Silencing of type Iγ phosphatidylinositol phosphate kinase suppresses ovarian cancer cell proliferation, migration and invasion

SIYU CAO[1,2], CHUNHUA CHEN[2], JUNLI XUE[3], YAN HUANG[2], XIAOFENG YANG[1] and KUN LING[2]

[1]Department of Gynecology and Obstetrics, The First Affiliated Hospital of the Medical School, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China; [2]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55901, USA; [3]Department of Oncology, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200120, P.R. China

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Abstract. Metastasis is the major cause of death in ovarian cancer patients. Given that the molecular mechanism underlying metastasis formation is critical for improving therapeutic development and clinical treatment, it must be fully understood. Recent studies have revealed that lipid kinase type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) participates in the metastasis of breast cancer and colon cancer by regulating cell migration and invasion. However, its role in the progression of ovarian cancer is unclear. Here we showed that PIPKIγ expression is upregulated in multiple epithelial ovarian cancer cell lines. Silencing of PIPKIγ impaired PI3K/AKT signaling and inhibited the aggressive behaviors of epithelial ovarian cancer cells, including proliferation, migration and invasion. Moreover, we found that PIPKIγ was required for the activation of signal transducer and activator of transcription 3 (STAT3) in epithelial ovarian cancer cells, indicating that STAT3 may also be engaged in the PIPKIγ-dependent aggressiveness of epithelial ovarian cancer cells. Our results, for the first time, identified PIPKIγ as a novel regulator in epithelial ovarian cancer cells that promotes cell proliferation, migration and invasion by activating multiple signaling pathways. Therefore, we propose that PIPKIγ could potentially be a therapeutic target for the early detection and treatment of epithelial ovarian cancer. Further studies employing *in vivo* models are necessary to test this possibility.

Correspondence to: Dr Kun Ling, Department of Biochemistry and Molecular Biology, Cancer Center, Mayo Clinic, Guggenheim 16-04, 200 First Street SW, Rochester, MN 55905, USA
E-mail: ling.kun@mayo.edu

Dr Xiaofeng Yang, Department of Gynecology and Obstetrics, The First Affiliated Hospital of the Medical School, Xi'an Jiaotong University, 277 West Yanta Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: yxf73@163.com

Abbreviations: PIPKIγ, type Iγ phosphatidylinositol phosphate kinase

Key words: type Iγ phosphatidylinositol phosphate kinase, ovarian cancer, proliferation, migration, invasion, PI3K/AKT, STAT3

Introduction

Ovarian cancer is the leading cause of death among all gynecologic malignancies, and the mortality rate of ovarian cancer continues to increase while the incidence rate remains high in recent decades according to recently published cancer statistics in China, 2015 (1). Therefore, efficient targets for early detection and treatment of ovarian cancers are urgently needed. Normal ovarian epithelial cells have a limited ability to proliferate and migrate for wound healing after ovulation or rupture of the mature follicle. However, epithelium-originated ovarian cancer cells are able to spread through the abdominal cavity forming multiple implants on the peritoneal surface. While early-stage epithelial ovarian cancers can be cured when they are still confined to the ovary upon diagnosis, a majority of epithelial ovarian cancers are diagnosed at the advanced stage after peritoneal dissemination has occurred, which is often too late for efficient treatment (2). In this context, it is critical to understand the molecular mechanisms driving epithelial ovarian cancer progression, especially the development of metastasis, for the identification of valuable drug targets and development of effective therapeutic strategies.

Previous studies have shed light on the multiple signaling pathways involved in epithelial ovarian cancer, including the PI3K/AKT pathway that commonly participates in the proliferation and survival of tumor cells (3-5). In addition to PI3K, other players in the phosphoinositide signaling pathway are also implicated in regulating cancer cells (6,7). For example, type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) generates phosphatidylinositol 4,5-biphosphate [PtdIns(4,5)P_2] as the substrate of PI3K to activate AKT and downstream signaling cascades, which then promote proliferation and survival (8). Moreover, PtdIns(4,5)P_2 is an important secondary messenger that regulates various cellular events including protein trafficking, actin reorganization, cell adhesion and migration (9). We recently observed that PIPKIγ is engaged in the metastasis

of breast cancer by regulating the proliferation, migration and invasion of breast cancer cells (6,10,11). In this context, we proposed that PIPKIγ may also contribute to epithelial ovarian cancer metastasis as both cancers share similar pathways. Interestingly, we found that PIPKIγ was highly expressed in the epithelial ovarian cancer cells. This lipid kinase was necessary for the activation of the PI3K/AKT pathway and regulated the migration and invasion of these cells. Furthermore, loss of PIPKIγ impaired signal transducer and activator of transcription 3 (STAT3) activation that is closely associated with the poor prognosis of ovarian carcinomas (12). Our data strongly suggest that PIPKIγ may have profound influence on facilitating the progression of epithelial ovarian cancer. These results endorse the potential of this lipid kinase as a novel therapeutic target for epithelial ovarian cancer treatment and call for further investigation.

Materials and methods

Cell culture. All five human epithelial ovarian cancer cell lines (OVCAR-7, OVCAR-8, PEO-1, PEO-4 and SKOV-3) were kindly provided by Dr William A. Cliby (Mayo Clinic, Rochester, MN, USA), and the immortalized OSE (OSE hTERT) cells were obtained from Dr Vijayalakshmi Shridhar (Mayo Clinic). All cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), while OSE hTERT cells were maintained in NOE complete media consisting of 50% v/v Medium 199 and 50% v/v MCDB (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15% FBS, 1% penicillin/streptomycin and 0.1% epithelial growth factor (EGF; Sigma-Aldrich). Cell cultures were maintained at 37˚C with 5% CO_2.

Antibodies. The rabbit polyclonal PIPKIγ antibody was generated and purified as described previously (6). Antibodies against phosphorylated AKT (pSer473), total AKT, phosphorylated ERK1/2 (pThr202/Tyr204), total ERK1/2, phosphorylated STAT3 (pTyr705), total STAT3, phosphorylated JAK2 (pTyr1007/1008) and total JAK2 were procured from Cell Signaling Technology (Danvers, MA, USA). Antibody for β-actin was purchased from Sigma-Aldrich.

Constructs and transfection. Two distinct siRNA sequences specifically targeting human PIPKIγ were: PIPKIγ-siRNA1 (ATCCGCGTCGTGGTCATGAACAACA) and PIPKIγ-siRNA2 (GCGTGGTCAAGATGCACCTCAAGTT). PIPKIγ siRNAs and control siRNA were synthesized at Invitrogen (Stealth RNAi). Mouse PIPKIγ constructs encoding isoform 1 and isoform 2 that are resistant to human PIPKIγ siRNAs were constructed as described previously (6).

For transient transfection of siRNAs, OVCAR-8 and SKOV-3 cells were plated in 6-well culture plates at $4x10^5$ cells/well, and then reversely transfected using Lipofectamine RNAiMAX (Invitrogen) for 48 h before further analyses. For the rescue experiments, SKOV-3 cells were transiently transfected with DNA plasmids using X-tremeGENE 9 (Roche) for 24 h, and then lifted and reversely transfected with siRNAs as described above.

Immunoblotting. The transfected OVCAR-8 and SKOV-3 cells were collected in 200 µl of 1X SDS lysis buffer (40 mM Tris-HCl, 1 mM EDTA, 150 mM KCl, 100 mM $NaVO_3$, 1% Triton X-100, 1 mM PMSF, pH 7.5) on ice. Proteins in the lysates were separated by electroporation using 10% SDS-PAGE gels and then transferred onto PVDF membranes. After being blocked with 5% non-fat milk in TBS-T at room temperature for 1 h, membranes were incubated with primary antibodies overnight in blocking buffer at 4˚C, followed by HRP-conjugated secondary antibody for 1 h at room temperature. Then membranes were incubated with Supersignal Chemiluminescent Substrate (Thermo Fischer Scientific, Waltham, MA, USA) and imaged using ChemiDOC imaging system (Bio-Rad, Hercules, CA, USA).

Cell viability assay. OVCAR-8 and SKOV-3 cells transfected with PIPKIγ-siRNA1, PIPKIγ-siRNA2 or control siRNA for 48 h were plated ($1x10^5$ cells/well) as triplicates in 96-well plates and cultured for 48 h at 37˚C in tissue culture incubator. Then 10 µl of 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for another 4 h. After replacing the culture medium with Stop Solution (40 mM HCL in isopropanol; 100 µl/well), the absorbance was measured at 590 nm on a plate reader.

Flow cytometry. For cell cycle analysis, the cells transfected with PIPKIγ-siRNA1, PIPKIγ-siRNA2, or control siRNA for 48 h were washed twice with phosphate-buffered saline (PBS) and then fixed with prechilled 70% ethanol at 4˚C overnight. Fixed cells were washed twice with PBS and then stained in 500 µl propidium iodide solution containing 50 µg/ml propidium iodide (eBioscience, USA), 0.1 mg/ml RNase A and 0.05% Triton X-100 at 37˚C for 1 h. The DNA content was determined by a flow cytometer (BD Biosciences, San Jose, CA, USA). Data were then analyzed using ModFit software (Verity Software, Topsham, ME, USA). For apoptosis analyses, cells were stained with 7-AAD (BioLegend, USA) at 4˚C for 15 min or fixed with fixation buffer (BioLegend) followed by caspase-3 staining (BD Biosciences). The cells were then anayzed by flow cytometer (BD Biosciences).

Cell migration assay. The migration assay was performed using modified Boyden chambers (Neuroprobe, Gaithersburg, MD, USA) according to the manufacturer's instructions (6). The polycarbonate membranes (8-µm pore size; Neuroprobe) were precoated with 10 µg/ml type I collagen for 3 h at 37˚C and placed on the top of the lower chamber filled with DMEM containing 10% FBS. Serum-starved cells ($3x10^5$) in serum-free medium were added to the upper chamber and incubated at 37˚C in a tissue culture incubator for 8 h. After the removal of non-migrated cells on top of the membrane with a cotton swab, the membranes were fixed with methanol and stained with 0.2% crystal violet. The number of cells that migrated to the lower side of the membrane were counted under a x20 magnification lens using a Nikon microscope (TE-2000) and averaged from at least 5 randomly selected fields. We set duplicates for each sample.

Cell invasion assay. Cell invasion assay was carried out in Matrigel-coated Transwells (BD Biosciences, USA) as previ-

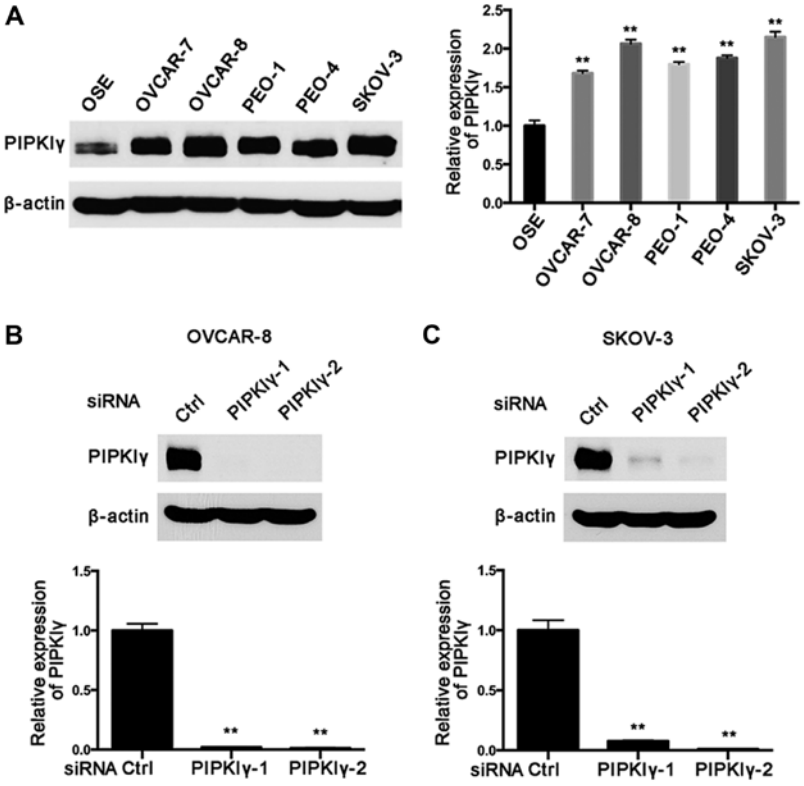

Figure 1. Human epithelial ovarian cancer cells exhibit elevated expression of PIPKIγ. (A) PIPKIγ levels in normal, immortalized OSE (OSE hTert) cells and commonly used epithelial ovarian cancer cell lines (OVCAR-7, OVCAR-8, PEO-1, PEO-4 and SKOV-3) were determined by immunoblotting using the indicated antibodies. Intensities of PIPKIγ bands were quantified by ImageJ and plotted (right panel). (B and C) Two distinct siRNAs (PIPKIγ-1 and PIPKIγ-2) and a control siRNA were transfected into OVCAR-8 (B) or SKOV-3 (C) cells for 48 h. Cells were then harvested and subjected to immunoblotting to examine the protein levels of PIPKIγ. After quantification using ImageJ, intensity of the PIPKIγ bands was normalized against actin in the same sample, then the PIPKIγ levels in cells treated with PIPKIγ siRNAs were normalized against the PIPKIγ levels in cells treated with control siRNA. Results from three independent experiments were statistically analyzed and presented as mean ± SD. **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase.

ously described (11). Transwells were incubated with DMEM for 4 h and the lower compartment was filled with DMEM containing 10% FBS. OVCAR-8 or SKOV-3 cells ($4x10^4$) were plated in the upper compartment and incubated at 37˚C in a tissue culture incubator for 24 h. The cells that invaded the Matrigel and reached the lower surface of the membrane were fixed with 4% polyformaldehyde and stained with 0.2% crystal violet. The number of invaded cells was counted under microscope as described above.

Quantitative RT-PCR. OVCAR-8 and SKOV-3 cells transfected with PIPKIγ-siRNA1, PIPKIγ-siRNA2or control siRNAfor 48 h were collected and washed in PBS, and then used to prepare total RNA using RNA kit (Invitrogen, Life Technologies). RNAs (2 mg) were reversely transcripted into cDNAs using M-MLV (Invitrogen, Life Technologies), followed by quantitative PCR using the following primers (BGI, USA). Matrix metalloproteinase (MMP)-2 forward, 5'-GTTCATTTGGCGGACTGT-3' and reverse, 5'-AGGGTG CTGGCTGAGTAG-3'; MMP-9 forward, 5'-AATCTCACCGA CAGGCAGCT-3' and reverse, 5'-CCAAACTGGATGACGAT GTC-3'; and GAPDH forward, 5'-GAAGGTGAAGGTCGG AGT-3' and reverse, 5'-CATGGGTGGAATCATATTGGAA-3'. PCR program consisted of 50˚C for 2 min, 95˚C for 5 min, followed by 49 cycles at 95˚C for 20 sec, 60˚C for 20 sec, 72˚C for 25 sec. The samples were then heated at 95˚C for 10 sec and 65˚C for 30 sec followed by gradual heating to 95˚C for 15 sec. The results were analyzed by CFX Manager software (13).

Statistical analysis. All experiments were repeated at least three times. Results were analyzed with Prism 6 and are presented as mean ± standard deviation (SD). The significance of group differences was identified as P<0.01 or P<0.05.

Results

PIPKIγ is upregulated in ovarian cancer cells. Abnormally altered expression of a protein in cancer cells often indicates a correlation between this protein and the development and/or progression of cancer (6). Thus, we firstly examined the expression of PIPKIγ in epithelial ovarian cancer cells. According to the Human Protein Atlas, both RNA and protein levels of PIPKIγ are relatively low in normal ovarian tissues, however higher PIPKIγ expression is detected in some epithelial ovarian carcinomas. To verify this, we examine PIPKIγ expression in five most commonly used human epithelial ovarian cancer cell lines, including OVCAR-7, OVCAR-8, PEO-1, PEO-4 and SKOV-3. As shown in Fig. 1A, PIPKIγ was found to be highly expressed in all the tested cell lines compared to that in the normal epithelial cell line OSE-hTERT. Notably, OVCAR-8 and SKOV-3 cells exhibited substantially elevated

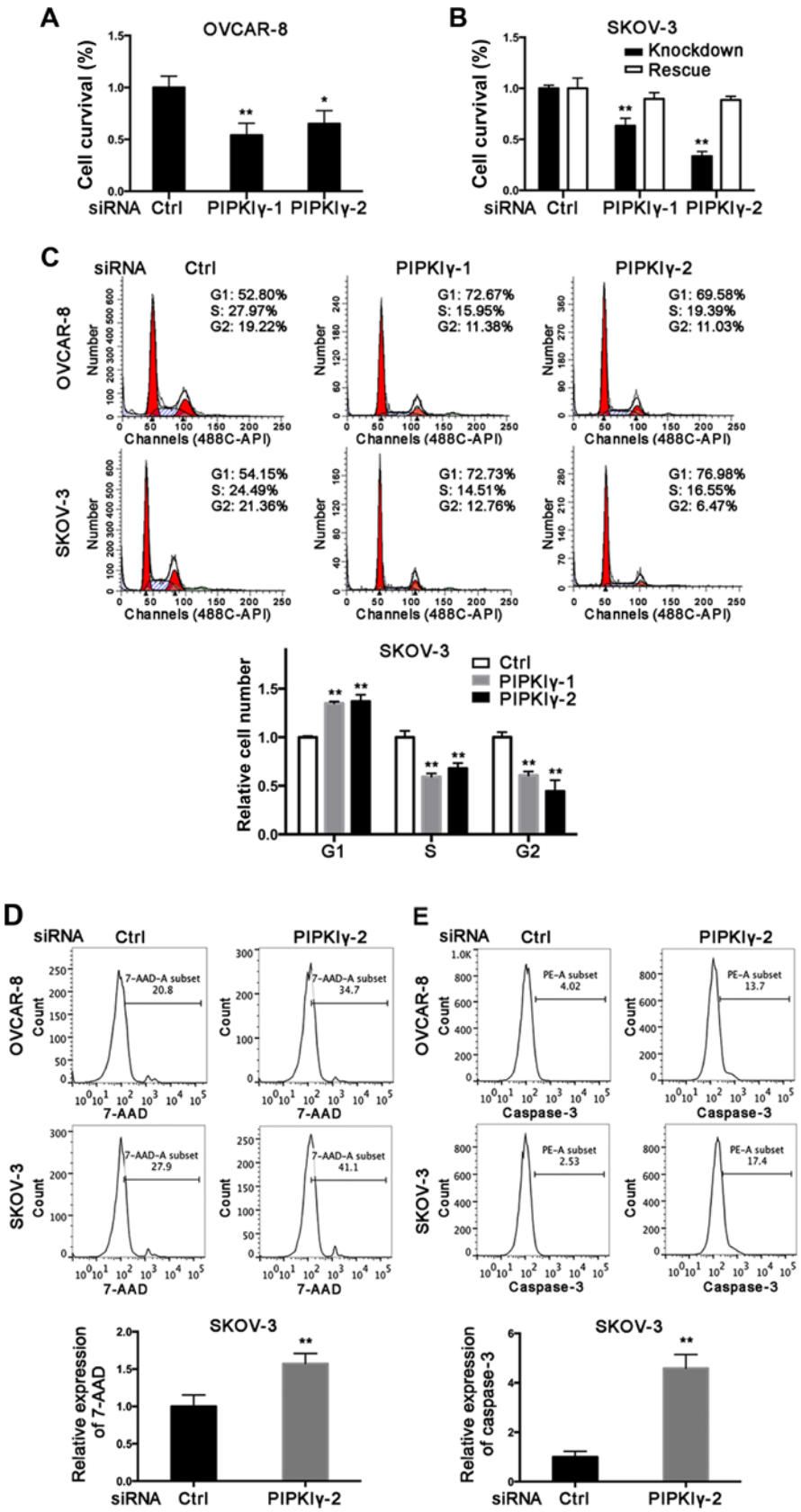

Figure 2. PIPKIγ deficiency inhibits the proliferation and survival of epithelial ovarian cancer cells. OVCAR-8 and SKOV-3 cells were transfected with the indicated siRNAs (control, PIPKIγ-1 and PIPKIγ-2) for 48 h, and then subjected to variant analyses to determine the cell viability, proliferation, and apoptosis. (A and B) Cell viability was determined by MTT assay in triplicates in the indicated OVCAR-8 (A) or SKOV-3 (B) cells. (B) To determine whether recovery of PIPKIγ expression could rescue cell viability, we introduced the expression of siRNA-resistant PIPKIγ isoform 1 and isoform 2 (rescue) in SKOV-3 cells for 24 h before siRNA treatment. (C) Cell cycle progression was evaluated by flow cytometry. The number of control or PIPKIγ-depleted SKOV-3 cells in G1, S and G2 phase was statistically analyzed from three independent experiments and plotted. (D and E) Apoptosis was determined by flow cytometry after 7-AAD (D) and caspase-3 (E) staining. Results for SKOV-3 cells were statistically analyzed from three independent experiments and plotted. Data are presented as mean ± SD. *P<0.05; **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase.

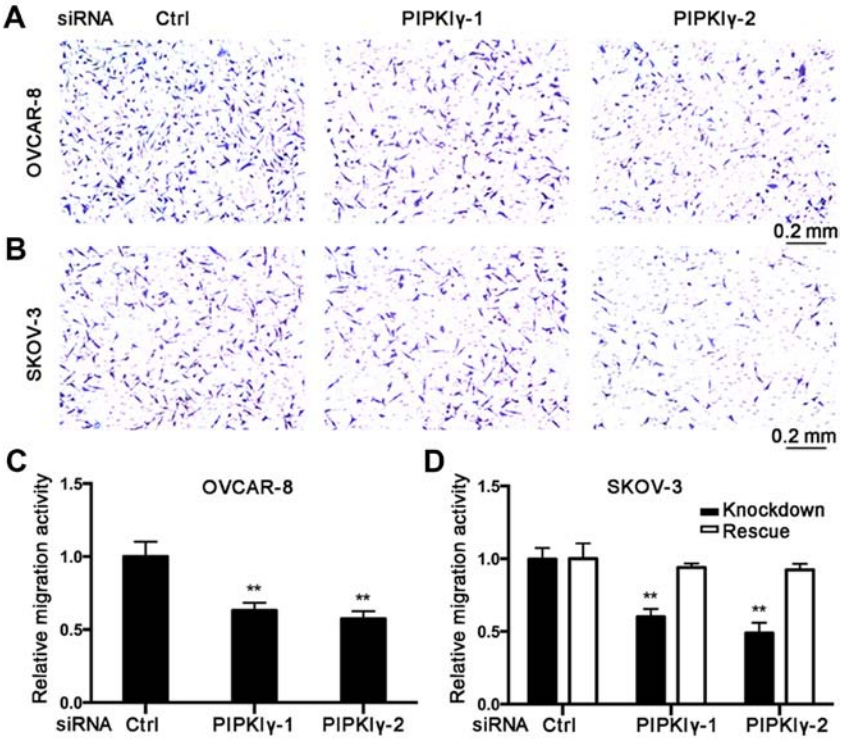

Figure 3. Loss of PIPKIγ suppresses the migration of epithelial ovarian cancer cells. Migration assay was performed using modified Boyden chambers in triplicates using OVCAR-8 (A) or SKOV-3 (B) cells transfected with the indicated siRNAs (control, PIPKIγ-1 and PIPKIγ-2). (A and B) Cells migrating across the membrane were fixed and stained, then imaged under a microscope. (C and D) Cells imaged in A and B were counted in five random fields under x20 magnification and averaged, and then statistically analyzed from three independent experiments and plotted. (D) Rescue experiments were conducted using SKOV-3 cells by introducing the expression of siRNA-resistant PIPKIγ isoform 1 and 2 by transient transfection, followed by transfection of control or PIPKIγ-specific siRNAs. Then cells were subjected to migration assay and quantified as described above. Data are presented mean ± SD. **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase.

PIPKIγ expression compared to that in the normal epithelial cells (Fig. 1A). These data suggest that upregulation of PIPKIγ may correlate with the development and/or progression of epithelial ovarian cancers.

Silencing of PIPKIγ impairs the viability of human epithelial ovarian cancer cells and promotes apoptosis. To investigate the role of PIPKIγ in epithelial ovarian cancer cells, we utilized two distinct PIPKIγ-specific siRNAs (PIPKIγ-1 and PIPKIγ-2) to knock down endogenous PIPKIγ in OVCAR-8 and SKOV-3 cells, as these two lines showed the highest expression of PIPKIγ and share similar characteristics. After confirming the knockdown efficiency of these siRNAs (Fig. 1B and C), we examined the aggressive behaviors of PIPKIγ-depleted cells in comparison with the control cells treated with the scrambled siRNA. As shown in Fig. 2A and B, reduction of PIPKIγ in the OVCAR-8 and SKOV-3 cells led to a significant decrease in cell survival as determined by MTT assay. Importantly, the decreased viability was rescued by introducing the expression of RNAi-resistant PIPKIγ back in PIPKIγ-depleted SKOV-3 cells (Fig. 2B), further demonstrating that PIPKIγ is indeed required for the viability of ovarian cancer cells. To delineate how PIPKIγ affects cell viability, we determined both cell cycle progression and apoptosis in the control and PIPKIγ-deficient cells. As shown in Fig. 2C, the cells treated with PIPKIγ RNAi exhibited a significant increase in G1 phase and less cells were observed in the S phase, indicating a delayed G1-to-S transition in both OVCAR-8 and SKOV-3 cells (Fig. 2C). Furthermore, we found that loss of PIPKIγ also induced higher apoptosis when we examined 7-AAD (Fig. 2D) and caspase-3 (Fig. 2E). These results indicate that PIPKIγ likely regulates multiple pathways and contributes to the growth of ovarian cancer cells by both promoting cell proliferation and inhibiting apoptosis.

Cell migration and invasion are suppressed in PIPKIγ-deficient human epithelial ovarian cancer cells. To further elucidate the role of PIPKIγ in regulating the malignant behaviors of epithelial ovarian cancer cells, we conducted *in vitro* cell migration and invasion assays. Using the Boyden chamber system, we found that the PIPKIγ-depleted cells migrated significantly slower responding to serum when compared to the control cells (Fig. 3). Results from the Transwell invasion assay showed that knockdown of PIPKIγ led to a substantially impaired invasive ability (Fig. 4). Furthermore, both migration and invasion capacities were almost completely rescued when the expression of PIPKIγ was recovered in the SKOV-3 cells (Figs. 3 and 4). Taken together, these results demonstrate that PIPKIγ indeed is required for the malignant behavior of epithelial ovarian tumor cells, indicating that inhibition of PIPKIγ may suppress the development of metastasis in epithelial ovarian cancer.

PIPKIγ is required for the activation of the PI3K/AKT pathway in human epithelial ovarian cancer cells. Since our results indicated that PIPKIγ regulates the proliferation and

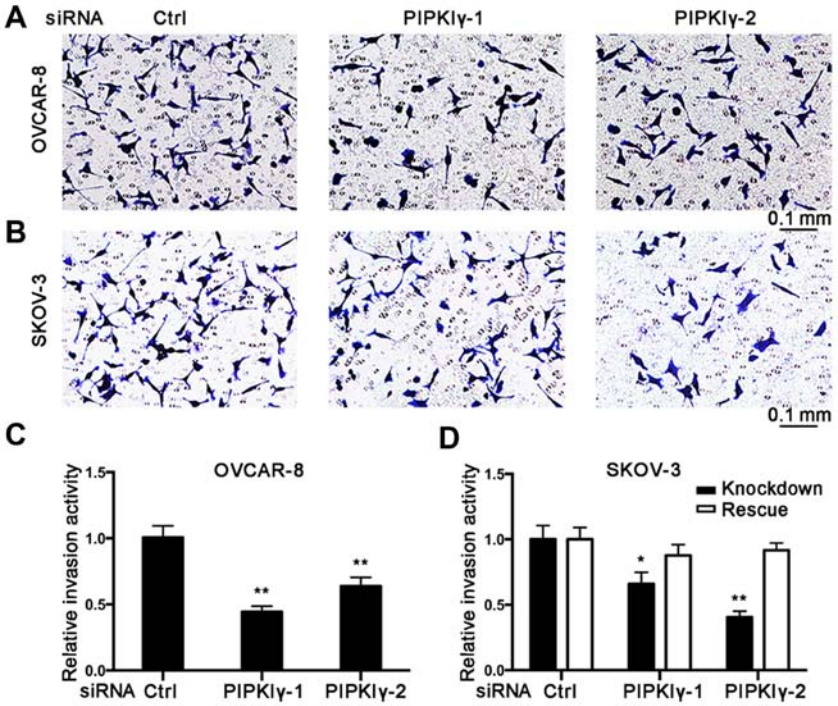

Figure 4. PIPKIγ is required for the invasion of epithelial ovarian cancer cells. OVCAR-8 (A) and SKOV-3 (B) cells were transfected with siRNAs (control, PIPKIγ-1 and PIPKIγ-2) separately for 48 h, and then subjected to invasion assay using Matrigel-coated Transwells in triplicates. Cells that invaded to the lower surface of the membrane were fixed and stained with 0.2% crystal violet, and then imaged under a microscope. (C and D) Cells that invaded to the matrix were quantified as described in Fig. 5. The invasion index was calculated as instructed by the manufacturer, statistically analyzed from three independent experiments, and plotted. (D) By introducing the expression of exogenous siRNA-resistant PIPKIγ isoform 1 and 2, SKOV-3 cell invasion was almost completely rescued. Data are presented mean ± SD. *P<0.05; **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase.

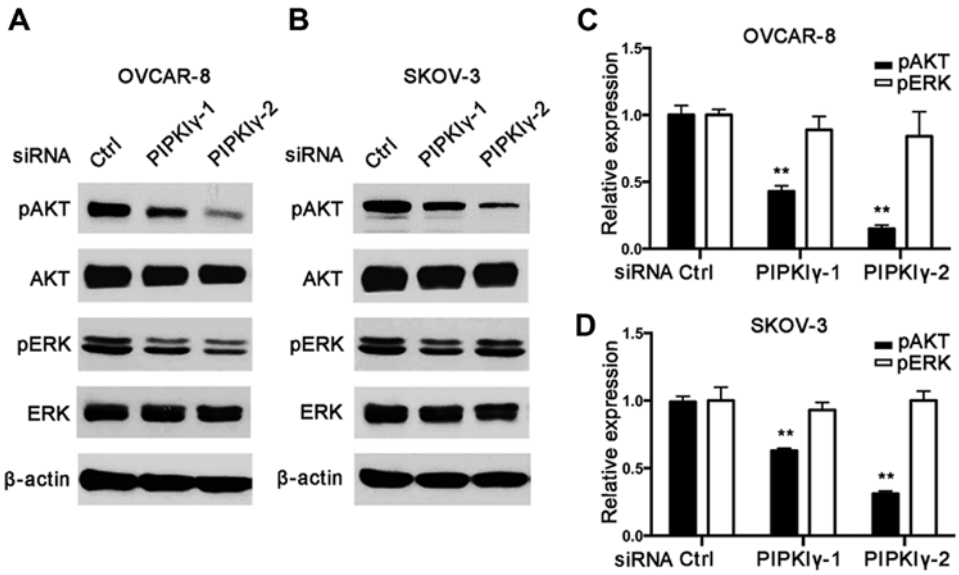

Figure 5. PIPKIγ depletion attenuates the PI3K/AKT pathway in epithelial ovarian cancer cells. (A and B) OVCAR-8 (A) and SKOV-3 (B) cells treated with control or PIPKIγ-specific siRNAs for 48 h were subjected to immunoblotting with the indicated antibodies: pAKT, Ser473-phosphorylated AKT; AKT, total AKT; pERK, Thr202/Tyr204-phosphorylated ERK; ERK, total ERK. β-actin was used as loading control. (C and D) Intensity of pAKT and pERK bands were normalized against the total AKT and total ERK of the same sample, respectively. The relative levels of pAKT and pERK in each sample were then statistically analyzed in both OVCAR-8 (C) and SKOV-3 (D) cells from three independent experiments. Data are presented as mean ± SD. **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase.

migration of epithelial ovarian cancer cells, we then tested whether this is through PI3K/AKT and/or MAPK/ERK pathways that often participate in ovarian carcinogenesis (14,15). As shown in Fig. 5, PIPKIγ-depleted cells exhibited much less activated AKT than the control cells; however, activation of the MAPK pathway appeared similar in the control and PIPKIγ-depleted cells. These results indicate that PIPKIγ is necessary for the activation of the PI3K/AKT pathway but not

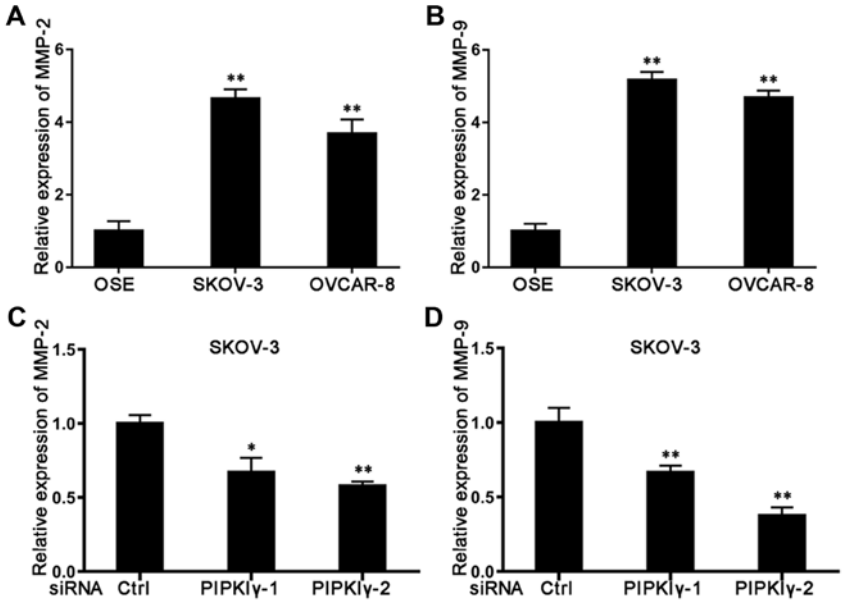

Figure 6. MMP-2 and MMP-9 are both downregulated in PIPKIγ-depleted SKOV-3 cells. (A and B) OSE, ORCAR-8 and SKOV-3 cells were subjected to quantitative PCR using MMP-2 (A) or MMP-9 (B) primers. (C and D) SKOV-3 cells transfected with control or the indicated PIPKIγ-specific siRNAs for 48 h were collected for RNA extraction followed by reverse transcription. cDNAs were then analyzed by quantitative PCR using MMP-2 (C) or MMP-9 (D) specific primers. Results from three independent experiments were then analyzed by CFX Manager software with GAPDH used as internal control, and then plotted as mean ± SD. *P<0.05; **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase; MMP, matrix metalloproteinase.

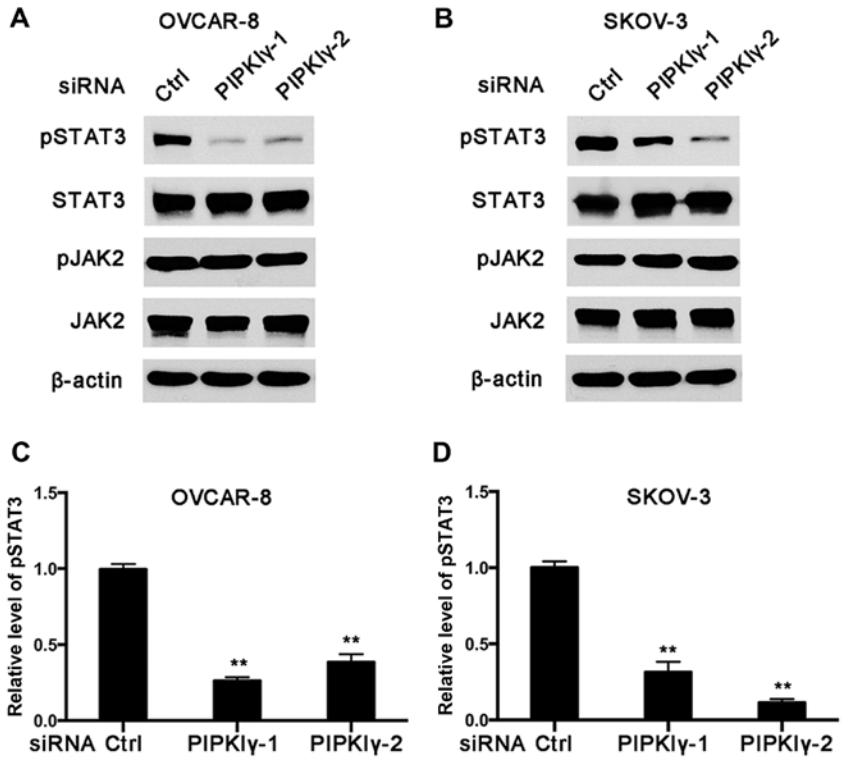

Figure 7. PIPKIγ mediates JAK2-independent STAT3 activation in ovarian epithelial cancer cells. (A and B) OVCAR-8 (A) and SKOV-3 (B) cells treated with the indicated siRNAs for 48 h, were then subjected to immunoblotting using the indicated antibodies: pSTAT3, Tyr705-phosphorylated STAT3; STAT3, total STAT3; pJAK2, Tyr1007/1008-phosphorylated JAK2; JAK2, total JAK2. β-actin was used as loading control. (C and D) Intensity of pSTAT3 bands was normalized against the total STAT3 of the same sample. The relative levels of pSTAT3 in each sample were then statistically analyzed in both OVCAR-8 (C) and SKOV-3 (D) cells from three independent experiments. Data are presented as mean ± S.D. **P<0.01. PIPKIγ, type Iγ phosphatidylinositol phosphate kinase; STAT3, signal transducer and activator of transcription 3.

the MAPK pathway, although MAPK is known to be closely related to migration in epithelial ovarian cancers (14,15). Our data suggest that inhibition of PIPKIγ blocks ovarian tumor cell proliferation and migration by downregulating the PI3K/

AKT pathway, which may subsequently interrupt the metastasis of epithelial ovarian cancer.

Expression of MMP-2 and MMP-9 is regulated by PIPKIγ. MMPs are known as a family of proteolytic enzymes that remodel the extracellular matrix to promote tumor metastasis. Among all 23 members in the MMP family, MMP-2, MMP-7 and MMP-9 are most closely associated with ovarian cancer tumor metastasis (16), and MMP-2 and MMP-9 are highly expressed in ovarian cancer ascites and tissues (17). When we utilized qRT-PCR to measure the expression of these MMPs, we found that both MMP-2 and MMP-9 were expressed at significantly higher levels in the epithelial ovarian cancer cells than levels noted in the normal OSE cells (Fig. 6A and B). Compared to normal SKOV-3 cells, depletion of PIPKIγ led to a reduction in both MMP-2 (Fig. 6C) and MMP-9 (Fig. 6D). However, levels of these MMPs remained the same in the control and PIPKIγ-depleted OVCAR-8 cells (data not shown), suggesting that PIPKIγ mediates a cell type-specific regulation in the expression of MMP-2 and MMP-9. Considering the role of MMP-2 and MMP-9 in metastasis formation, our results suggest that PIPKIγ may promote cell invasion by regulating the expression of these MMPs, and therefore may potentially facilitate metastasis in certain epithelial ovarian cancers.

PIPKIγ activates the STAT3 pathway in ovarian cancer cells. STAT3 is activated by phosphorylation of a tyrosine residue by activated EGFR, JAK or Src (18). Constitutively phosphorylated and activated in 70% of ovarian cancers, aberrant STAT3 signaling is significantly correlated with the development of ovarian cancer (18,19). Currently, targeting the STAT3 pathway has become a major focus of drug development, and numerous STAT3 inhibitors have been shown to be effective in suppressing tumor cell migration (18-20). Importantly, it has been recently reported that elevated STAT3 expression in ovarian cancer ascites promotes tumor invasion and metastasis (21). In this context, we explored whether PIPKIγ, which is required for migration and invasion of epithelial ovarian cancer cells, regulates the activation of STAT3. As shown in Fig. 7, the level of Tyr705-phosphorylated STAT3 was notably decreased in the PIPKIγ-depleted cells compared to that noted in the control cells in both the OVCAR-8 and SKOV-3 cell lines. However, the levels of phosphorylated JAK2 and total JAK2 appeared comparable between the control and PIPKIγ-depleted cells (Fig. 7A and B). These results indicate that PIPKIγ is required for the basal activity of STAT3 in non-stimulated ovarian cancer cells, which is independent of JAK2. Considering the important role of the STAT3 pathway in the progression of ovarian cancer, our findings reveal PIPKIγ as a novel JAK2-independent regulator of STAT3, which may be an important mechanism underlying PIPKIγ-dependent survival and migration/invasion of ovarian cancer cells.

Discussion

In recent years, lipid kinases have been intensively explored in the tumor metastasis of ovarian cancer. Among them, the PI3K pathway, which is deregulated in epithelial ovarian cancers, has been extensively studied (4,22,23). Recent genomic analyses have revealed that components of the PI3K pathway are often mutated or altered in many human cancers (24-30), which supports PI3K as one of the most prospecting targets for therapeutic intervention in cancers (31). Correspondingly, a number of PI3K inhibitors have shown antitumor activities when applied alone or combined with chemotherapies (24,25). However, only a small portion of patients benefit from each single PI3K inhibitor, as distinct PI3K isoforms play different roles in cellular signaling and oncogenic transformation (32-36). In addition, side effects resulting from inhibition of other pathways such as RAF/MAPK (37) and the development of acquired resistance (38) further increase the complexity of the clinical application of PI3K inhibitors. These limitations call for new drug targets and novel therapeutic strategies, such as the combination of multiple targeted therapies. As reported previously, PIPKIγ that functions upstream of PI3K targets focal adhesion and regulates growth factor-induced cell migration and invasion of breast cancer cells (39,40). Here we report for the first time that PIPKIγ is also required for ovarian cancer cells to proliferate, survive, migrate and invade *in vitro*. Our results suggest that activation of the PI3K/AKT and STAT3 pathways both require PIPKIγ in epithelial ovarian cancer cells, indicating a molecular connection between PIPKIγ and conventional survival and metastasis pathways.

Among all of the four subtypes of ovarian cancers, the majority of tumors are derived from the ovarian surface epithelium. Utilizing several epithelial ovarian cancer cell lines, we found that epithelial ovarian cancer cells displayed elevated expression of PIPKIγ, indicating a correlation between PIPKIγ and malignancy of these cells. Indeed, proliferation, migration and invasion of these cells were significantly suppressed when PIPKIγ was depleted, accompanied by enhanced apoptosis. Since the PI3K/AKT pathway has been implicated in the survival and metastasis of epithelial ovarian cancers (41), the effects following the knockdown of PIPKIγ likely resulted from the inhibition of AKT activation, for AKT activity was repressed by depleting PIPKIγ in these cells.

Moreover, our results suggest that other signaling cascades important for ovarian cancer progression could be regulated by PIPKIγ, such as STAT3. In addition to a transcription factor, phosphorylated STAT3 can also localize to focal adhesions and promote ovarian cancer cell motility (12). Importantly, a recent study revealed that STAT3 expression is elevated in ovarian cancer ascites and promotes the progression/metastasis of ovarian cancer *in vivo* (21). This study demonstrated that phosphorylation of Tyr705 in STAT3, which indicates the constitutive activation of STAT3, is directly correlated with the extent and severity of ovarian cancer. In this context, our finding that deficiency of PIPKIγ severely impaired Tyr705 phosphorylation in STAT3 in both OVCAR-8 and SKOV-3 cells reveals STAT3 as a novel regulator in ovarian cancer cells. Notably, this PIPKIγ-dependent STAT3 activation was independent of JAK1/2. Therefore, we reason that the PIPKIγ-dependent Tyr705 phosphorylation of STAT3 is likely mediated by EGFR or Src kinases; both are important for PIPKIγ functions (11,40). Since STAT3, upon phosphorylated by Src, targets focal adhesions (12), it is important to explore whether this requires PIPKIγ in future research.

STAT3 is an important transcription factor regulating the expression of a wide variety of proteins including MMPs, which

are frequently upregulated in malignant tumor cells (42,43). In ovarian cancers, MMP-2 and MMP-9 are two of the most commonly elevated MMPs and contribute to the development of tumor metastasis and poor prognosis (44-48). We previously reported the substantial downregulation of MMP-9 in PIPKIγ-depleted breast cancer cells (45). Here we further showed that PIPKIγ-deficient SKOV-3 cells indeed exhibited lower mRNA levels of both MMP-2 and MMP-9. Although it has been suggested that the expression of MMP-2 and MMP-9 in ovarian epithelial cells can be regulated by STAT3 (49,50) and PIPKIγ depletion inhibits STAT3 activity similarly in OVCAR-8 cells as in SKOV-3 cells, no significant MMP-2 or MMP-9 reduction was detected in OVCAR-8 cells after PIPKIγ depletion. This suggests some complexity in the regulation of MMP-2 and MMP-9 in different types of epithelial ovarian cancer cells. Nevertheless, loss of PIPKIγ undeniably caused substantially reduced invasion in the OVCAR-8 and SKOV-3 cells. We reason that in SKOV-3 cells, PIPKIγ likely promotes invasion via the STAT3/MMPs axis; whereas in OVCAR-8 cells, other signaling cascades regulated by PI3K/AKT and/or STAT3 contribute more to cell invasion. Considering the structural similarity between invadopodia and focal adhesions and the role of STAT3 in focal adhesion assembly (12), it should be tested whether invadopodium assembly may be disturbed by the inhibited STAT3 phosphorylation in PIPKIγ-depleted cells. The underlying mechanism should be explored in the future.

In the present study, we mainly focused on the role of PIPKIγ in epithelial ovarian cancer cells and provide initial evidence supporting the contribution of PIPKIγ in epithelial ovarian cancer cell proliferation, migration and invasion, which is consistent with previous studies that revealed the role of PIPKIγ in oncogenic growth and cancer metastasis (6,11,51). Importantly, our results establish a solid base for further *in vivo* and translational studies to confirm whether PIPKIγ could be a valuable drug target alone or combined with other therapeutic strategies targeting ovarian cancer.

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