Integrity of SOS1/EPS8/ABI1 Tri-Complex Determines Ovarian Cancer Metastasis

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Abstract

Ovarian cancer is mainly confined in peritoneal cavity and its metastasis is often associated with the formation of malignant ascites. As lysophosphatidic acid (LPA) is present at high levels in ascites of ovarian cancer patients and potently stimulates cell migration, we reason that LPA-stimulated cell migration may play an important role in ovarian cancer metastasis. Here, we show that only ovarian cancer cell lines with LPA migratory response undergo peritoneal metastatic colonization. LPA-stimulated cell migration is required for metastatic colonization because knockdown of LPA receptor subtype 1 (LPAR1) abolishes this event. However, the difference in metastatic potentials is not caused by the absence of LPAR1 because both metastatic and nonmetastatic lines express similar levels of LPAR1. Instead, we find that LPA can activate Rac only in metastatic cells and that metastatic colonization of ovarian cancer cells necessitates Rac activity. These results thus suggest that LPA-induced Rac activation is a prerequisite for ovarian cancer metastasis. In metastatic cells, Rac activation is facilitated by SOS1/EPS8/ABI1 tri-complex and the integrity of this tri-complex is essential for LPA-stimulated cell migration and metastatic colonization. We show that at least 1 member of SOS1/EPS8/ABI1 tri-complex is absent in nonmetastatic ovarian cancer cells and reexpressing the missing one conferred them with metastatic capability. Importantly, coexpression of SOS1, EPS8, and ABI1, but not of any individual member of SOS1/EPS8/ABI1 tri-complex, correlates with advanced stages and shorter survival of ovarian cancer patients. Our study implicates that the integrity of SOS1/EPS8/ABI1 tri-complex is a determinant of ovarian cancer metastasis. Cancer Res; 70(23); 9979–90. ©2010 AACR.

Introduction

Ovarian cancer has the highest mortality rate among gynecologic cancers mainly due to complication of metastasis (1). Unlike other solid tumors that rely on the vasculature for metastasis, ovarian cancer is predominantly confined within the abdominal cavity and spread by direct extension to adjacent organs and/or disseminate throughout the peritoneal cavity (2, 3). A widely recognized behavior of ovarian cancer is its ability to seed the abdominal cavity with tumor implants, subsequent migration of tumor cells into peritoneum and underlying organs, and the formation of ascites (3, 4). Apparently, the ability of ovarian cancer cells to migrate is essential for ovarian cancer metastasis.

Cell migration is a complex process involving the reorganization of actin cytoskeleton that is facilitated by the members of the Rho GTases including Rac, Cdc42, and Rho (5–7). In the process of cell migration, Rac facilitates the formation of actin-rich membrane ruffle, called lamellipodia, at the leading edge of migrating cells. Cdc42 regulates cell polarity and filopodia formation, thus controlling the direction of cell movement. Rho controls the formation of stress fibers and maintains focal adhesions at the rear of the cells (6). Ras has also been implicated to play an important role in cell migration because microinjection of Ras-neutralizing antibody blocks cell migration (7) and forced expression of constitutively active Ras mutant enhances ovarian cancer cell migration (8). As Ras can activate Rac through Tiam1 (9), β-PIX (10), or SOS1/EPS8/ABI1 tri-complex (11, 12), it is likely that Ras may facilitate cell migration by regulating Rac activity.

Lysophosphatidic acid (LPA) is a growth factor–like phospholipid and has been uniquely linked to ovarian malignancies. For example, LPA triggers protease production/activation (13, 14) and Cox-2 expression (15), thus facilitating ovarian cancer cell invasion. It also promotes angiogenesis by inducing the expression of various proangiogenic factors including VEGF (16), IL8 (17), and Gro-α (18). Moreover, LPA can be produced and secreted into peritoneal cavity by both ovarian cancer cells (19, 20) and mesothelial cells...
(21). Importantly, LPA is present at high levels in ascites of ovarian cancer patients (22–25) and potently stimulates ovarian cancer cell migration (8, 26, 27). Therefore, ascite-borne LPA is likely to play a critical role in ovarian cancer metastasis by facilitating cell migration.

In this study, we demonstrated an excellent correlation between LPA migratory responses and metastatic potentials in a panel of ovarian cancer cell lines. Preventing LPA-stimulated cell migration by silencing LPA receptor subtype 1 (LPAR1) diminished peritoneal metastatic colonization of ovarian cancer cells, implicating the importance of LPA-stimulated cell migration in ovarian cancer metastasis. LPA activates Rac only in metastatic cells, and a signaling pathway consisting of Ras-SOS1/EPS8/ABI1 tri-complex mediates LPA-induced Rac activation. Interestingly, 1 or more members of SOS1/EPS8/ABI1 tri-complex are absent in nonmetastatic ovarian cancer cells; however, reexpressing the missing member converts the nonmetastatic lines to metastatic ones. Finally, we show that SOS1/EPS8/ABI1 coexpression, but not any one alone, correlates with advanced clinical stage and shorter survival of ovarian cancer patients.

Materials and Methods

Cells, shRNAs, and other reagents

All cells were maintained in DMEM containing 10% fetal calf serum at 37°C in a humidified incubator supplied with 5% CO2. The shRNA sequences for each target genes were designed using web-based Block-iT program (Invitrogen) and subcloned into pLV-shRNA vector (Biosettia). Information for shRNA sequences, antibodies, and other reagents are described in Supplementary Data.

Transwell cell migration assay

Transwell cell migration was assayed as previously described (28). Briefly, the lower phase of Transwell was coated with 10 μg/mL of collagen I or laminin, and serum-free medium with or without 10 μmol/L of LPA was added to lower chambers. Serum-starved cells (105 per well) were added to Transwells and allowed to migrate for 4 hours. Cells that remained in Transwells were removed with cotton swabs, and cells attached on the lower phase of Transwells were stained with crystal violet solution. The stained cells were solubilized with 10% acetic acid and quantitated on a microplate reader.

Peritoneal metastatic colonization assay

Peritoneal metastatic colonization assays were performed as previously described (29, 30). Briefly, cells in the log phase were trypsinized and resuspended in PBS. Six-week-old athymic female nude mice (Hsd: athymic Nude-Foxn1nu, Harlan Sprague Dawley) were intraperitoneally injected with 106 cells per mouse. To determine the role of LPAR1, SOS1, EPS8, and ABI1 in metastatic colonization, SK-OV3 and HEY cells expressing shRNAs against these genes were intraperitoneally injected into mice. To determine the importance of Rac activity, cells expressing constitutively active Rac1 (RacG12V) or dominant-negative Rac1 (Rac1T17N) were injected into mice. Five weeks after injection, the mice were sacrificed and autopsied. Visible metastatic implants were also collected and weighed. All procedures were approved by the Institution Animal Care Committee at Medical College of Georgia.

Real-time quantitative PCR

Total RNA was extracted from cells using Trizol (Invitrogen), treated by DNase I and reverse transcribed with SuperScriptase II (Invitrogen). Generated cDNA was subjected to real-time PCR to measure LPAR1, LPAR2, LPAR3, and GAPDH levels with the respective TaqMan probes (Applied Biosystem). The expression levels were standardized by comparing the Ct values of target with that of GAPDH.

Rac, Cdc42, and Rho activity assays

Activation of Rac/Cdc42, and Rho was measured with the Rac/Cdc42 and the Rho activity assay kits (Cellbio Labs). To determine the effect of LPA on Rac, Cdc42, or RhoA activity, cells (2 × 106 cells per 10-cm dish) were serum-starved for 2 days and then stimulated with 10 μmol/L of LPA for various times. The cells were lysed and analyzed for Rac, Cdc42, or RhoA activity. To determine the effect of constitutively active Ras on Rac activity, HEY, IGROV1, OVCAR3, and SK-OV3 cells were infected with empty or H-RasG12V-containing retrovirus for 2 days followed by 2-day puromycin selection. The cells were lysed and analyzed for Rac activity. To determine how silencing Tiam1, SOS1, EPS8, and ABI1 affected LPA-induced Rac activation, HEY and SK-OV3 cells were incubated with lentiviral vectors containing the respective shRNAs for 2 days and starved for another 2 days. The cells were stimulated with 10 μmol/L of LPA for 2 or 5 minutes followed by the analysis of Rac activity. To determine the effect of forced expression of SOS1, EPS8, and ABI1 on LPA-induced Rac activation, TOV21G, IGROV1, and OVCAR3 cells were infected with lentiviral vectors encoding SOS1, EPS8, and ABI1, respectively, for 2 days and starved for another 2 days. The cells were stimulated with 10 μmol/L of LPA for 5 minutes followed by the analysis of Rac activity.

Immunofluorescence

Cells were cultured on 10 μg/mL of collagen I–coated coverslips overnight in serum-free condition and then treated with 10 μmol/L of LPA for 30 minutes. The cells were fixed with 3% paraformaldehyde and permeabilized with 1% Triton X-100 followed by 1-hour incubation with rhodamine-conjugated phalloidin. The polymerized actin was visualized using a fluorescence microscope (Axiovert 200M; Zeiss).

Histochemistry

Specimens were collected from 64 epithelial ovarian cancer patients who underwent initial treatment at Zhongnan...
Hospital of Wuhan University from July 2002 to July 2006 (their clinicopathologic parameters are described in Supplementary Table S1). Patients were followed up until November 2009. Overall survival was defined from initial treatment until cancer-related death or the last follow-up. Samples were processed in compliance with a protocol approved by the Ethics Committee of Wuhan University. All experiments conformed to the legal mandates implemented in China, and written informed consent was received from each patient. The immunohistochemistry of SOS1, EPS8, and ABI1 was performed as previously described (31). Briefly, 4 μm thick sections of formalin-fixed/paraffin-embedded were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide treatment. Sections were blocked with rabbit serum and incubated with antibodies against SOS1, EPS8, or ABI1. Antigens were visualized by streptavidin-biotin-peroxidase complex method. Immunostaining was evaluated by 2 pathologists without knowledge of patients’ clinical information. All 3 antigens were found to be localized in cytoplasm. Extent of immunostaining was graded on the basis of the percentage of cells displaying staining. “−” is considered as negative staining (<10%); “+”, “++”, and “+++” were considered as positive staining (10%–25%, 25%–50%, and >50%, respectively).

Statistical analysis
Statistical analyses of cell migration, metastatic implant weights, and LPAR1 mRNA levels were performed by ANOVA and Student’s t test. The chi-square test and Fisher’s exact test were used to compare covariates between SOS1/EPS8/ABI1 tri-complex staining and clinicopathologic parameters. Survival curves were plotted according to the estimate of Kaplan and Meier. The log-rank test was used to determine the significance of differences in survival distribution. Statistical analyses were aided by SPSS (release 15.0; SPSS Inc.). All statistical tests were 2-sided, and P < 0.05 was considered to be significant.

Results
LPA migratory response is associated with ovarian cancer metastatic colonization
The fact that LPA levels are elevated in ascites of ovarian cancer patients and LPA serves as a motility stimulator prompted us to hypothesize that LPA-stimulated cell migration is necessary for ovarian cancer metastasis. To test it, we first determined whether LPA migratory responses correlated with metastatic potentials in ovarian cancer cells. Transwell migration assay showed that LPA increased cell migration in ES2, HEY, OVCAR433, OVCAR5, and SK-OV3 lines on both collagen I- and laminin-coated surfaces but did poorly in HEC1A, IGROV1, OVCAR3, and TOV21G lines (Fig. 1A and Supplementary Fig. S1). With the aid of a well-established peritoneal seeding model (29, 30), we found that animals receiving LPA-responsive lines (ES2, HEY, OVCAR433, OVCAR5, and SK-OV3) displayed overt metastatic implants on omentum, liver, diaphragm, and mesentery (Fig. 1B and Supplementary Fig. S2). In contrast, metastatic colonization was not detected in animals receiving LPA-unresponsive lines (HEC1A, IGROV1, OVCAR3, and TOV21G) (Fig. 1B and Supplementary Fig. S2). These results show that LPA migratory response correlates with metastatic potential in ovarian cancer cells. LPAR1 is known to mediate LPA-stimulated cell migration in various cell types including ovarian cancer cells (32–34). We lentivirally introduced LPAR1, LPAR2, or LPAR3 shRNA into metastatic SK-OV3 and HEY cells and confirmed that these shRNAs specifically reduced their respective target expression (Supplementary Fig. S3). Knockdown of LPAR1 greatly inhibited LPA-stimulated cell migration in SK-OV3 and HEY cells (Fig. 1C), whereas LPAR2 shRNA displayed slight inhibition and LPAR3 shRNA exhibited no effect on LPA-stimulated cell migration (Supplementary Fig. S3). In the following experiment, we intraperitoneally injected control (luciferase shRNA) or LPAR1-knockdown cells into nude mice. Five weeks later, mice receiving control cells developed metastatic implants in their peritoneal cavities. However, mice receiving LPAR1-knockdown cells had much less metastatic colonization and the weight of implants was reduced by greater than 90% in both SK-OV3 and HEY cells (Fig. 1D and Supplementary Fig. S4). These results suggest that LPA-stimulated cell migration is required for ovarian cancer metastasis. Rac is activated only by LPA in metastatic ovarian cancer cells and required for metastatic colonization
To determine what caused the difference in LPA migratory responses between metastatic and nonmetastatic cells, we initially measured the levels of LPAR1 mRNA in these lines. Except HEC1A that did not have detectable LPAR1 mRNA, metastatic and nonmetastatic lines exhibited similar LPAR1 mRNA levels (Supplementary Fig. S5). Subsequently, we lentivirally overexpressed LPAR1 in IGROV1, OVCAR3, and SK-OV3 cells. Although SK-OV3 cells with LPAR1 overexpression displayed greater LPA migratory response than the parental cells, enforced LPAR1 expression could not render nonmetastatic IGROV1 and OVCAR3 cells responding to LPA for cell migration or undergoing metastatic colonization (Supplementary Fig. S5). These results indicate that the defective LPA migratory response in nonmetastatic lines occurs downstream of LPAR1. Because of the critical role of cytoskeleton reorganization in cell migration (26), we next examined the effect of LPA on cytoskeleton reorganization. Cells were treated with LPA followed by staining with rhodamine-conjugated phalloidin to detect F-actin, which was observed on the plasma membrane of unstimulated cells (Fig. 2A). Exposure to LPA led to dramatic actin reorganization in metastatic HEY and SK-OV3 cells but did little in nonmetastatic IGROV1 and OVCAR3 cells (Fig. 2A). As cytoskeleton reorganization is facilitated by Rac, Cdc42, and Rho (5), we examined the effect of LPA on their activities in these lines. Rho was similarly activated by LPA in all lines (Fig. 2B). Cdc42 was slightly activated by LPA in OVCAR3 cells and unaffected in other lines (Fig. 2B). In contrast, LPA significantly activated Rac in metastatic HEY and SK-OV3 cells but not in nonmetastatic IGROV1 and
OVCAR3 cells (Fig. 2B). These results suggest that the inability of LPA to activate Rac may account for lacking LPA-induced cytoskeleton reorganization and cell migration in nonmetastatic cells.

To further investigate the association between Rac- and LPA-stimulated cell migration, we inhibited Rac activity by introducing dominant-negative Rac1 (Rac1T17N) in metastatic SK-OV3 and HEY cells. Forced Rac1T17N expression

Figure 1. LPA-stimulated cell migration and metastatic colonization of ovarian cancer cells. A, cell migration was analyzed using Transwells with or without 10 μmol/L of LPA contained in the lower chambers. The lower phase of Transwells was coated with 10 μg/mL of collagen I. Images are stained cells on the lower phases. Cell migration is presented as the OD_{600 nm}. Data are means ± SE. n = 3. *, P < 0.001 versus 0 μmol/L of LPA; #, P < 0.05 versus 0 μmol/L of LPA. B, cells from various ovarian cancer lines were intraperitoneally injected to nude mice for 5 weeks to allow metastatic colonization. Images are the views of various areas in peritoneal cavity. Arrows point to metastatic implants. Metastatic implants were collected and weighed. Data are means ± SE. n = 6. C, SK-OV3 and HEY cells were lentivirally transduced with luciferase or LPAR1 shRNA and subsequently analyzed for LPA-stimulated cell migration. Results are presented as fold increase of cell migration [(LPA-stimulated cell migration)/(basal cell migration)]. Data are means ± SE. n = 3. *, P < 0.001 versus sh-Luc. D, control or LPAR1 knockdown cells were intraperitoneally injected to nude mice for 5 weeks. Metastatic implants were collected and weighed. Data are means ± SE. n = 6. *, P < 0.001 versus sh-Luc.
Figure 2. Rac regulates LPA-stimulated cell migration and metastatic colonization. A, cells were stimulated with 10 μmol/L of LPA for 30 minutes followed by immunostaining with rhodamine-conjugated phalloidin. Cytoskeleton reorganization was visualized under a fluorescence microscope. B, cells were stimulated with 10 μmol/L of LPA for various times and then analyzed for Rac, Cdc42, and RhoA activities. C, SK-OV3 and HEY cells were lentivirally transduced with empty vector (control) or vector-containing Rac1T17N, whereas IGROV1, OVCAR3, and TOV21G cells were lentivirally transduced with empty vector (control) or vector containing Rac1G12V. Transduced cells were analyzed for LPA-stimulated cell migration. Results are presented as fold increase of cell migration [(LPA-stimulated cell migration)/(basal cell migration)]. Data are means ± SE. n = 3. *, P < 0.001 versus control; **, P < 0.005 versus control; #, P < 0.05 versus control. D, Rac1T17N-expressing SK-OV3 and HEY cells or Rac1V12G-expressing OVCAR3 and IGROV1 cells were injected into nude mice. Five weeks after injection, metastatic implants were collected and weighed. Data are the mean ± SE. n = 6. *, P < 0.001 versus control; **, P < 0.005 versus control.
completely abolished LPA-stimulated cell migration in these cells (Fig. 2C). In parallel, we expressed constitutively active Rac1 (Rac1G12V) in nonmetastatic IGROV1, OVCAR3, and TOV21G lines and found that enforced Rac1G12V expression rendered them responsive to LPA for cell migration (Fig. 2C).

We next injected dominant-negative Rac1 (Rac1T17N)-expressing HEY or SK-OV3 cells into mice and found that Rac1T17N expression eliminated 100% and 98.35% metastatic colonization in mice, respectively (Supplementary Fig. S6A and Fig. 2D). Meanwhile, we injected constitutively active Rac1 (Rac1G12V)-expressing IGROV1 and OVCAR3 cells into mice. Although there was no detectable metastatic implant in mice receiving control cells, implants were readily seen in mice receiving Rac1G12V-expressing cells (Supplementary Fig. S6B and Fig. 2D). These results implicate that Rac is a key metastasis regulator.

**SOS1/EPS8/ABI1 tri-complex is involved in LPA-induced Rac activation and metastatic colonization**

To characterize the signaling pathway mediating LPA-induced Rac activation, we turned our attention on Ras, as LPA can activate Ras in metastatic ovarian cancer cells (8, 26) and Ras can activate Rac in various cell types (9, 11). We introduced dominant-negative H-Ras (H-RasT17N) into HEY and SK-OV3 cells and found that inhibiting Ras activity abrogated LPA-induced Rac activation (Fig. 3A), suggesting that Ras mediates LPA-induced Rac activation. We next treated nonmetastatic IGROV1 and OVCAR3 cells with LPA and observed that LPA effectively activated Ras in both cell lines (Supplementary Fig. S7), ruling out the possibility that inability of LPA to activate Ras led to the defect in Rac activation. In further experiment, we introduced constitutively active Ras (H-RasG12V) into ovarian cancer cells. H-RasG12V elevated Rac activity in metastatic HEY and SK-OV3 cells, and LPA treatment did not further increase Rac activity (Fig. 3A). In contrast, H-RasG12V could not activate Rac in nonmetastatic IGROV1 or OVCAR3 cells (Fig. 3A). These results suggest that Ras can activate Rac only in metastatic ovarian cancer cells.

Ras can activate Rac through Tiam1 (9), β-PIX (10), or SOS1/EPS8/ABI1 tri-complex (11). We determined their potential involvement by individually silencing their expression in HEY and SK-OV3 cells (Fig. 3B and Supplementary Fig. S8A and D). Although Tiam1 shRNAs and β-PIX siRNA pool effectively inhibited their respective target expression, they did not alter LPA- or H-RasG12V–induced Rac activation (Supplementary Fig. S8A and D). In contrast, SOS1, EPS8, and ABI1 shRNAs all blocked LPA- or RasG12V–induced Rac activation (Fig. 3B). Transwell migration assay also showed that knockdown of SOS1, EPS8, or ABI1, but not Tiam1, greatly decreased LPA-induced cell migration in SK-OV3 and HEY cells (left panel, Fig. 3C). Similarly, disrupting SOS1/EPS8/ABI1 tri-complex blocked RasG12V-induced migration of SK-OV3 (right panel, Fig. 3C). The inhibitory effect of SOS1/EPS8/ABI1 shRNAs on Rac activation and cell migration was specific, as forcing the expression of respective murine counterparts largely restored these 2 events (data not shown). When SOS1-, EPS8-, or ABI1-knockdown cells were analyzed for peritoneal metastatic colonization, we detected a significant reduction in the weight of metastatic implants in mice receiving knockdown cells compared with mice receiving the control cells (Fig. 3D and Supplementary Fig. S9).

**SOS1/EPS8/ABI1 tri-complex is not intact in nonmetastatic ovarian cancer cells**

To further investigate the importance of SOS1/EPS8/ABI1 tri-complex in metastasis, we performed immunoblotting to examine the expression of SOS1, EPS8, and ABI1 in ovarian cancer lines. All members of this tri-complex were expressed in metastatic ES2, HEY, OVCAR433, OVCAR5, and SK-OV3 lines (Fig. 4A). In contrast, at least 1 member of this tri-complex was absent in nonmetastatic lines: EPS8 in IGROV1, ABI1 in OVCAR3, SOS1 in TOV21G line, and all three in HEK1 line (Fig. 4A). To determine whether the absence of SOS1/EPS8/ABI1 tri-complex member was responsible for the defect in LPA migratory response and metastatic colonization, SOS1, EPS8, and ABI1 were expressed in TOV21G, IGROV1, and OVCAR3 cells, respectively. LPA both activated Rac (Fig. 4B) and stimulated cell migration in TOV21G/SOS1, IGROV1/EPS8, and OVCAR3/ABI1 cells (Fig. 4C). We next injected TOV21G/SOS1, IGROV1/EPS8, OVCAR3/ABI1, and the respective empty-vector control cells into nude mice followed by analyzing metastatic colonization. There was no detectable metastatic implant in mice receiving control cells (Fig. 4D and Supplementary Fig. S10). In contrast, implants were readily visible in the peritoneal cavities of mice that received TOV21G/SOS1, IGROV1/EPS8, or OVCAR3/ABI1 cells (Fig. 4D and Supplementary Fig. S10). These results further demonstrate the necessity of an intact SOS1/EPS8/ABI1 tri-complex in ovarian cancer metastasis.

**Coexpression of EPS8, ABI1, and SOS1 correlates with clinical malignancy of human ovarian cancer patients**

As laboratory study may not recapitulate clinical ovarian malignancy, we extended our study by examining the expression of SOS1, EPS8, and ABI1 in 64 ovarian cancer specimens (Supplementary Table S1). Immunohistochemistry showed that SOS1, EPS8, and ABI1 were positive in 48.4%, 62.5%, and 75% of the ovarian cancer specimens, respectively (Fig. 5A and B). When the expression status of the individual member of SOS1/EPS8/ABI1 tri-complex was analyzed, we found that none of them was statistically correlated to age, histologic types, pathologic grades, clinical stages, or patient survival (Supplementary Table S2 and Fig. 5B and C).

Our results with the established cell lines indicate that the integrity of SOS1/EPS8/ABI1 tri-complex is essential for ovarian cancer metastasis. We thus focused on the specimens that were immunoreactive for all 3 members of this tri-complex. Of 64 samples, 26 specimens were stained positive for all 3 proteins and designated as “co-positive” (Supplementary Table S1). The remaining specimens were negative for 1 or more of these proteins and designated as “co-negative.” The status of SOS1/EPS8/ABI1 coexpression was independent of age, histologic types, or pathologic grade (Table 1). However, the “co-positive” percentage increased significantly along with the more advanced stages (stage I,
Figure 3. SOS1/EPS8/ABI1 tri-complex participates in LPA-stimulated cell migration and metastatic colonization. A, HEY, SK-OV3, IGROV, and OVCAR3 cells were transduced with empty vector or retroviral vector-containing H-RasT17N or H-RasG12V, then stimulated with 10 μmol/L of LPA for 5 minutes, and analyzed for Rac activity. B, HEY and SK-OV3 cells were transduced with lentiviral vector containing luciferase (control), Tiam1, SOS1, EPS8, or ABI1 shRNA for 4 days. An aliquot of transduced cells was subjected to immunoblotting to detect Tiam1, SOS1, EPS8, and ABI1 with the respective antibodies (only SK-OV3 cells were shown). Transduced cells were also stimulated with 10 μmol/L of LPA for 2 or 5 minutes or infected with retrovirus containing H-RasG12V for 2 days followed by the analysis of Rac activity. C, transduced SK-OV3 and HEY cells were assayed for LPA- or H-RasG12V–induced cell migration. Results are presented as fold increase of cell migration [(LPA-stimulated cell migration)/(basal cell migration)]. Data are means ± SE. n = 3. *, P < 0.001 versus sh-Luc; #, P < 0.005 versus sh-Luc. D, transduced SK-OV3 and HEY cells were intraperitoneally injected to nude mice for 5 weeks. Metastatic implants were collected and weighed. Data are means ± SE. n = 6. *, P < 0.001 versus sh-Luc.
Discussion

High levels of LPA is present in ascites of ovarian cancer patients (22–25) and can potently stimulate ovarian cancer cell migration (8, 27). In this study, we found that only ovarian cancer cell lines with LPA migratory response undergo peritoneal metastatic colonization (Fig. 1A and B), suggesting a potential link between LPA-stimulated cell migration and ovarian cancer metastasis. Although recent studies indicated that multiple LPA receptors may contribute to LPA-stimulated cell migration (21, 35), we showed that silencing LPAR1 largely abrogated both LPA-stimulated cell migration and metastatic colonization (Fig. 1C and D). The discrepancy may be caused by the means to silence LPA receptors, as synthetic siRNAs were used in these early studies whereas we used shRNAs for LPAR1 knockdown. A recent study reports that direct administration of LPA in vivo led to enhanced ovarian cancer metastasis and reduced animal survival (36). Enforced LPA receptor expression has also been shown to increase ovarian cancer aggressiveness in the xenograft model (35). These findings all support the notion that LPA migratory response is a prerequisite for ovarian cancer metastasis.

Cell migration requires reorganization of actin cytoskeleton that is regulated by the members of Rho GTPases including Rac (37). We show that LPA only induces cytoskeletal reorganization in metastatic ovarian cancer cells (Fig. 2A), indicating that members of Rho GTPase family may not be properly activated by LPA in nonmetastatic cells. This possibility is substantiated, as LPA significantly activates Rac only in metastatic cells (Fig. 2B). Inhibiting Rac activity abolished LPA-stimulated cell migration and peritoneal metastatic colonization of metastatic cells, whereas constitutively active Rac1 confers nonmetastatic cells with LPA migratory response and the ability to metastasize (Fig. 2C and D). Rac activation has been shown to correlate with hepatocellular carcinoma and breast cancer metastasis (38, 39). Our findings extend the importance of Rac activation in ovarian cancer metastasis.

Tiam1, β-PIX, and SOS1/EPS8/ABI1 tri-complex can mediate Ras-induced Rac activation (9–11). We show that silencing any member of SOS1/EPS8/ABI1 tri-complex, but not
Tiam1 or β-PIX, abrogates LPA- or Ras-induced Rac activation, cell migration, and peritoneal metastatic colonization (Fig. 3B–D). SOS1/EPS8/ABI1 tri-complex has been shown to mediate EGF- or PDGF-induced Rac activation and actin remodeling (40). This complex is also involved in phosphoinositide 3-kinase–induced Rac activation (41). Our studies present another example of the usage of SOS1/EPS8/ABI1 tri-complex for Rac activation. Our observation with ovarian cancer cells is different from that of an early report in which LPA is shown to activate Rac through Tiam1 in murine fibroblasts (32). This difference may be attributed to species and cell lineage difference.

As silencing any member of SOS1/EPS8/ABI1 tri-complex is sufficient to diminish ovarian cancer cell migration and metastatic colonization, it indicates that the integrity of SOS1/EPS8/ABI1 tri-complex may determine ovarian cancer metastatic potentials. Indeed, metastatic lines express all 3 members of this tri-complex whereas at least 1 member is absent in nonmetastatic lines (Fig. 4A). Introducing the missing member in the respective line renders it capable

Figure 5. SOS1/EPS8/ABI1 coexpression correlates with shorter survival of ovarian cancer patients. A, immunohistochemistry of SOS1, EPS8, and ABI1 on ovarian cancer specimens. Scale bars, 200 μm. B, percentage of various stage ovarian cancer specimens positive for the expression of SOS1, EPS8, and ABI1. S/E/B: coexpression of SOS1, EPS8, and ABI1. C, Kaplan–Meier survival curves for 61 ovarian cancer patients, stratified on the basis of individual SOS1, EPS8, or ABI1 expression or SOS1/EPS8/ABI1 coexpression.
of undergoing metastatic colonization (Fig. 4D). EPS8 has been reported to promote fibrosarcoma and oral squamous carcinoma cell migration (42, 43). ABI1 can positively regulate breast cancer cell migration and invasion (44). Moreover, depletion of SOS1 blocks prostate cancer cell migration and invasion (45). Although only a single member was focused in these studies, they support an essential role of SOS1/EPS8/ABI1 tri-complex in cell migration and metastasis.

Recent studies indicate the clinical relevance of the expression of SOS1 and EPS8 in cancer progression. The level of SOS1 is elevated in prostate cancer specimens with high Gleason scores (45). EPS8 overexpression is often detected in advanced stage of thyroid cancer (46), pancreatic cancer (47), oral squamous carcinoma (43), and pituitary tumors (48). To our knowledge, no study has been reported on the status of ABI1 in clinical cancer specimens. In this study, we detected the expression of SOS1, EPS8, and ABI1 in clinical ovarian cancer specimens. In this study, we detected the expression of SOS1, EPS8, and ABI1 in clinical ovarian cancer specimens (Fig. 5A). However, the expression of any individual member of SOS1/EPS8/ABI1 tri-complex alone is not statistically associated with any clinicopathologic parameters of patients (Supplementary Table S2). Instead, we find that SOS1/EPS8/ABI1 coexpression is significantly correlated with advanced stage and shorter survival (Table 1, Fig. 5B and C). This observation is in excellent agreement with the findings generated from the established cell lines that intact SOS1/EPS8/ABI1 tri-complex is required for ovarian cancer metastasis.

Our study demonstrates the importance of SOS1/EPS8/ABI1 tri-complex in ovarian cancer metastasis. Although we show it with the established ovarian cancer cell lines that may not fully simulate clinical setting, the consistency seen in multiple cell lines, the convergence of loss- and gain-of-function findings, and especially the excellent correlation observed between SOS1/EPS8/ABI1 coexpression and advanced disease stage/shorter patient survival strongly argue against any confounding influence derived from our experimental studies. Our studies suggest that the integrity of SOS1/EPS8/ABI1 tri-complex determines ovarian cancer metastasis and that therapeutic approach may be developed by targeting this tri-complex.

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<th>Table 1. Correlation between the status of SOS1/ABI1/EPS8 coexpression and clinicopathologic parameters of ovarian cancer patients</th>
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<td>II</td>
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<td>III</td>
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<td>IV</td>
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<tr>
<td>aMedium age = 51 (27–69).</td>
</tr>
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</table>

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