

# Lysophosphatidic acid receptor 2 and Gi/Src pathway mediate cell motility through cyclooxygenase 2 expression in CAOV-3 ovarian cancer cells

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Abbreviations: COX, cyclooxygenase; LPA, lysophosphatidic acid; MMP, matrix metalloproteinase; PI3 kinase, phosphatidylinositide 3-kinase; siRNA, small interfering RNA

## Abstract

Lysophosphatidic acid (LPA) is a bioactive phospholipids and involves in various cellular events, including tumor cell migration. In the present study, we investigated LPA receptor and its transactivation to EGFR for cyclooxygenase-2 (COX-2) expression and cell migration in CAOV-3 ovarian cancer cells. LPA induced COX-2 expression in a dose-dependent manner, and pretreatment of the cells with pharmacological inhibitors of Gi (pertussis toxin), Src (PP2), EGF receptor (EGFR) (AG1478), ERK (PD98059) significantly inhibited LPA-induced COX-2 expression. Consistent to these results, transfection of the cells with selective Src siRNA attenuated COX-2 expression by LPA. LPA stimulated CAOV-3 cell migration that was abrogated by pharmacological inhibitors and antibody of EP2.

Higher expression of LPA2 mRNA was observed in CAOV-3 cells, and transfection of the cells with a selective LPA2 siRNA significantly inhibited LPA-induced activation of EGFR and ERK, as well as COX-2 expression. Importantly, LPA2 siRNA also blocked LPA-induced ovarian cancer cell migration. Collectively, our results clearly show the significance of LPA2 and Gi/Src pathway for LPA-induced COX-2 expression and cell migration that could be a promising drug target for ovarian cancer cell metastasis.

**Keywords:** cell movement; cyclooxygenase-2; lysophosphatidic acid; ovarian neoplasms; proto-oncogene proteins pp60 (c-src); receptors, lysophosphatidic acid

## Introduction

Ovarian cancer represents the fourth leading cause of cancer-related death for women in the Western world (Kohn *et al.*, 2003). Main reasons for the low survival rate of patients with ovarian cancer are caused by lack of effective early detection and treatment, as well as the highly metastatic nature of the disease. While most solid tumors metastasize through blood vessels or lymphatic nodes, ovarian cancer metastasizes through direct dissemination from the primary site(s) into the peritoneal cavity (Gardner *et al.*, 1995). The bioactive lipid lysophosphatidic acid (LPA) is detectable in the majority of patients with both early- and late-state ovarian cancer, reaching concentrations of 80 μM in both the plasma and ascites of ovarian cancer patients (Xu *et al.*, 1995; Fang *et al.*, 2000). Ovarian cancer cells also produce LPA, thereby maintaining an LPA-rich microenvironment (Fang *et al.*, 2002). Upon binding to G protein coupled plasma membrane receptors (LPA1, LPA2, and LPA3), LPA exerts diverse biological effects, including cell proliferation/survival, induction of neurite retraction, inhibition of gap junctional communication, and stimulation of cell motility (Moolenaar *et al.*, 1997). More recently, LPA4 (GPR23/P2Y9) (Noguchi *et al.*, 2003) and LPA5 (Kotarsky *et al.*, 2006; Lee *et al.*, 2006) have been identified as a subfamily of LPA receptors. They are structurally distinct from other LPA receptors.

Recent studies on LPA knockout mice indicate that each LPA receptor has distinct function (Contos *et al.*, 2002). Furthermore, LPA1 is widely expressed in human tissues, while the distribution of LPA2 and LPA3 is more restricted (An *et al.*, 1998; Bandoh *et al.*, 1999). Among LPA receptors, LPA2 is aberrantly expressed in various cancer cells, including ovarian cancer cells (Fujita *et al.*, 2003). Therefore, expression of LPA receptor might be closely related to the development of various cancers.

Cyclooxygenase-2 (COX-2) is responsible for the synthesis of PGE2 and other prostaglandins from arachidonic acid (Cao *et al.*, 2002). Expression of COX-2 is elevated in ovarian cancer (Klimp *et al.*, 2001), and LPA promotes aggressive behavior in ovarian cancer cells through induction of COX-2 expression (Symowicz *et al.*, 2005). Despite the importance of COX-2 in LPA-induced invasiveness, the detailed underlying mechanism is remained to be solved. Therefore, we undertook to investigate the role of LPA receptors in LPA-induced COX-2 expression and cell migration and present herein strong evidence that LPA2 and Gi/Src transactivation to EGFR are responsible for COX-2 expression and aggressiveness in ovarian cancer cells.

## Materials and Methods

### Reagents

LPA was purchased from Avanti Polar Lipids (Alabaster, AL). Pertussis toxin (Glycerol Solution), AG1478, PD98059, and GM6001 were from Calbiochem (San Diego, CA). VPC32183, AH6809 and AH23848 were from Sigma-Aldrich (St. Louis, MO). Antibodies for LPA2, COX-2, EGFR, phospho-ERK, phospho-Tyr, EP2, c-Src and ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH antibody was obtained from Lab Frontier (Seoul, Korea). Enhanced chemiluminescence reagents and Sepharose 4B were from Amersham-Pharmacia Biotech (Piscataway, NJ). LipofectAMINE 2000 was obtained from Invitrogen (Carlsbad, CA). All other reagents used were of the purest grade available.

### Cell culture

CAOV-3, OVCAR-3, PA-1, 2774 and SKOV-3 ovarian cancer cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in the complete medium that consists of RPMI 1640 (CAOV-3, OVCAR-3, 2774 and SKOV-3) or EMEM (PA-1), supplemented with 10% FBS in a humidified atmosphere containing

5% CO<sub>2</sub> at 37°C. For experimental purposes, the cells ( $5 \times 10^5$ ) were plated in six-well culture dishes. After 1 day, the cells were serum starved overnight before use.

### Reverse transcription-PCR (RT-PCR)

Total cellular RNA (1 µg) isolated from cultured cell line was used for reverse transcription. The cDNA was then subjected to PCR amplification with primer sets for COX-2, LPA1, LPA2, LPA3, and GAPDH: COX-2 forward, 5'-TGGGAAGCCTCTCTAACCTCTCCT-3'; COX-2 reverse 5'-CTTTGACT-GTGGGAGGATACATCTC-3'; LPA1 forward, 5'-TAGGGTGGTCATTGTGGTCA-3'; LPA1 reverse, 5'-GGCAGAGTTGAATTCAAGCAAG-3'; LPA2 forward, 5'-TGGCCTACCCCTTCCATGTTCC-3'; LPA2 reverse, 5'-ATTGACCAGTGAGTTGGCCT-3'; LPA3 forward, 5'-GTCCATAGCAACCTGACCAAA-3'; LPA3 reverse, 5'-GTTCTCCTGAGAGAAGCAGCA-3'; GAPDH forward, 5'-CATTTCCAGGAGCGAGA-3'; GAPDH reverse, 5'-CTGCTTCACCACCTTCTTG-AT-3'. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide, and their intensity was compared using EagleSight Software V. 3.2 (Stratagene, La Jolla, CA).

### Immunoblotting

Quiescent cells were treated with 25 µM LPA for the indicated period. After solubilizing the cells with 250 µl of ice-cold buffer (Lee *et al.*, 2005), lysates were resolved by 10% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked at 4°C overnight in a covered container and allowed to react with a specific antibody in 1:1,000 dilution, and detection of specific proteins was carried out by enhanced chemiluminescence following the manufacturer's instruction.

### Immunoprecipitation

Lysates containing 200 µg of proteins in extraction buffer were incubated with 4 µg/ml anti-EGFR antibody at room temperature for 5 h. The immunocomplex was precipitated with protein A Sepharose CL-4B beads (Amersham Biosciences, Piscataway) overnight at 4°C. The beads were washed with PBS containing Tween 20, resuspended in SDS buffer, and boiled for 5 min. The protein samples were then immunoblotted with anti-p-Tyr antibody in 1:1,000 dilution.

### Estimation of PGE2 and MMP-2

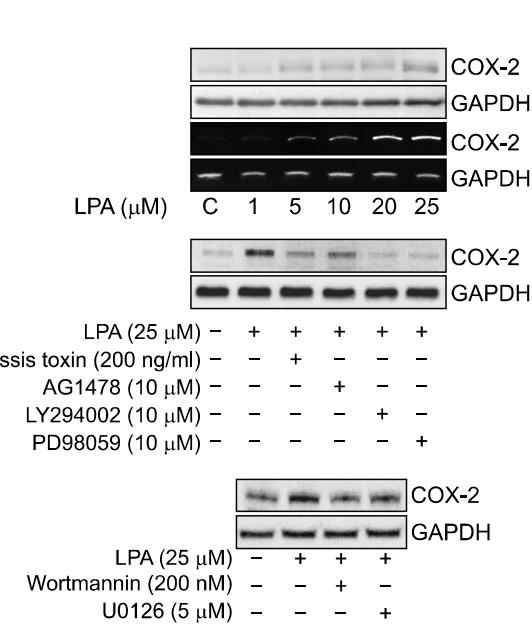
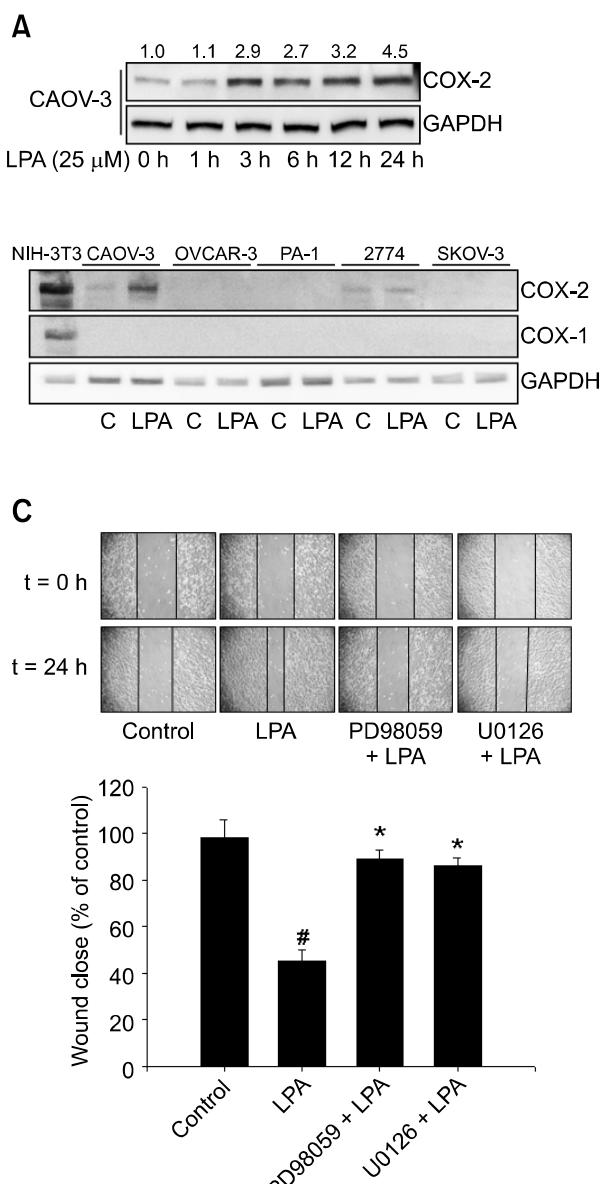
The levels of PGE2 and MMP-2 from the con-

ditioned media of LPA-treated CAOV-3 cells were detected by using the ELISA kits for PGE2 (Cayman Chemical Company, Ann Arbor, MI) and MMP-2 (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. To measure the MMP activity, gelatin zymography was utilized as described previously (Lakka *et al.*, 2000). After stimulating with or without LPA for 24 h, the cells were washed with PBS and incubated with fresh serum-free medium. After 24 h, conditioned medium was collected and centrifuged, and protein concentrations were determined. Equal amounts of protein were subjected to 0.1% gelatin SDS-PAGE under nonreducing conditions. Gels were washed in 2.5% Triton X-100 and incubated

overnight in Tris-CaCl<sub>2</sub> buffer. The gels were then stained with 0.5% Coomassie blue for 1 h and destained in 20% methanol and 10% acetic acid. The clear bands represent gelatinase activity.

### Small interfering RNA (siRNA)

siRNA corresponding to the LPA<sub>2</sub> gene was designed and synthesized by Invitrogen life technologies (Carlsbad, CA). The following sequences were used: 5'-UGUGGAACAAUGAGGAAGAGGU-AGGC-3'. siRNA duplexes were used for transfection at a final concentration of 100 pmol. The c-Src siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and is a pool of 4



**Figure 1.** LPA induces COX-2 expression through Gi, EGFR and ERK. (A) The cells were serum starved overnight, and stimulated with LPA for indicated times. Cell lysates were analyzed by immunoblotting against anti-COX-1 or anti-COX-2 antibody. As a control, the lysate of NIH-3T3 cells was analyzed in the same gel. (B) The cells were pretreated with or without pharmacological inhibitors of Gi (pertussis toxin), PI3 kinase (LY294002 and wortmannin), EGFR (AG1478), ERK (PD98059 and U0126), followed by stimulation with indicated concentration of LPA. The cell lysates were analyzed by either RT-PCR or immunoblotting. (C) The cells were pretreated with or without indicated pharmacological inhibitors, followed by stimulation with LPA and the cell migration was analyzed at time 0 and 24 h. Results show a representative of three experiments and the mean  $\pm$  S.D. of three experiments.  $^{\#}P < 0.05$  vs. control,  $^{*}P < 0.05$  vs. LPA treatment.

target-specific 20-25 nt siRNAs designed to knock down gene expression. Cells were plated onto six-well culture dishes a day before the transfection and then transiently transfected with LipofectAMINE 2000 according to the manufacturer's instruction.

### **Wound healing assay**

The cells were grown to confluence in six-well plates for 2 days and serum starved overnight. A scrape in the form of a cross was made through the confluent monolayer with a plastic pipette tip. The cells were treated with or without inhibitors, followed by LPA treatment. For siRNA experiment, the cells were first transfected with or without siRNA. Then the confluent cells were serum starved overnight, and a scrape was made before LPA treatment. Several wounded areas were marked for orientation, observed, and then photographed by phase contrast microscopy at 24 h after the scratch.

### **Statistical analysis**

Results are expressed as the mean  $\pm$  S.D. and an analysis was done by one-way Student's t-test. *P*-values less than 0.05 were considered statistically significant.

## **Results**

### **LPA induces COX-2 expression**

Ovarian cancer leads to more fatalities than any other form of gynecological cancer in the developed world. As a possible biomarker for ovarian cancer cells, LPA enhances tumor cell motility, proliferation, angiogenesis, survival, invasion and metastasis through LPA receptors (Fang *et al.*, 2000). It has been recently reported that LPA stimulates the expression of COX-2 that promotes aggressive behavior in ovarian cancer DOV13 cells (Symowicz *et al.*, 2005). To determine the effect of LPA on COX-2 expression in our system, the serum-starved various ovarian cancer cells were stimulated by 25  $\mu$ M LPA for 3 h, and COX expression levels were analyzed by immunoblotting (Figure 1A). Among the tested ovarian cancer cells, COX-1 expression was not detectable even in the presence of LPA. While COX-2 was expressed in both CAOV-3 and 2774 cells as the basal level, LPA induced COX-2 expression only in CAOV-3 cells. Furthermore, LPA induced both COX-2 mRNA and protein expression in a dose-dependent manner in CAOV-3 cells (Figure 1B).

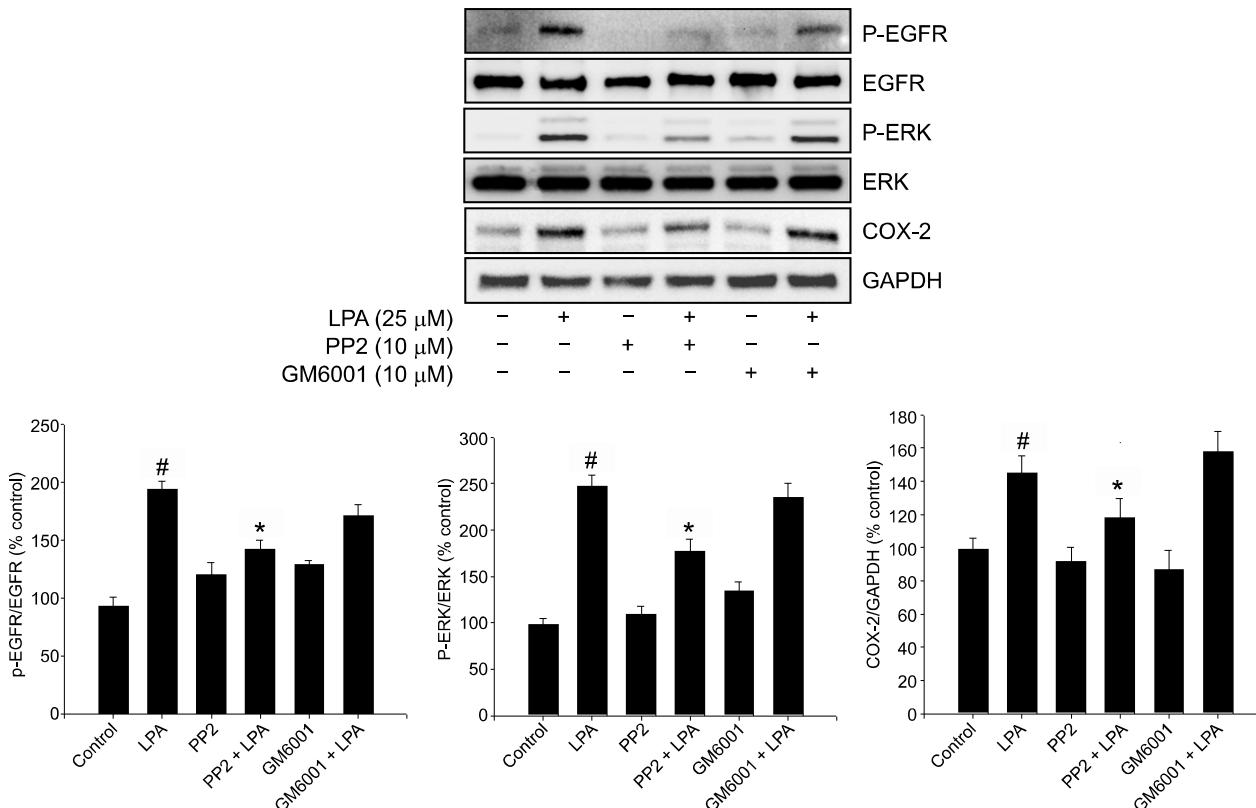
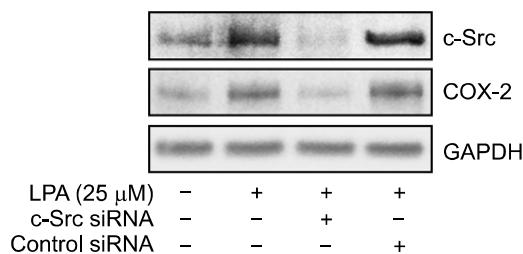
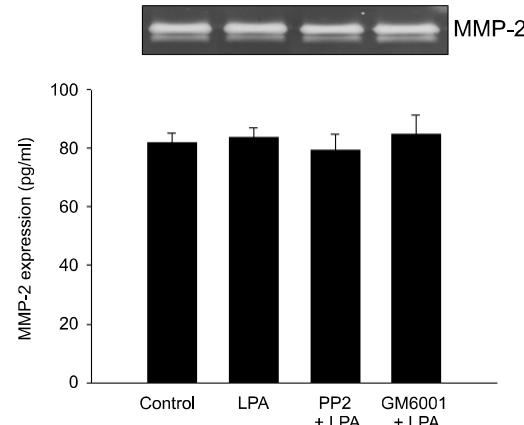
Next, we treated CAOV-3 cells with various pharmacological inhibitors to determine the intracellular signaling pathway of COX-2 expression by LPA. Induction of COX-2 protein by LPA was completely inhibited by pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3 kinase, LY294002 and wortmannin), ERK (PD98059 and U0126), Gi (pertussis toxin) and EGFR (AG1478), suggesting the critical roles of PI3 kinase ERK, Gi and EGFR for COX-2 expression (Figure 1B). To determine the effect of LPA on CAOV-3 cell migration, the cells were stimulated with or without LPA, and the cell migration was measured by wound healing analysis. LPA strongly closed the wound after 24 h of scratch (Figure 1C). However, LPA-induced cell migration was abrogated when the cells were pretreated with PD98059 or U0126. Therefore, these results clearly show the important role of ERK for LPA-induced cell migration in CAOV-3 cells.

### **LPA transactivates EGFR through Gi and Src**

Although the role of EGFR for LPA-induced COX-2 expression was previously studied (Symowicz *et al.*, 2005), little is known how EGFR is transactivated by LPA receptor in ovarian cancer cells. To examine EGFR activation by LPA, CAOV-3 cells were treated with pharmacological inhibitors of MMP (GM6001) or Src (PP2), followed by stimulation with LPA. LPA-induced phosphorylation of EGFR and ERK was significantly inhibited by PP2 treatment (Figure 2A). In addition, PP2 treatment also abrogated COX-2 expression by LPA. However, GM6001 treatment did not show any remarkable inhibition of EGFR phosphorylation and COX-2 expression by LPA. These results indicate that Src but not MMP pathway is necessary for LPA-induced EGFR transactivation and COX-2 expression. To confirm the importance of Src on LPA-induced COX-2 expression, the cells were transfected with selective c-Src siRNA, and the expression of COX-2 was analyzed. As shown in Figure 2B, LPA-induced COX-2 expression was profoundly inhibited by c-Src siRNA. Next, CAOV-3 cells were treated with LPA with or without PP2 and GM6001, and MMP-2 expression and activation were monitored to substantiate our data showing COX-2 expression by LPA independent to MMP. As shown in Figure 2C, LPA did not induce the expression and activation of MMP-2.

### **PGE2 is involved in LPA-induced cell migration**

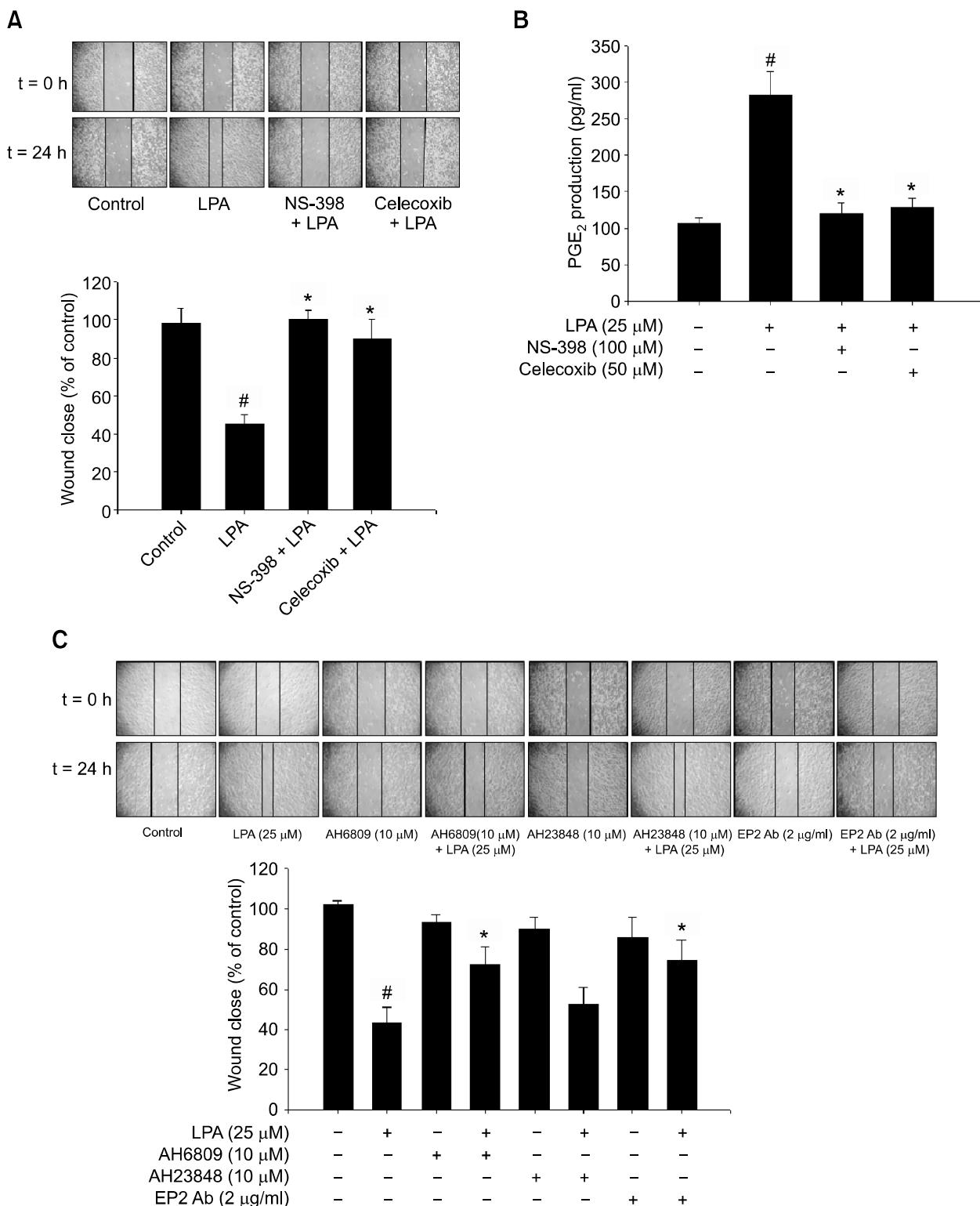
One of the underlying mechanisms of COX-2 induced cell migration is through the production of

**A****B****C**

**Figure 2.** Src is involved in EGFR transactivation. The cells were pretreated with pharmacological inhibitors of Src (PP2) or MMP (GM6001) for 1 h, followed by stimulation with 25 μM LPA. (A) The cell lysates were immunoblotted as described in materials and methods. Anti-p-Tyr antibody was utilized to detect P-EGFR, after the cell lysate was immunoprecipitated with EGFR antibody. Results show a representative blot of three experiments and the mean ± S.D. of three experiments. (B) The cells were transfected with either selective c-Src or negative control siRNA, followed by stimulation with or without 25 μM LPA. (C) Conditioned media was analyzed by gelatin zymography (upper gel) and ELISA (lower graph) to detect MMP-2 activity and total expression, respectively. Results show a representative gel and ELISA of three experiments. #P < 0.05 vs. control, \*P < 0.05 vs. LPA treatment.

PGE<sub>2</sub>, leading to either activation of EGFR (Buchanan *et al.*, 2003) or MAPK/ERK (Krysan *et al.*, 2005). PGE<sub>2</sub> also has been implicated in

tumorigenesis and tumor progression in several cancers (Eberhart *et al.*, 1994; Kargman *et al.*, 1995; Hida *et al.*, 1998). To test the role of COX-2

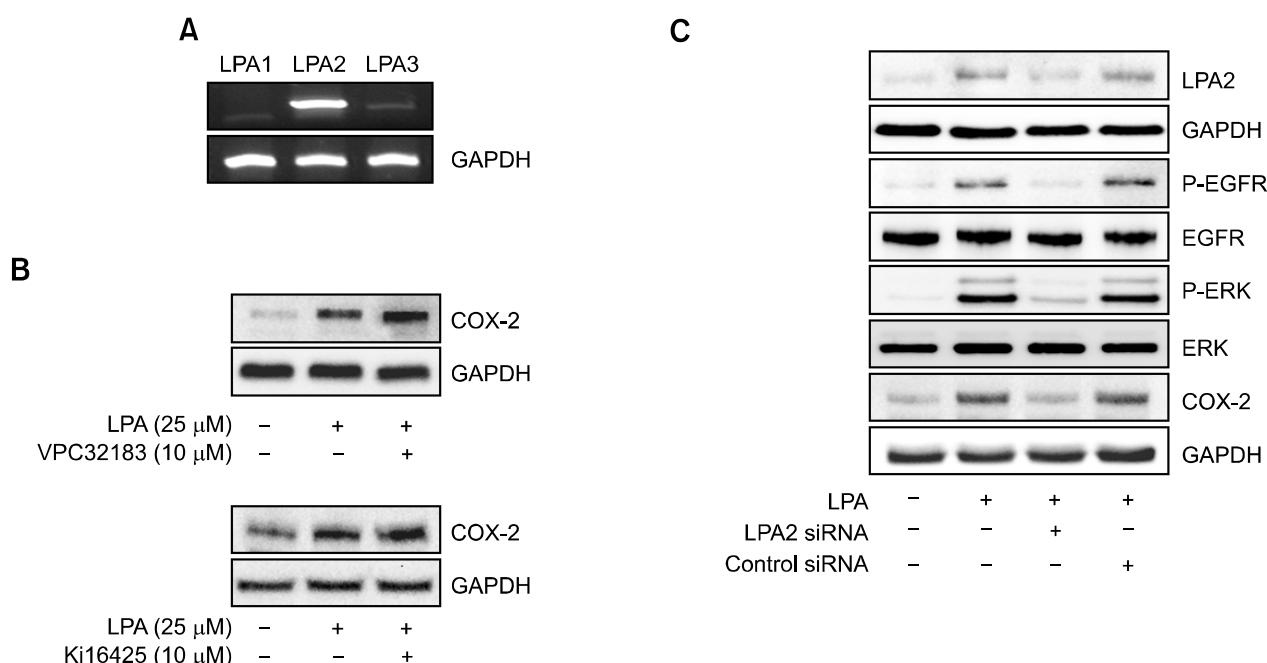


**Figure 3.** EP2 is involved in LPA-induced cell migration. (A) The cells were pre-incubated with or without pharmacological inhibitors of COX-2 (100  $\mu$ M NS-398, 50  $\mu$ M celecoxib), followed by 25  $\mu$ M LPA stimulation. The cell migration was assessed by wound healing at time 0 h and 24 h. (B) Pre-incubated cells with COX-2 inhibitors were stimulated with LPA, and the amount of PGE2 was assessed by ELISA. (C) The cells were pre-incubated with pharmacological inhibitors of EP2 (AH6809), EP4 (AH23848) or anti-EP2 blocking antibody, followed by LPA stimulation. The cell migration was analyzed by wound healing at time 0h and 24 h. Results show a representative of five experiments and the mean  $\pm$  S.D. of five experiments.  $^{\#}P < 0.05$  vs. control,  $^{*}P < 0.05$  vs. LPA treatment.

on LPA-induced cell migration, the cells were pretreated with COX-2 selective inhibitors. As shown in Figure 3A, LPA-induced cell migration was profoundly inhibited by NS-398 and celecoxib, suggesting the importance of COX-2 for LPA-induced CAOV-3 cell migration. Next, we determined the effect of LPA on PGE2 production. Like other ovarian cancer HEY or OVCAR-433 cells (Spinella *et al.*, 2004), CAOV-3 cells secrete more than 100 pg/ml of PGE2 even without any treatment. When the cells were stimulated with LPA, PGE2 secretion was dramatically increased. However, LPA-induced PGE2 production was abrogated in the presence of COX-2 inhibitors (Figure 3B). To gain an insight whether and which receptor of PGE2 is involved in LPA-induced CAOV-3 cell motility, the cells were treated with pharmacological antagonist for EP2 (AH6809) and EP4 (AH23848). As shown in Figure 3C, pretreatment of the cells with AH6809 but not AH23848 significantly inhibited LPA-induced cell migration. Furthermore, treatment of the cells with anti-EP2 antibody profoundly inhibited LPA-induced cell migration. These results suggest the importance of EP2 receptor of PGE2 for CAOV-3 cell migration by LPA.

### LPA<sub>2</sub> is involved in LPA-induced COX-2 expression

There are at least three types of LPA receptors mediating LPA-induced biological phenomenon, and their differential expression were reported in various cancer cells, including ovarian cancer cells (Wang *et al.*, 2007). To examine the mRNA expression of these LPA receptors in CAOV-3 cells, RT-PCR analysis was utilized with specific LPA receptor primers. As shown in Figure 4A, higher mRNA expression of LPA<sub>2</sub> was observed compared to that of LPA<sub>1</sub> and LPA<sub>3</sub>. To test which type of receptor is responsible for LPA-induced COX-2 expression, the cells were incubated with VPC32183 or Ki16425 that inhibits LPA<sub>1</sub> and LPA<sub>3</sub>. As shown in Figure 4B, VPC32183 and Ki16425 did not inhibit LPA-induced COX-2 expression, suggesting that LPA<sub>1</sub> and LPA<sub>3</sub> are not involved in COX-2 expression by LPA. Next, we determined the role of LPA<sub>2</sub> on LPA-induced COX-2 expression by utilizing LPA<sub>2</sub> specific siRNA. Unlike negative control siRNA, transfection of LPA<sub>2</sub> siRNA efficiently reduced the expression of LPA<sub>2</sub> protein (Figure 4C), indicating the specificity of LPA<sub>2</sub> siRNA. Then, the cells were transfected with LPA<sub>2</sub> siRNA, followed by stimulation with LPA. LPA<sub>2</sub>



**Figure 4.** LPA<sub>2</sub> is responsible for phosphorylation of EGFR and ERK, as well as COX-2 expression. (A) Total RNA of CAOV-3 cells were reverse transcribed and amplified utilizing specific primers for each LPA receptors with LPA or not as described in Materials and methods. (B) The cells were pre-treated with or without pharmacological inhibitors of LPA<sub>1</sub> & LPA<sub>3</sub> (VPC32183, Ki16425), followed by stimulation with indicated concentration of LPA. The cell lysates were analyzed by immunoblotting. Results show a representative blot of three experiments. (C) The cells were transfected with either LPA<sub>2</sub> specific siRNA or negative control siRNA, followed by stimulation with or without 25 μM LPA. The cell lysates were subject to immunoblotting against each antibody. Results show a representative blot of three experiments.

siRNA completely blocked LPA-induced COX-2 protein expression (Figure 4C), suggesting that LPA2 is necessary for COX-2 expression by LPA. Moreover, transfection of the cells with LPA2 siRNA inhibited LPA-induced phosphorylation of EGFR and ERK that are critical for COX-2 expression by LPA. Therefore, these results clearly demonstrate the essential roles of LPA2 on activation of EGFR and ERK, leading to COX-2 expression.

### LPA2 is responsible for CAOV-3 cell migration

LPA receptors are implicated in the cell motility of various types of cells. LPA1 is linked to MC3T3E1 osteoblast motility (Masiello *et al.*, 2006), while LPA2 is involved in RAFTK/PyK2 phosphorylation and PC12 cell motility (Park *et al.*, 2006). Since LPA2 is overexpressed in CAOV-3 cells and involved in COX-2 expression, we tested the role of LPA2 on cell migration. The cells were transfected with either LPA2 specific siRNA or negative control siRNA, followed by stimulation with or without LPA. Transfection of the cells with LPA2 siRNA but not negative control siRNA abrogated

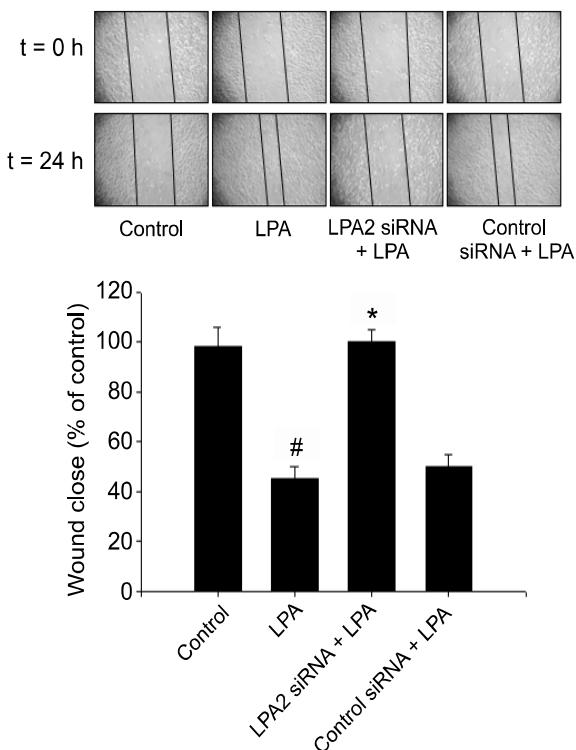
LPA-induced cell migration, indicating the critical role of LPA2 on CAOV-3 cell migration (Figure 5).

### Discussion

Tumor cell invasion and metastasis is a complex, multi-step process during which tumor cells spread from the primary tumor to distant organs (Yang *et al.*, 2006). Accumulated clinical and experimental evidence indicates that COX-2 is involved in the metastatic phenotypes of cancers, including ovarian cancer. In the present study, we for the first time demonstrate the importance of LPA receptor and Gi/Src signaling for LPA-induced EGFR activation, COX-2 expression, as well as motility stimulation in CAOV-3 ovarian cancer cells.

There are several signaling pathways for EGFR transactivation by LPA receptor. One of them involves Gi and Src. Pretreatment of the adrenal glomerulosa cells with PP2, a Src inhibitor, inhibited EGFR transactivation and ERK activation by LPA (Shah *et al.*, 2005). Another well-known pathway for EGFR transactivation is matrix metalloproteinase (MMP) activation by LPA receptor. MMP stimulates proteolytic cleavage of transmembrane precursors, such as pro-HB-EGF, which subsequently activates the EGFR family (Prenzel *et al.*, 1999; Gschwind *et al.*, 2002). In human bronchial epithelial cells, activation of Lyn and MMP but not Src are required for EGFR transactivation by LPA (Zhao *et al.*, 2006). In the present study, we showed that Src, but not MMP pathway is involved in LPA-induced EGFR activation and COX-2 expression. Pretreatment of the cells with PP2 abolished phosphorylation of EGFR and ERK, as well as COX-2 expression by LPA (Figure 2A). Furthermore, LPA-induced COX-2 expression was also significantly inhibited by Src siRNA pretreatment. Previous report claimed that MMP activation for proteolytic cleavage of transmembrane precursors was a critical step for EGFR activation in human colon cancer cells (Shida *et al.*, 2005). This difference could be due to the types of cancer cells tested.

The relative expression of LPA receptor isoforms depends on the type of cells. While higher expression of LPA-1 was reported in OVCAR-3 cells, LPA3 is the major receptor type in SKOV-3 cells (Park *et al.*, 2007). In the present study, we showed that LPA2 expression is higher than other types of LPA receptor in CAOV-3 cells. These results are in good accord with previous report (Fang *et al.*, 2004). The roles of LPA receptor on cancer cell motility are well documented. LPA1 is required for the motility of breast, prostate, and melanoma



**Figure 5.** LPA2 is involved in LPA-induced cell motility. The cells were transfected with either LPA2 or negative control siRNA, followed by stimulation with or without 25  $\mu$ M LPA. Results show a representative of four experiments and the mean  $\pm$  S.D. of four experiments.  $^{\#}P < 0.05$  vs. control,  $^{*}P < 0.05$  vs. LPA treatment.

cells (Hama *et al.*, 2004). Human pancreatic cancer cells also use LPA1 for LPA-induced cell migration (Yamada *et al.*, 2004). However, the LPA receptor responsible for LPA-induced ovarian cancer cell migration is controversial. While LPA2 was reported to mediate LPA-induced human ovarian cancer cell migration (Xu *et al.*, 2004; Symowicz *et al.*, 2005), Sugimoto *et al.* recently claimed that LPA1 is critical for motility stimulation by LPA in CHO cells (2006). Furthermore, little is known which LPA receptor is responsible for COX-2 expression to stimulate ovarian cancer cell motility. We therefore determined the receptor responsible for LPA-induced COX-2 expression and motility stimulation. Our data clearly demonstrate that LPA2 is critical for COX-2 expression, leading to ovarian cancer cell motility stimulation by LPA. First, much higher expression of LPA2 than LPA1 and LPA3 was shown in CAOV-3 cells. Second, transfection of the cells with LPA2 selective siRNA inhibited phosphorylation of ERK and EGFR, as well as COX-2 expression. Finally and most importantly, transfection of selective LPA2 siRNA abolished motility stimulation by LPA.

In conclusion, our data clearly show that LPA stimulates COX-2 expression and cell motility through LPA2/Gi/Src/EGFR/ERK signaling cascade in ovarian cancer cells. Our results will help to understand the molecular mechanism for LPA-induced motility and useful approach for inhibition of motility in ovarian cancer progression.

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### References

- An S, Bleu T, Hallmark OG, Goetzl EJ. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* 1998;273:7906-10
- Bando K, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H, Inoue K. Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* 1999;274:27776-85
- Buchanan FG, Wang D, Bargiacchi F, DuBois RN. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* 2003;278:35451-7
- Cao Y, Prescott SM. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. *J Cell Physiol* 2002;190:279-86
- Contos JJ, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J. Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Biol* 2002;22:6921-9
- Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;107:1183-8
- Fang X, Gaudette D, Furui T, Mao M, Estrella V, Eder A, Pustilnik T, Sasagawa T, Lapushin R, Yu S, Jaffe RB, Wiener JR, Erickson JR, Mills GB. Lysophospholipid growth factors in the initiation, progression, metastases, and management of ovarian cancer. *Ann N Y Acad Sci* 2000;905:188-208
- Fang X, Schummer M, Mao M, Yu S, Tabassam FH, Swaby R, Hasegawa Y, Tanyi JL, LaPushin R, Eder A, Jaffe R, Erickson J, Mills GB. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. *Biochim Biophys Acta* 2002;1582:257-64
- Fang X, Yu S, Bast RC, Liu S, Xu HJ, Hu SX, LaPushin R, Claret FX, Aggarwal BB, Lu Y, Mills GB. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J Biol Chem* 2004;279:9653-61
- Fujita T, Miyamoto S, Onoyama I, Sonoda K, Mekada E, Nakano H. Expression of lysophosphatidic acid receptors and vascular endothelial growth factor mediating lysophosphatidic acid in the development of human ovarian cancer. *Cancer Lett* 2003;192:161-9
- Gardner MJ, Jones LM, Catterall JB, Turner GA. Expression of cell adhesion molecules on ovarian tumour cell lines and mesothelial cells, in relation to ovarian cancer metastasis. *Cancer Lett* 1995;91:229-34
- Gschwind A, Prenzel N, Ullrich A. Lysophosphatidic acid-induced squamous cell carcinoma cell proliferation and motility involves epidermal growth factor receptor signal transactivation. *Cancer Res* 2002;62:6329-36
- Hama K, Aoki J, Fukaya M, Kishi Y, Sakai T, Suzuki R, Ohta H, Yamori T, Watanabe M, Chun J, Arai H. Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1. *J Biol Chem* 2004;279:17634-9
- Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugiura T, Takahashi T. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 1998;58:3761-4
- Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res* 1995;55:2556-9
- Klimp AH, Hollema H, Kempinga C, van der Zee AG, de Vries EG, Daemen T. Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. *Cancer Res* 2001;61:

7305-9

Kohn EC, Mills GB, Liotta L. Promising directions for the diagnosis and management of gynecological cancers. *Int J Gynaecol Obstet* 83 Suppl 2003;1:203-9

Kotarsky K, Boketoft A, Bristulf J, Nilsson NE, Norberg A, Hansson S, Owman C, Sillard R, Leeb-Lundberg LM, Olde B. Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. *J Pharmacol Exp Ther* 2006;318:619-28

Krysan K, Reckamp KL, Dalwadi H, Sharma S, Rozengurt E, Dohadwala M, Dubinett SM. Prostaglandin E2 activates mitogen-activated protein kinase/Erk pathway signaling and cell proliferation in non-small cell lung cancer cells in an epidermal growth factor receptor-independent manner. *Cancer Res* 2005;65:6275-81

Lakka SS, Jasti SL, Kyritsis AP, Yung WK, Ali-Osman F, Nicolson GL, Rao JS. Regulation of MMP-9 (type IV collagenase) production and invasiveness in glioma by the extracellular signal-regulated kinase and jun amino-terminal kinase signaling cascades. *Clin Exp Metastasis* 2000;18:245-52

Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* 2006;281:23589-97

Lee J, Jung ID, Chang WK, Park CG, Cho DY, Shin EY, Seo DW, Kim YK, Lee HW, Han JW, Lee HY. p85 beta-PIX is required for cell motility through phosphorylations of focal adhesion kinase and p38 MAP kinase. *Exp Cell Res* 2005;307:315-28

Masiello LM, Fotos JS, Galileo DS, Karin NJ. Lysophosphatidic acid induces chemotaxis in MC3T3-E1 osteoblastic cells. *Bone* 2006;39:72-82

Moolenaar WH, Kranenburg O, Postma FR, Zondag GC. Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr Opin Cell Biol* 1997;9:168-73

Noguchi K, Ishii S, Shimizu T. Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* 2003;278:25600-6

Park KS, Lee HY, Lee SY, Kim MK, Kim SD, Kim JM, Yun J, Im DS, Bae YS. Lysophosphatidylethanolamine stimulates chemotactic migration and cellular invasion in SK-OV3 human ovarian cancer cells: involvement of pertussis toxin-sensitive G-protein coupled receptor. *FEBS Lett* 2007;581:4411-6

Park SY, Schinkmann KA, Avraham S. RAFTK/Pyk2 mediates LPA-induced PC12 cell migration. *Cell Signal* 2006;18:1063-71

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. EGF receptor transactivation by

G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 1999;402:884-8

Shah BH, Baukal AJ, Shah FB, Catt KJ. Mechanisms of extracellularly regulated kinases 1/2 activation in adrenal glomerulosa cells by lysophosphatidic acid and epidermal growth factor. *Mol Endocrinol* 2005;19:2535-48

Shida D, Kitayama J, Yamaguchi H, Yamashita H, Mori K, Watanabe T, Nagawa H. Lysophosphatidic acid transactivates both c-Met and epidermal growth factor receptor, and induces cyclooxygenase-2 expression in human colon cancer LoVo cells. *World J Gastroenterol* 2005;11:5638-43

Spinella F, Rosano L, Di Castro V, Natali PG, Bagnato A. Endothelin-1-induced prostaglandin E2-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. *J Biol Chem* 2004;279:46700-6

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

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

Wang P, Wu X, Chen W, Liu J, Wang X. The lysophosphatidic acid (LPA) receptors their expression and significance in epithelial ovarian neoplasms. *Gynecol Oncol* 2007;104:714-20

Xu J, Lai YJ, Lin WC, Lin FT. TRIP6 enhances lysophosphatidic acid-induced cell migration by interacting with the lysophosphatidic acid 2 receptor. *J Biol Chem* 2004;279:10459-68

Xu Y, Gaudette DC, Boynton JD, Frankel A, Fang XJ, Sharma A, Hurteau J, Casey G, Goodbody A, Mellors A, et al. Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. *Clin Cancer Res* 1995;1:1223-32

Yamada T, Sato K, Komachi M, Malchinkhuu E, Tobo M, Kimura T, Kuwabara A, Yanagita Y, Ikeya T, Tanahashi Y, Ogawa T, Ohwada S, Morishita Y, Ohta H, Im DS, Tamoto K, Tomura H, Okajima F. Lysophosphatidic acid (LPA) in malignant ascites stimulates motility of human pancreatic cancer cells through LPA1. *J Biol Chem* 2004;279:6595-605

Yang J, Mani SA, Weinberg RA. Exploring a new twist on tumor metastasis. *Cancer Res* 2006;66:4549-52

Zhao Y, He D, Saatian B, Watkins T, Spannhake EW, Pyne NJ, Natarajan V. Regulation of lysophosphatidic acid-induced epidermal growth factor receptor transactivation and interleukin-8 secretion in human bronchial epithelial cells by protein kinase Cdelta, Lyn kinase, and matrix metalloproteinases. *J Biol Chem* 2006;281:19501-11