells before LPA-induced cell migration assays were conducted. As shown in Supplementary Fig. S2C,[3] similar sensitivities to the inhibitors tested were also observed in SKOV3 cells, suggesting that ID8 is a good system to recapitulate human EOC cells in terms of LPA responsiveness.

LPA Stimulated Invasion of ID8 Cells and $LPA_{1/3}$ Was Likely to Be Involved

LPA dose-dependently induced cell invasion of ID8 cells, which peaked at 1 to 4 μmol/L (Fig. 4A). Similar to LPA-induced cell migration, AACOCF3 also inhibited cell invasion induced by LPA (Fig. 4B). Similar to human EOC cells, ID8 cells expressed the three major LPA receptors, LPA_{1-3} (Fig. 4C). A $LPA_{1/3}$-specific inhibitor, Ki16425, almost complete inhibited LPA-induced cell invasion in ID8 cells (Fig. 4D), suggesting that either LPA_1, LPA_3, or both of them are involved in LPA-induced invasion in these cells.

LPA Enhanced Tumorigenesis/Metastasis of ID8 Cells and Ascites Formation in Syngeneic Mice

ID8 cells i.p. injected resulted in the formation of numerous tumors as reported previously (33). However, these tumors were small in size (<0.05 mm^3). Daily injections of LPA (100 μmol/L × 200 μL PBS/mouse) for 8 weeks enhanced tumorigenesis of ID8 cells in C57/BL6 syngeneic and immunocompetent mice as evidenced by (*a*) early onset of initial tumors detected, (*b*) early onset of ascites formation (60-70 days in the LPA group versus 80-100 days in the PBS group) with larger ascites volumes, (*c*) formation of tumors that were >1 mm^3, and (*d*) reduced mouse survival times (Fig. 5A-C; Table 1A). In the LPA group, 80% of the mice formed ascites at 77 ± 7 days compared with only 20% of mice formed ascites in the PBS group at the same time. Also, the volumes of ascites formed in the PBS group were less (10 ± 5 mL in the LPA group versus 6 ± 2 mL in the PBS group). However, at later days, most of the mice in the PBS group also developed ascites (n = 21 and 19 in the LPA and

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

the PBS groups, respectively; Fig. 5A). Both groups presented tumors on the peritoneal wall, diaphragm, omentum, and mesentery. Tumors were also found on the surface of the spleen, liver, kidney, and small intestine in 10% mice from both groups of mice. Thus, LPA did not significantly affect the location of tumor formation. However, LPA did significantly stimulate tumor growth, with tumors >0.05 mm^3 found only in the LPA group. Approximately 30% of tumors in the LPA-treated group were 3 ± 2 mm^3, mainly located on the peritoneal wall and the diaphragm (Fig. 5B). The representative H&E staining of the tumors from the PBS and LPA groups are shown in Fig. 5C. In the PBS group, tumors were small and not highly invasive, although the mesothelial layer was disorganized (the normal tissue layers are shown by arrows and the tumor areas are labeled by "tumor"). In contrast, LPA-treated tumors were bigger and the tumor covered the entire slide.

To track the tumor development in living mice, we generated a luciferase-expressing ID8 cell line and used them in the i.p. injection mouse model. The mice were imaged every 2 weeks. At week 4 post-injection, tumor development was detected in the LPA group but not in the PBS group (Supplementary Figs. S3A and B).[3] We found an excellent correlation between the intensity of the bioluminescent signal and the tumor load. Even very small tumors could be detected by the imaging. For the ID8 model, tumors mostly grew on the peritoneal wall, including the ventral (top) and both sides of the body, which were very well imaged. These results showed the promoting roles of LPA in tumorigenesis and/or metastasis. Enhanced tumorigenesis/metastasis likely results in early mouse death. Therefore, we determined mouse survival in LPA- and PBS-treated mice. Fifty percent of the mice receiving LPA died in 75 ± 3 versus 95 ± 5 days in the PBS group, suggesting LPA accelerated cancer-associated mouse deaths (Fig. 5D).

Although the i.p. injection model has been considered as an experimental metastasis model of EOC, it is hard or impossible to distinguish primary tumor and metastatic tumor growth in this model and the initial steps of EOC metastasis have been bypassed. Thus, we used an orthotopic mouse model to further test the effect of LPA. ID8 cells (5×10^5) were orthotopically injected into the bursa of a mouse ovary and LPA was administered by daily i.p. injection for 4 weeks (Table 1B). We found that LPA treatment for a shorter time (4 versus 8 weeks) in the early stage of cancer development was sufficient to induce differences in tumorigenesis and/or metastasis. The orthotopic model results showed that LPA significantly affected tumor growth at the primary site and enhanced tumor metastasis to the distant organ, such as diaphragm, and ascites formation ($n = 7$ in the PBS group and $n = 8$ in the LPA group). Although tumor development in the orthotopic model was slower than that in the i.p. injection model, LPA still accelerated mortality of mice compared with the PBS group (50% of mouse death at 128 versus 160 days in the control group; Table 1B).

Discussion

All cell types in the immune system, including T cells, B cells, natural killer cells, macrophages, and dendritic cells, participate in tumorigenesis and metastasis. Therefore, it is necessary to include these cells and to examine their

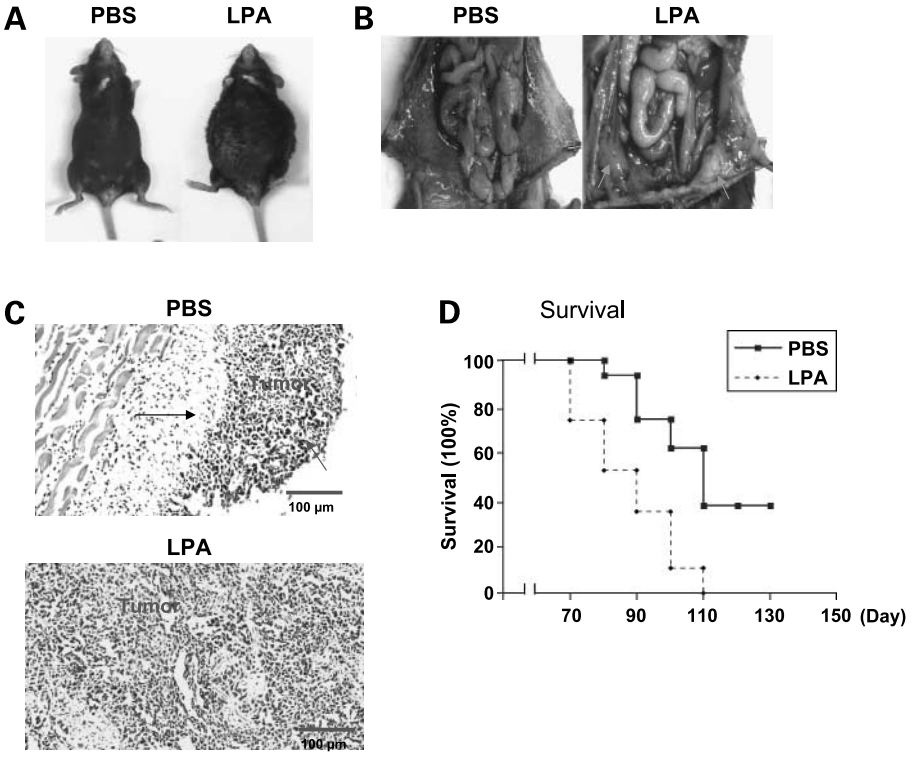

Figure 5. LPA enhanced tumor growth of ID8 cells and ascites formation in female C57/BL6 mice. ID8 cells (5×10^6) in PBS were i.p. injected into mice and LPA (100 μmol/L of 200 μL PBS) were i.p. injected daily for 4 to 8 weeks ($n = 19$ in PBS group and $n = 21$ in LPA group). **A,** mice in the LPA group developed severe ascites compared with control mice. **B,** large peritoneal tumors in peritoneum observed in the LPA groups (*arrows*). **C,** H&E staining of tumor from mice in LPA and PBS groups. **D,** LPA decreased survival rate of mice when compared with that in the control group analyzed by the Kaplan-Meier survival analysis.

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

Table 1.

(A) Summary of mice i.p. injected with ID8 cells

	PBS	LPA
Tumor injection route	i.p.	i.p.
LPA injection route	Pumps + daily injection	Pumps + daily injection
No. mice	$n = 19$	$n = 21$
	From 4 independent experiments	From 4 independent experiments
Tumors >0.05 mm^3	None	3-5/mouse, 6-50 mm^3
Average ascites forming time and volume	80-100 d	60-70 d
	6 ± 2 mL	10 ± 5 mL
Average survival	95 ± 5 d	75 ± 3 d
		** $P = 0.00444$

(B) Summary of orthotopic mice model

	PBS	LPA
Tumor injection route	Orthotopic	Orthotopic
LPA injection route	Daily injection	Daily injection
No. mice	$n = 7$	$n = 8$
Average survival	160 d	128 d
		* $P = 0.012$
Metastatic organs	Diaphragm, peritoneal wall, mesentery, and omentum	Diaphragm, peritoneal wall, mesentery, omentum, liver, and kidney
Metastatic tumor size	<2 mm	>5 mm
	Detected in 30% mice	Detected in 50% mice
Primary tumor	0.169 ± 0.112 mm^3	0.371 ± 0.23 mm^3
		* $P = 0.0333$
Ascites	4 of 7 developed ascites; no mice developed ascites before 16 wk	8 of 8 developed ascites; 3 of 8 developed ascites early (at 13 wk)

effects in a preclinical cancer model. Because human xenografts cannot be done in immunocompetent mice, identification of mouse cells that closely resemble the properties and signaling pathways of their human counterparts is critical. In this work, we have compared the cellular functional responses to LPA in mouse EOC cells and human EOC cells. Both human and mouse EOC cells similarly responded to LPA in colony formation, cell migration, and cell invasion assays. In addition, the signaling pathways involved in LPA-induced cell migration are similar in ID8 and SKOV3 cells. In particular, we have found that both human SKOV3 EOC and mouse ID8 EOC cell lines require COX-1, but not COX-2, in LPA-induced cell migration. Although COX-2 has been shown to be involved in one human EOC cell line, Dov13 cells (15), the majority of human ovarian cancer tissues and mouse EOC models have elevated COX-1, but not COX-2, expression (29–32). These data suggest that the ID8 cell line is a suitable model for testing the effects of LPA on ovarian cancer cells and evaluating LPA-targeted therapeutics in immunocompetent mouse models.

We have used two models (i.p. and orthotopic injection) to observe the effects of LPA *in vivo*. Consistent with our xenographic models using human EOC cells (10, 11), our results suggest that LPA affects both tumorigenesis and metastasis *in vivo*. These results are also highly consistent with the effects of LPA on colony formation, cell migration, and invasion *in vitro* (44).

There are two major strategies to target LPA for EOC treatment. The first is to target LPA signaling pathways including its receptors and downstream signaling molecules and the second is to target LPA production and/or degradation. We have shown that, similar to human EOC cells, ID8 cells express at least three major LPA receptors, $LPA_{1\text{-}3}$, with one or more of these receptors likely to be involved in cell invasion induced by LPA (Fig. 4). To date, at least six LPA receptors have been identified (27). These receptors may have overlapping or even opposing effects *in vitro* and *in vivo*. Although many LPA receptor-specific antagonists have been developed or are under development, specifically targeting of these receptors *in vivo* is still challenging. Alternatively, downstream signaling pathways/molecules can be identified and tested for blocking the effects of LPA *in vivo*. For example, phosphatidylinositol 3-kinase has been considered to be an effective cancer target for LPA-induced and other factor-induced tumor development (45–47). We show here that phosphatidylinositol 3-kinase is also likely to be involved in LPA-induced migration of mouse EOC cells. Moreover, to our knowledge, our work is the first to show that COX-1 and 15-LOX are involved in LPA-induced migration of both human and mouse EOC cells. The potential complete inhibition of LPA-induced cell migration when both of these pathways are inhibited provides promising results that warrant further testing *in vivo* using the mouse model developed here.

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

Targeting downstream signaling molecules may result in more specific inhibition of LPA effects related to tumor metastasis, whereas upstream targets, such as LPA receptors, may be involved in a broader cellular effects, some of which should not be inhibited in normal physiology. Interestingly, PLA_2s are not only involved in LPA signaling but also involved in LPA production (48). Thus, targeting these enzymes may present special advantages.

Ascites formation is a major burden for patients with ovarian cancer. It represents a tumor microenvironment and contains large number of tumor and other cells as well as promoting growth factors, cytokines, and lipids factors. We present here the first evidence that LPA has a promoting activity in ascites formation *in vivo*. This activity may be related to its ability to stimulate VEGF secretion, because VEGF has been shown to be an important factor for the regulation of blood vessel permeability and ascites formation (49, 50). Therefore, the mouse model established here may be used to study the mechanisms of ascites formation *in vivo*.

In summary, we have established a mouse model using ID8 to test the *in vivo* effect of LPA on EOC. Both our *in vitro* and *in vivo* results suggest that ID8 cells closely recapitulate responses to LPA. In addition, the similarity in the effects of LPA on tumorigenesis and metastasis observed in immunocompromised and immunocompetent mice suggest that the promoting functions of LPA are not diminished by the presence of endogenous T or B cells. Thus, this is a valuable system to identify and test therapeutics that target LPA in EOC and to study the mechanisms by which ascites forms in EOC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The imaging work was supported in part by the Indiana Genomics Initiative. The Indiana Genomics Initiative is supported in part by the Lilly Endowment.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.

2. Sengupta S, Wang Z, Tipps R, Xu Y. Biology of LPA in health and disease. Semin Cell Dev Biol 2004;15:503–12.

3. Mills GB, Moolenaar WH. The emerging role of lysophosphatidic acid in cancer. Nat Rev Cancer 2003;3:582–91.

4. Xu Y, Sengupta S, Singh S, Steinmetz R. Novel lipid signaling pathways in ovarian cancer cells. Cell Sci Rev 2006;3:168–97.

5. Huang MC, Lee HY, Yeh CC, Kong Y, Zaloudek CJ, Goetzl EJ. Induction of protein growth factor systems in the ovaries of transgenic mice overexpressing human type 2 lysophosphatidic acid G protein-coupled receptor (LPA2). Oncogene 2004;23:122–9.

6. Boucharaba A, Serre CM, Gres S, et al. Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer. J Clin Invest 2004;114:1714–25.

7. Boucharaba A, Serre CM, Guglielmi J, Bordet JC, Clezardin P, Peyruchaud O. The type 1 lysophosphatidic acid receptor is a target for therapy in bone metastases. Proc Natl Acad Sci U S A 2006;103:9643–8.

8. Umezu-Goto M, Tanyi J, Lahad J, et al. Lysophosphatidic acid production and action: validated targets in cancer? J Cell Biochem 2004;92:1115–40.

9. Baker DL, Fujiwara Y, Pigg KR, et al. Carba analogs of cyclic phosphatidic acid are selective inhibitors of autotaxin and cancer cell invasion and metastasis. J Biol Chem 2006;281:22786–93.

10. Kim KS, Sengupta S, Berk M, et al. Hypoxia enhances lysophosphatidic acid responsiveness in ovarian cancer cells and lysophosphatidic acid induces ovarian tumor metastasis *in vivo*. Cancer Res 2006;66:7983–90.

11. Sengupta S, Kim KS, Berk MP, et al. Lysophosphatidic acid downregulates tissue inhibitor of metalloproteinases, which are negatively involved in lysophosphatidic acid-induced cell invasion. Oncogene 2007;26:2894–901.

12. Sugimoto N, Takuwa N, Yoshioka K, Takuwa Y. Rho-dependent, Rho kinase-independent inhibitory regulation of Rac and cell migration by LPA1 receptor in G_i-inactivated CHO cells. Exp Cell Res 2006;312:1899–908.

13. Ren J, Xiao YJ, Singh LS, et al. Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. Cancer Res 2006;66:3006–14.

14. So J, Wang FQ, Navari J, Schreher J, Fishman DA. LPA-induced epithelial ovarian cancer (EOC) *in vitro* invasion and migration are mediated by VEGF receptor-2 (VEGF-R2). Gynecol Oncol 2005;97:870–8.

15. Symowicz J, Adley BP, Woo MM, Auersperg N, Hudson LG, Stack MS. Cyclooxygenase-2 functions as a downstream mediator of lysophosphatidic acid to promote aggressive behavior in ovarian carcinoma cells. Cancer Res 2005;65:2234–42.

16. Bian D, Su S, Mahanivong C, et al. Lysophosphatidic acid stimulates ovarian cancer cell migration via a Ras-MEK kinase 1 pathway. Cancer Res 2004;64:4209–17.

17. Zhao X, Wang D, Zhao Z, et al. Caspase-3-dependent activation of calcium-independent phospholipase A_2 enhances cell migration in non-apoptotic ovarian cancer cells. J Biol Chem 2006;281:29357–68.

18. Schwartz BM, Hong G, Morrison BH, et al. Lysophospholipids increase interleukin-8 expression in ovarian cancer cells. Gynecol Oncol 2001;81:291–300.

19. Fang X, Yu S, Bast RC, et al. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. J Biol Chem 2004;279:9653–61.

20. Hu YL, Tee MK, Goetzl EJ, et al. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. J Natl Cancer Inst 2001;93:762–8.

21. Lee Z, Swaby RF, Liang Y, et al. Lysophosphatidic acid is a major regulator of growth-regulated oncogene α in ovarian cancer. Cancer Res 2006;66:2740–8.

22. So J, Navari J, Wang FQ, Fishman DA. Lysophosphatidic acid enhances epithelial ovarian carcinoma invasion through the increased expression of interleukin-8. Gynecol Oncol 2004;95:314–22.

23. Wang D, Zhao Z, Caperell-Grant A, et al. S1P differentially regulates migration of human ovarian cancer and human ovarian surface epithelial cells. Mol Cancer Ther 2008;7:1993–2002.

24. Sengupta S, Xiao YJ, Xu Y. A novel laminin-induced LPA autocrine loop in the migration of ovarian cancer cells. FASEB J 2003;17:1570–2.

25. Yamada T, Sato K, Komachi M, et al. Lysophosphatidic acid (LPA) in malignant ascites stimulates motility of human pancreatic cancer cells through LPA1. J Biol Chem 2004;279:6595–605.

26. Amano S, Akutsu N, Ogura Y, Nishiyama T. Increase of laminin 5 synthesis in human keratinocytes by acute wound fluid, inflammatory cytokines and growth factors, and lysophospholipids. Br J Dermatol 2004;151:961–70.

27. Murph MM, Nguyen GH, Radhakrishna H, Mills GB. Sharpening the edges of understanding the structure/function of the LPA1 receptor: expression in cancer and mechanisms of regulation. Biochim Biophys Acta 2008;1781:547–57.

28. Rao CV. Regulation of COX and LOX by curcumin. Adv Exp Med Biol 2007;595:213–26.

29. Daikoku T, Tranguch S, Trofimova IN, et al. Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. Cancer Res 2006;66:2527–31.

30. Urick ME, Johnson PA. Cyclooxygenase 1 and 2 mRNA and protein expression in the *Gallus domesticus* model of ovarian cancer. Gynecol Oncol 2006;103:673–8.

31. Rocconi RP, Kirby TO, Seitz RS, et al. Lipoxygenase pathway receptor expression in ovarian cancer. Reprod Sci 2008;15:321–6.

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1106

32. Roffeis J, Hornung D, Kuhn H, Walther M. 15-Lipoxygenase-2 is differentially expressed in normal and neoplastic ovary. Eur J Cancer Prev 2007;16:568–75.

33. Roby KF, Taylor CC, Sweetwood JP, et al. Development of a syngeneic mouse model for events related to ovarian cancer. Carcinogenesis 2000;21:585–91.

34. Xu Y, Fang XJ, Casey G, Mills GB. Lysophospholipids activate ovarian and breast cancer cells. Biochem J 1995;309:933–40.

35. Do TV, Symowicz JC, Berman DM, et al. Lysophosphatidic acid down-regulates stress fibers and up-regulates pro-matrix metalloproteinase-2 activation in ovarian cancer cells. Mol Cancer Res 2007;5:121–31.

36. Huang MC, Graeler M, Shankar G, Spencer J, Goetzl EJ. Lysophospholipid mediators of immunity and neoplasia. Biochim Biophys Acta 2002;1582:161–7.

37. Fishman DA, Liu Y, Ellerbroek SM, Stack MS. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. Cancer Res 2001;61:3194–9.

38. Gil OD, Lee C, Ariztia EV, et al. Lysophosphatidic acid (LPA) promotes E-cadherin ectodomain shedding and OVCA429 cell invasion in an uPA-dependent manner. Gynecol Oncol 2008;108:361–9.

39. Smicun Y, Gil O, Devine K, Fishman DA. S1P and LPA have an attachment-dependent regulatory effect on invasion of epithelial ovarian cancer cells. Gynecol Oncol 2007;107:298–309.

40. Wang FQ, Smicun Y, Calluzzo N, Fishman DA. Inhibition of matrilysin expression by antisense or RNA interference decreases lysophosphatidic acid-induced epithelial ovarian cancer invasion. Mol Cancer Res 2006;4:831–41.

41. Capo-Chichi CD, Smith ER, Yang DH, et al. Dynamic alterations of the extracellular environment of ovarian surface epithelial cells in premalignant transformation, tumorigenicity, and metastasis. Cancer 2002;95:1802–15.

42. Atsumi G, Murakami M, Kojima K, Hadano A, Tajima M, Kudo I. Distinct roles of two intracellular phospholipase A_2s in fatty acid release in the cell death pathway. Proteolytic fragment of type IVA cytosolic phospholipase $A_{2\alpha}$ inhibits stimulus-induced arachidonate release, whereas that of type VI Ca^{2+}-independent phospholipase A_2 augments spontaneous fatty acid release. J Biol Chem 2000;275:18248–58.

43. Zhou H, Das S, Murthy KS. Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by m3 and m2 receptors. Am J Physiol Gastrointest Liver Physiol 2003;284:G472–80.

44. Xu Y, Casey G, Mills GB. Effect of lysophospholipids on signaling in the human Jurkat T cell line. J Cell Physiol 1995;163:441–50.

45. Hu L, Zaloudek C, Mills GB, Gray J, Jaffe RB. *In vivo* and *in vitro* ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). Clin Cancer Res 2000;6:880–6.

46. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005;4:988–1004.

47. Lu Y, Wang H, Mills GB. Targeting PI3K-AKT pathway for cancer therapy. Rev Clin Exp Hematol 2003;7:205–28.

48. Aoki J, Inoue A, Okudaira S. Two pathways for lysophosphatidic acid production. Biochim Biophys Acta 2008;1781:513–8.

49. Nagy JA, Masse EM, Herzberg KT, et al. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. Cancer Res 1995;55:360–8.

50. Byrne AT, Ross L, Holash J, et al. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res 2003;9:5721–8.

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Lysophosphatidic acid stimulates cell migration, invasion, and colony formation as well as tumorigenesis/metastasis of mouse ovarian cancer in immunocompetent mice

Hui Li, Dongmei Wang, Hong Zhang, et al.

Mol Cancer Ther 2009;8:1692-1701. Published OnlineFirst June 9, 2009.