PDZ and LIM domain protein 4 suppresses the growth and invasion of ovarian cancer cells *via* inactivation of STAT3 signaling

Yanyan Jia, Huirong Shi*, Yuan Cao, Wenlong Feng, Meijuan Li, Xueru Li

Department of Obstetrics and Gynecology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

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ABSTRACT

Aims: PDZ and LIM domain protein 4 (PDLIM4) is frequently repressed in cancer tissues. However, the expression and role of PDLIM4 in ovarian cancer has not been addressed.

Main methods: In this study, we examined the expression and prognostic significance of PDLIM4 in ovarian cancer. The function of PDLIM4 in ovarian cancer cell growth, invasion, and tumorigenesis was further explored.

Key findings: PDLIM4 is downregulated in ovarian cancer compared to adjacent normal ovarian tissues. Downregulation of PDLIM4 is correlated with advanced tumor stage and lymph node metastasis. Low PDLIM4 expression is significantly associated with shorter overall survival in patients with ovarian cancer ($P = 0.0136$). Biologically, PDLIM4 overexpression suppresses the proliferation, colony formation, migration, and invasion of both CAOV3 and SKOV3 ovarian cancer cells, compared to empty vector-transfected cells. Consistently, *in vivo* data show that PDLIM4 overexpression inhibits the growth of SKOV3 xenograft tumors. Mechanistic investigation reveals that overexpression of PDLIM4 blocks the phosphorylation of STAT3 and represses STAT3-dependent transcriptional activation. Moreover, ectopic expression of PDLIM4 downregulates the expression of CCND1 and MMP9 in ovarian cancer cells. Rescue experiments demonstrate that overexpression of constitutively active STAT3 reverses PDLIM4-induced anticancer effects on ovarian cancer cells.

Significance: Overall, PDLIM4 downregulation is associated with aggressive tumor features and poor prognosis in ovarian cancer patients. PDLIM4 suppresses ovarian cancer cell growth and invasion by inhibiting STAT3 signaling. This study provides a potential therapeutic target for ovarian cancer.

1. Introduction

Ovarian cancer is one of the most lethal gynecologic malignancies worldwide [1]. The prognosis of advanced ovarian cancer is poor, with a 5-year survival rate of 10–30% [2,3]. Metastasis is a major cause of ovarian cancer-related deaths [4]. Surgery in combination with chemotherapy is the primary treatment for ovarian cancer [5,6]. Understanding the exact mechanism underlying the invasion and metastasis of ovarian cancer is of significance in treating advanced ovarian cancer.

Several signaling pathways in particular the STAT3 pathway have been reported to be involved in the progression of ovarian cancer [7,8]. STAT3 as a member of the STAT family of transcription factors shows the ability to enhance ovarian cancer invasion and metastasis [9]. The STAT3 pathway contributes to the stem cell-like properties of ovarian cancer cells [10,11]. Upon stimulation with cytokines and growth factors, STAT3 can be phosphorylated by receptor-associated Janus kinases (JAK), leading to STAT3 nuclear translocation and transactivation

of target genes such as CCND1 and MMP9 [12,13]. Therefore, STAT3 signaling is regarded as a promising therapeutic target for ovarian cancer.

PDZ and LIM domain protein 4 (PDLIM4) is a member of the PDLIM family that is composed of conserved PDZ and LIM domains [14]. PDLIM4 has been documented to participate in actin cytoskeleton remodeling and cell motility [15]. Several lines of evidence have indicated the involvement of PDLIM4 in tumor progression [16–18]. For instance, PDLIM4 overexpression suppresses the growth and clonogenicity of colon cancer cells [16]. PDLIM4 is downregulated in prostate cancer relative to adjacent benign tissues [17], and shows the ability to arrest cell cycle progression in prostate cancer cells [18]. Despite these studies, the expression and role of PDLIM4 in ovarian cancer has not been addressed.

In this study, we examined the expression and prognostic significance of PDLIM4 in ovarian cancer. We also characterized the function of PDLIM4 in ovarian cancer cell growth, invasion, and

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tumorigenesis. In addition, the signaling pathway(s) involved is determined.

2. Materials and methods

2.1. Tissue specimens

In this study, we collected 92 ovarian cancer and 35 adjacent normal ovarian tissues from the patients with ovarian cancer who underwent surgery at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). None of them had received any anti-cancer therapy before surgery. Clinicopathological information was retrieved from patient medical records. The median age of these patients was 58 years (range, 26–84 years). The collection and application of patient specimens were approved by the Human Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Every patient provided written informed consent for research purpose.

2.2. Immunohistochemical (IHC) staining and scoring

Paraffin-embedded tissue sections were dewaxed, hydrated, and blocked. The sections were then incubated rabbit anti-human PDLIM4 polyclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA; 1:50 dilution) at 4 °C overnight. After washing, the slices were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) for 30 min. The antibody complexes were visualized by incubation with diaminobenzidine chromogen. The staining results were evaluated by two pathologists without knowledge of the clinical outcome. The staining intensities were graded from 0 to 3, where 0 was defined as negative; 1 weak; 2 moderate; and 3 strong. The percentage of immune-reactive cells was graded from 0 to 4. Specially, no staining was scored as 0; 1–10% of cells stained as 1; 11–40% as 2; 41–70% as 3; and 71–100% as 4. The total score was determined as the product of staining intensity and percentage scores, ranging from 0 to 12. An IHC score of 2 or greater was considered high expression for PDLIM4, while an IHC score of < 2 was regarded as low PDLIM4 expression.

2.3. Cell culture and transfection

Human ovarian cancer cell lines CAOV3, OVCAR3, and SKOV3 were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Human ovarian surface epithelial cells were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in Ovarian Epithelial Cell Medium (ScienCell Research Laboratories). All the cell lines used in this study were authenticated by short tandem repeat profiling.

Full-length *PDLIM4* cDNA was cloned to pcDNA3.1(+) vector. A plasmid expressing constitutively active STAT3 (CA-STAT3) was obtained from Addgene Inc. (Cambridge, MA, USA). Cell transfection was performed using Lipofectamine 3000 (Invitrogen). For generation of PDLIM4 stably transfected cells, SKOV3 cells transfected with the PDLIM4-expressing plasmid were selected in the presence of 600 μg/mL G418 (Invitrogen) for 2 weeks.

2.4. Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted using Trizol reagents (Invitrogen) and reversely transcribed to cDNA. qPCR was performed with the following primers: PDLIM4 forward, 5′-CAAGGCACGGGACAAGCTCTAC-3′, PDLIM4 reverse, 5′-AGCAGGGACCTTAAGAAGCAG-3′; CCND1 forward, 5′-GCTGTGCATCTACACCCGACA-3′, CCND1 reverse, 5′-TTGAGCTTGT TCACCAGGAG-3′; MMP9 forward, 5′-ACGCAGACATCGTCATCC-3′, MMP9 reverse, 5′-AACCGAGTTGAACCACG-3′; GAPDH forward,

5′-CGACCACTTTGTCAAGCTCA-3′, GAPDH reverse, 5′-AGGGGTCTAC ATGGCAACTG-3′. The relative mRNA expression was determined by the $2^{-\Delta\Delta Ct}$ method [19].

2.5. Western blot analysis

Cells were lysed in Radio-immuno-protein assay buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Roche, Indianapolis, IN, USA). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% fat-free milk and incubated overnight at 4 °C with primary antibodies recognizing PDLIM4 (Thermo Fisher Scientific), phosphorylated STAT3 (p-STAT3), STAT3, and GAPDH (Cell Signaling Technology, Danvers, MA, USA). The membranes were then incubated with HRP-conjugated secondary antibodies, and developed by the chemiluminescence method (ECL, Amersham Biosciences, Piscataway, NJ, USA). Protein signals were quantified by densitometric analysis.

2.6. Cell proliferation assay

Cells were seeded into 12-well plates (4×10^5 cells/well) and allowed to grow for 3 days. Cells were counted every day, and growth curves were plotted.

2.7. Colony formation assay

Cells were seeded onto 12-well plates at a density of 400 cells/well and cultured for 2 weeks. After staining with 0.5% crystal violet, colonies (> 50 cells) were manually counted and scored.

2.8. Cell migration and invasion assays

Cell migration and invasion capacities were measured using the wound-healing and Transwell invasion assays, respectively [20]. In brief, for the wound-healing assay, cells were seeded in 60-mm culture dishes and grown to 100% confluency, and a scratch wound was created using a pipette tip. The cells were then cultured in fresh DMEM supplemented with 10 μg/mL mitomycin C (Sigma-Aldrich, St. Louis, MO, USA). Images were taken at 0 and 48 h after scratching. The percentage of wound closure was calculated from three independent experiments. For the invasion assay, a 24-transwell chamber (8-μm pore size) was used. Cells in serum-free culture medium were seeded into the upper chamber, and media with 10% FBS was added to the lower chamber as a chemoattractant. After a 24-h incubation, the number of invaded cells were counted in five microscopic fields.

2.9. STAT3 reporter assay

STAT3 reporter assay was performed as described previously [21]. In brief, cells were transfected with a STAT3 reporter plasmid (Signosis, Santa Clara, CA, USA) together with empty vector or PDLIM4-expressing plasmid using Lipofectamine 3000 (Invitrogen). The *Renilla* luciferase reporter plasmid, pRLTK was co-transfected to control for transfection efficiency. Twenty-four hours after transfection, cells were lysed. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.10. Animal experiments

All animal experiments were approved by the Institutional Use and Care of Animals Committee of Zhengzhou University. Female nude mice (aged 5 weeks) were subcutaneously injected with PDLIM4-over-expressing or control SKOV3 cells (2×10^6 cells/mouse). Tumor length (L) and width (W) were measured every week for 4 weeks, and tumor volume was calculated based on the following formula:
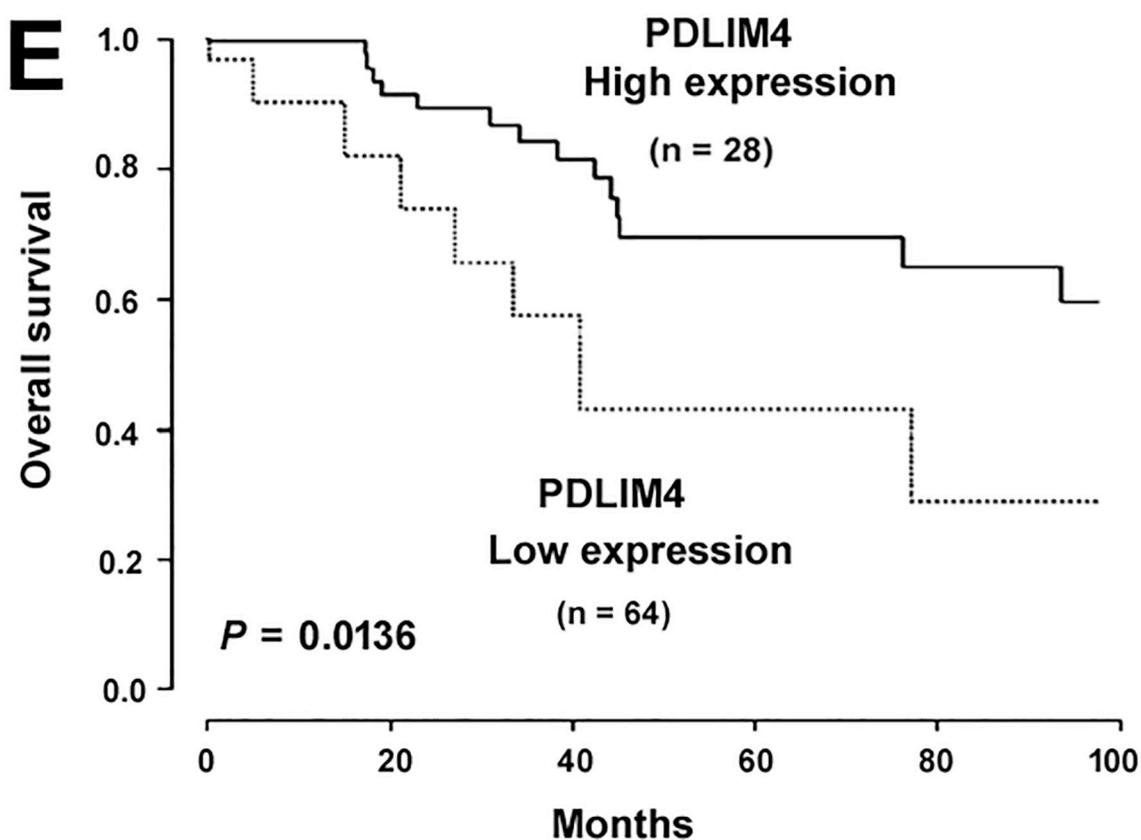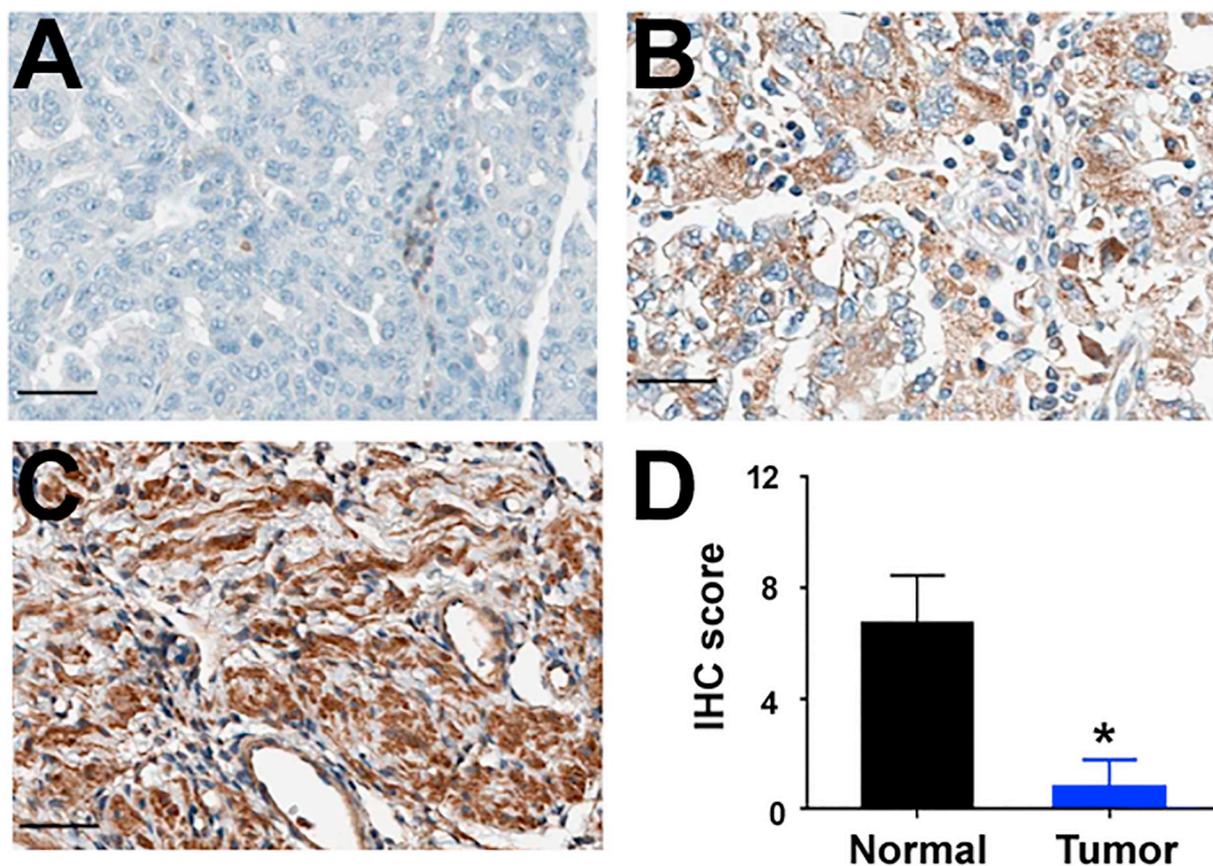
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Fig. 1. Downregulation of PDLIM4 correlates with poor prognosis in ovarian cancer. (A–D) Immunohistochemistry of PDLIM4 in ovarian cancer and normal ovarian tissues. Representative images show low (A) and high (B) expression of PDLIM4 in ovarian cancer tissues, as well as high PDLIM4 expression in normal tissues (C). Scare bar = 50 μm. (D) IHC score for PDLIM4 was lower in the ovarian cancer relative to normal tissues. *$P < 0.05$. (E) Overall survival was determined according to PDLIM4 expression status by the Kaplan-Meier method and the log-rank test.

Table 1
Association of PDLIM4 levels with clinicopathological parameters in patients with ovarian cancer.

Parameter	n	PDLIM4 expression		P
		Low (n = 64)	High (n = 28)	
Age, years				0.438
< 60	34	22	12	
≥ 60	58	42	16	
Tumor stage				0.003
I/II	32	16	16	
III/IV	60	48	12	
Histological type				0. 641
Serous	78	55	23	
Non-serous	14	9	5	
Lymph node metastasis				< 0.0001
Absent	34	15	19	
Present	58	49	9	

Volume = (L × W^2) / 2. After last measurement, mice were sacrificed, and xenograft tumors were weighed. Tumor samples were processed for immunostaining using anti-Ki-67 (Abcam) or anti-p-STAT3 (Cell Signaling Technology) antibodies. Nuclei were counterstained with haematoxylin.

2.11. Statistical analysis

Data are expressed as means ± standard deviation. Differences were analyzed by the Student's *t*-test or one-way analysis of variance with Tukey *post hoc* test. Survival analysis was performed with the Kaplan-Meier method and the log-rank test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Downregulation of PDLIM4 correlates with poor prognosis in ovarian cancer

Immunohistochemistry was performed to examine the expression of PDLIM4 in 92 ovarian cancer and 35 adjacent normal ovarian tissues. Positive PDLIM4 staining (86%) was readily observed in normal ovarian tissues, while only 30% of ovarian cancer tissues showed positive staining for PDLIM4 (Fig. 1A–D). As shown in Table 1, PDLIM4 expression levels were significantly correlated with advanced tumor stage ($P = 0.003$) and lymph node metastasis ($P < 0.0001$). The prognostic impact of PDLIM4 expression on patients' survival was assessed using the Kaplan-Meier method. The results demonstrated that low PDLIM4 expression was significantly associated with shorter overall survival ($P = 0.0136$; Fig. 1E).

3.2. PDLIM4 suppresses ovarian cancer cell growth

We examined the level of endogenous PDLIM4 in several ovarian cancer cell lines. The results showed that compared to normal ovarian epithelial cells, the mRNA and protein levels of PDLIM4 were significantly reduced in CAOV3, OVCAR3, and SKOV3 ovarian cancer cells (Fig. 2A and B). To explore the biological role of PDLIM4 in ovarian cancer cells, we performed PDLIM4 overexpression experiments in both CAOV3 and SKOV3 cells (Fig. 2C). Cell proliferation assay demonstrated that enforced expression of PDLIM4 significantly suppressed the proliferation of CAOV3 and SKOV3 cells, compared to empty vector-

transfected cells ($P < 0.05$; Fig. 2D). Colony formation assay further showed that PDLIM4-overexpressing ovarian cancer cells exhibited decreased growth (Fig. 2E).

3.3. PDLIM4 overexpression restrains ovarian cancer cell migration and invasion

Next, we evaluated the effect of PDLIM4 overexpression on ovarian cancer cell migration and invasion. *In vitro* wound-healing assay showed that CAOV3 and SKOV3 cells with PDLIM4 overexpression had a significant decline in the migration capacity relative to control cells ($P < 0.05$; Fig. 3A). Similarly, the invasiveness of CAOV3 and SKOV3 cells was substantially decreased in the setting of PDLIM4 overexpression (Fig. 3B).

### 3.4. PDLIM4 blocks STAT3 activation in ovarian cancer cells

A previous study [22] has demonstrated that PDLIM4 suppresses Src kinase activation in HCT116 colon cancer cells. However, the activation of Src remained unchanged in PDLIM4-overexpressing CAOV3 and SKOV3 cells relative to corresponding controls (data not shown), suggesting an Src-independent mechanism involved in PDLIM4 action in ovarian cancer. To determine the mechanism by which PDLIM4 exerts suppressive activity in ovarian cancer cells, we investigated the changes in several key signaling pathways, which are involved in the progression of ovarian cancer [23–25]. Western blot analysis demonstrated that the phosphorylation of STAT3 was markedly suppressed by PDLIM4 overexpression (Fig. 4A). However, no obvious change in the phosphorylation status of FAK, ERK, Akt, mTOR, and NF-κB was observed (data not shown). Consistent with the inactivation of STAT3, we observed a significant decline in the abundance of CCND1 and MMP9 transcripts in PDLIM4-overexpressing cells (Fig. 4B). Furthermore, ectopic expression of PDLIM4 suppressed STAT3-dependent luciferase reporter expression in ovarian cancer cells (Fig. 4C). These results suggest that PDLIM4 overexpression interferes with the transcriptional activity of STAT3.

3.5. Overexpression of constitutively active STAT3 rescues ovarian cancer cells from PDLIM4-induced anticancer activity

Next, we validated the role of STAT3 signaling in PDLIM4-mediated anti-tumor activity. To this end, we performed rescue experiments by overexpressing constitutively active STAT3 in SKOV3 cells. As shown in Fig. 5A, overexpression of constitutively active STAT3 significantly restored STAT3-dependent transcriptional activity. Most interestingly, PDLIM4-mediated suppression of cell proliferation (Fig. 5B), colony formation (Fig. 5C), and invasion (Fig. 5D) were reversed by ectopic expression of constitutively active STAT3. Collectively, our data demonstrate that the roles of PDLIM4 in ovarian cancer cell growth and invasion are mediated by the STAT3 pathway.

3.6. PDLIM4 inhibits the growth of ovarian cancer cells in vivo

Next, we investigated whether PDLIM4 exerts anti-tumor effect *in vivo*. We found that xenograft tumors formed by PDLIM4-overexpressing SKOV3 cells grew significantly slower than those by empty vector-transfected cells (Fig. 6A). The tumor weight was reduced in the PDLIM4 overexpression group (Fig. 6B). Immunohistochemistry staining confirmed a decline in the percentage of Ki-67-positive (Fig. 6C) and p-STAT3-positive (Fig. 6D) tumor cells in the PDLIM4
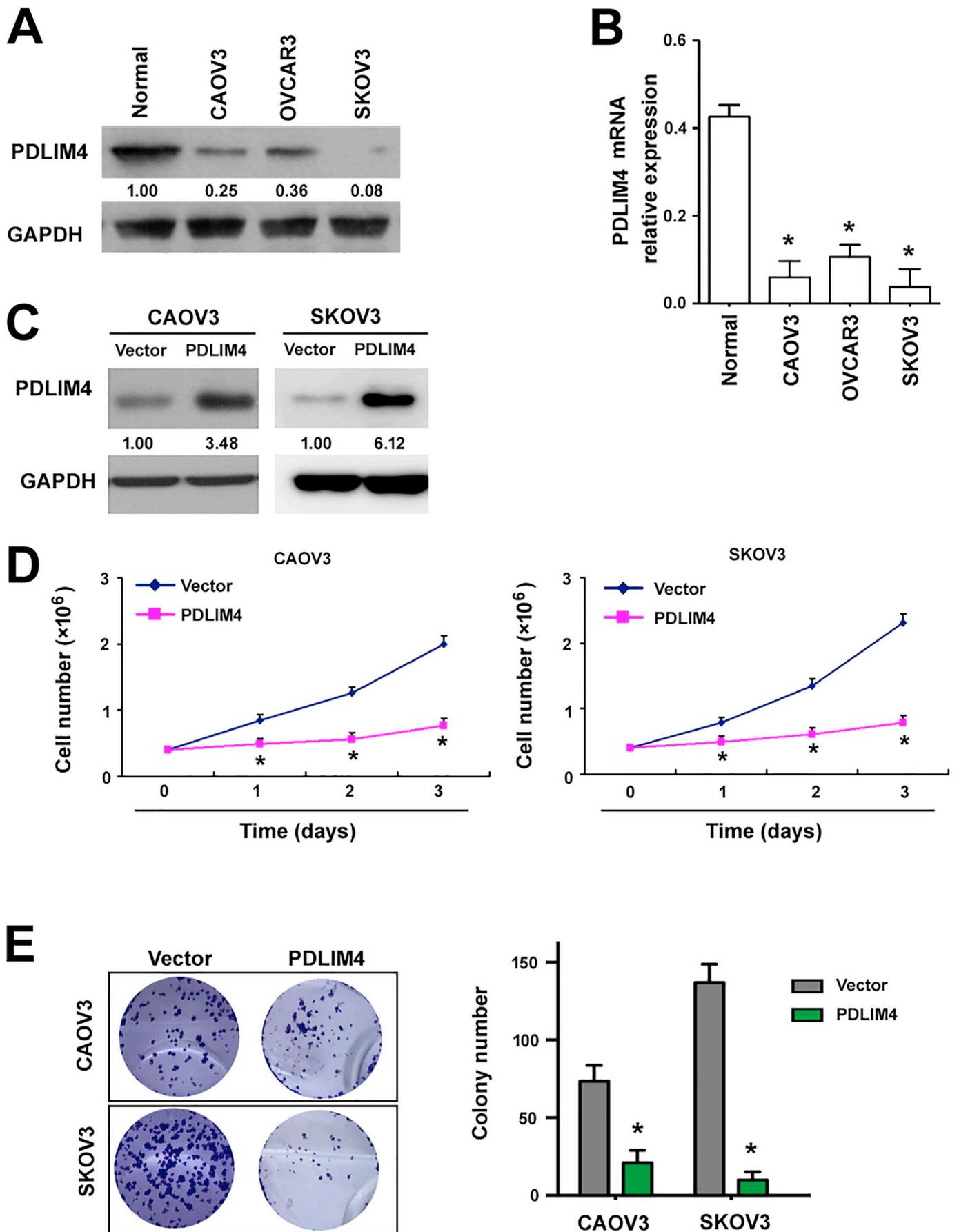
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Fig. 2. PDLIM4 suppresses ovarian cancer cell growth. (A) Western blot and (B) qPCR analysis of endogenous PDLIM4 expression in ovarian cancer cell lines. *$P < 0.05$ *vs.* normal ovarian epithelial cells. (C) PDLIM4 was overexpressed in both CAOV3 and SKOV3 cells, as validated by Western blot analysis. (D) Cell proliferation assay. Overexpression of PDLIM4 significantly suppressed the proliferation of CAOV3 and SKOV3 cells. (E) Colony formation assay. Colonies were counted after culturing for 2 weeks. *$P < 0.05$ *vs.* vector-transfected cells.
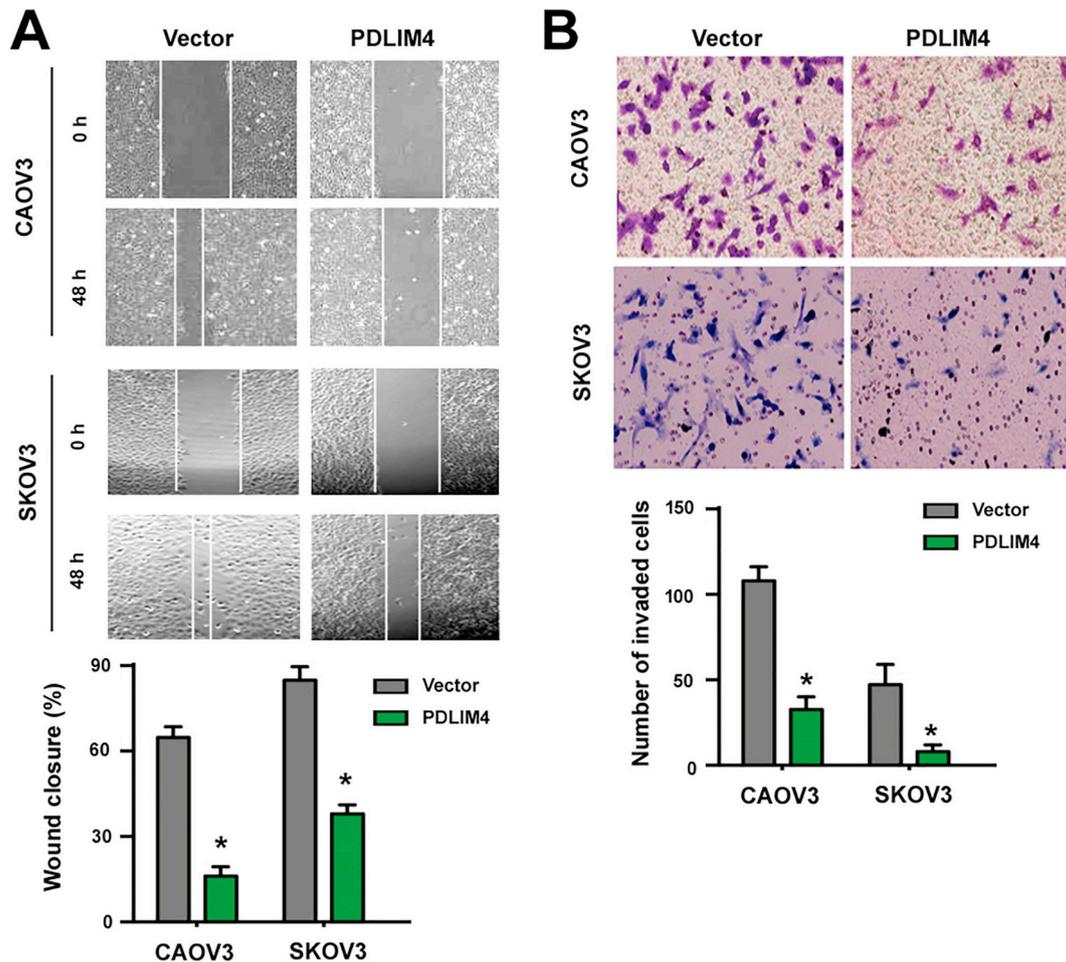
Fig. 3. PDLIM4 overexpression restrains ovarian cancer cell migration and invasion. (A) Cell migration was determined by *in vitro* wound-healing assay. (B) Cell invasion was determined by Transwell invasion assay. *$P < 0.05$ *vs.* vector-transfected cells.

overexpression group. Taken together, PDLIM4 suppresses the growth of ovarian cancer cells in mouse models.

4. Discussion

Epigenetic silencing of PDLIM4 *via* hypermethylation is frequently detected in cancers [17,26,27]. For instance, Morris et al. [27] reported that > 30% of renal cell carcinoma has promoter hypermethylation on PDLIM4 gene and reduced expression of PDLIM4. Botezatu et al. [26] demonstrated that PDLIM4 promoter methylation is increased in thyroid cancer relative to normal tissues. Feng et al. [28] documented that PDLIM4 promoter is hypermethylated in primary and metastatic breast cancer samples. Consistently, we also show that PDLIM4 is downregulated in ovarian cancer relative to adjacent normal ovarian tissues. The downregulation of PDLIM4 is associated with lymph node metastasis. Most importantly, patients with reduced PDLIM4 expression have significantly shorter overall survival than those with higher tumoral PDLIM4 levels. Therefore, PDLIM4 may serve as a novel prognostic factor for ovarian cancer.

Biologically, PDLIM4 acts as a tumor suppressor in ovarian cancer cells. We provide evidence that ectopic expression of PDLIM4 significantly inhibits the proliferation and colony formation of ovarian

cancer cells. Moreover, overexpression of PDLIM4 impairs the migratory and invasive capacities of ovarian cancer cells. *In vivo* tumorigenic studies further confirm that enforced expression of PDLIM4 leads to a reduction in the growth of xenograft tumors from ovarian cancer cells. Collectively, these observations indicate that PDLIM4 plays a tumor-suppressive role in ovarian cancer. In agreement with our findings, Vanaja et al. [18] reported that PDLIM4 exerts tumor-suppressive effects on prostate cancer cells. It is conceivable that PDLIM4 acts as a tumor suppressor in some types of cancers including ovarian cancer and prostate cancer. The tumor-suppressive activity of PDLIM4 provides an explanation for the association of reduced PDLIM4 expression with aggressive parameters of ovarian cancer.

Mechanistically, PDLIM4 overexpression leads to inhibition of STAT3 signaling in ovarian cancer cells. Activation of the STAT3 pathway contributes to the aggressiveness of ovarian cancer cells [9,11]. Pharmacological blockade of STAT3 signaling causes anti-tumor effects against ovarian cancer [10,29]. Of note, we show that ectopic expression of PDLIM4 selectively blocks the phosphorylation activation of STAT3 without altering the activation of FAK, ERK, Akt, mTOR, and NF-κB. The results suggest that STAT3 signaling plays a fundamental role in mediating the activity of PDLIM4. In support of this hypothesis, we show that overexpression of constitutively active STAT3 reverses the

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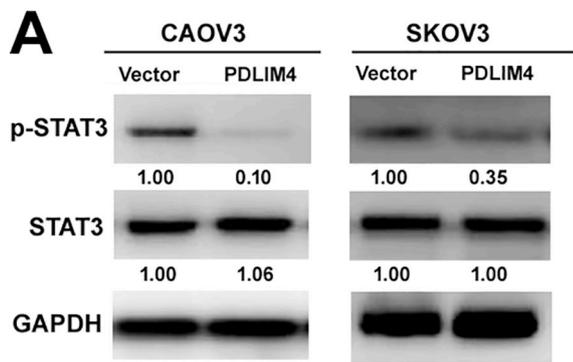
Fig. 4. PDLIM4 blocks STAT3 activation in ovarian cancer cells. (A) Western blot analysis of indicated proteins. (B) qPCR analysis of CCND1 and MMP9 transcripts in PDLIM4-overexpressing cells. (C) STAT3 reporter assays showed that ectopic expression of PDLIM4 suppressed STAT3-dependent luciferase reporter expression in ovarian cancer cells. *P < 0.05 vs. vector-transfected cells.
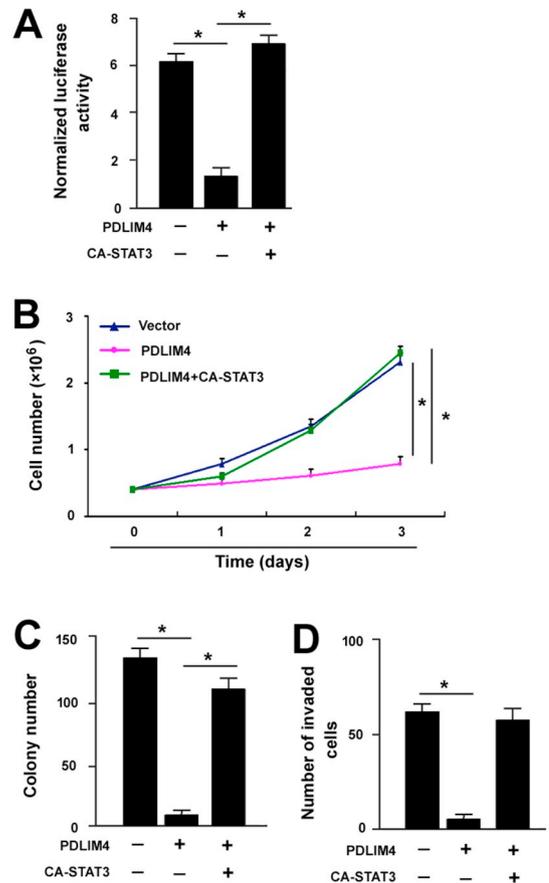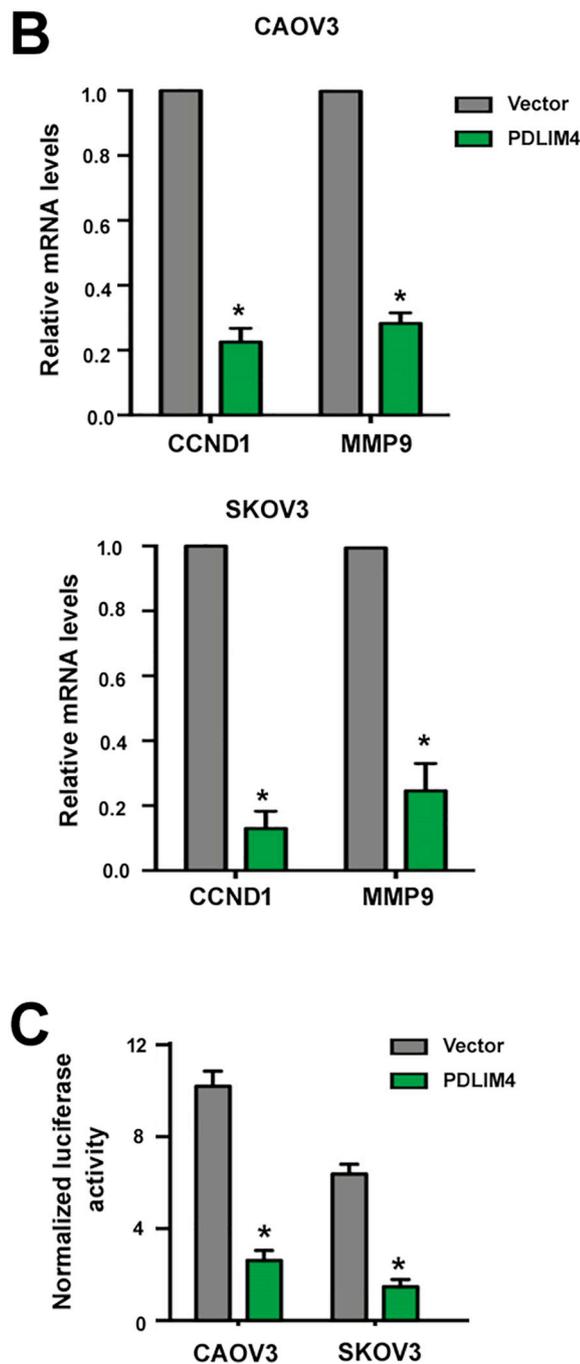
Fig. 5. Overexpression of constitutively active STAT3 rescues ovarian cancer cells from PDLIM4-induced anticancer activity. (A) Overexpression of constitutively active STAT3 significantly restored STAT3-dependent transcriptional activity. (B) Cell proliferation, (C) colony formation, and (D) invasion were determined after ectopic expression of constitutively active STAT3. *P < 0.05.

suppressive effects of PDLIM4 on ovarian cancer growth and invasion. A previous study has indicated that PDLIM2 can repress the expression of NF-κB and STAT3 in cancer cells [30]. It has been suggested that PDLIM2 acts as a nuclear ubiquitin E3 ligase, which can target STAT3 protein to degradation [31]. These observations provide a possible mechanism for the inhibition of STAT3 signaling by PDLIM4. However, more work is required to address how PDLIM4 blocks the activation of STAT3 signaling.

STAT3 is known to transactivate a lot of genes involved in tumor growth and progression [32]. We demonstrate that overexpression of PDLIM4 suppresses the transcriptional activity of STAT3 as determined by luciferase reporter assays. Moreover, the target genes CCND1 and MMP9 were suppressed by PDLIM4 overexpression. CCND1 is an important cell cycle regulator and contributes to the progression of the G1 phase [33]. MMP9 is involved in the breakdown of extracellular matrix and thus promotes cell migration and invasion [34]. Taken together, these results suggest that PDLIM4-mediated anti-tumor effect against ovarian cancer is causally linked to inactivation of STAT3 signaling and downregulation of STAT3 target genes including CCND1 and MMP9.
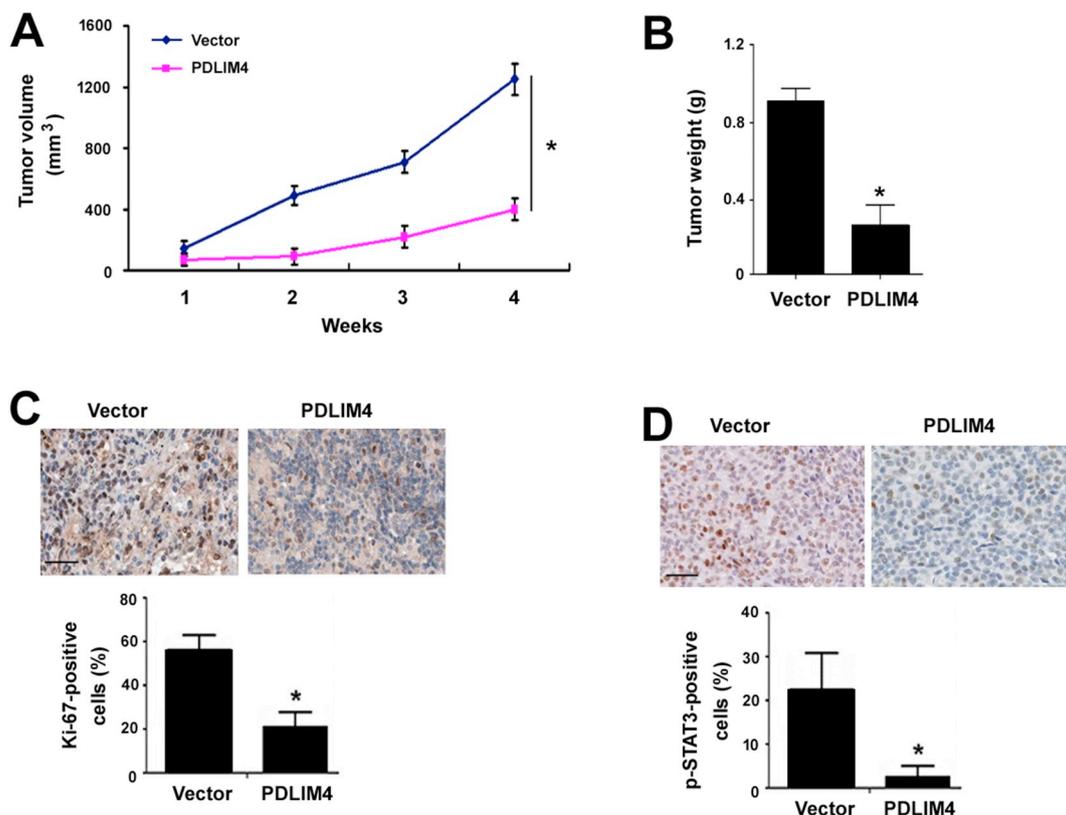
Fig. 6. PDLIM4 inhibits the growth of ovarian cancer cells *in vivo*. (A) Tumor volume was calculated every week for 4 weeks. (B) Tumor weight was determined 4 weeks after cell injection. (C, D) Immunohistochemistry staining of (C) Ki-67 and (D) p-STAT3 in the xenograft tumors. Scare bar = 100 μm. *$P < 0.05$.

5. Conclusion

In summary, our data indicate that PDLIM4 is downregulated in ovarian cancer and associated with shorter overall survival of patients with ovarian cancer. Enforced expression of PDLIM4 can remarkably inhibit the proliferation, invasion, and tumorigenesis of ovarian cancer cells. Mechanistic investigation reveals that PDLIM4 blocks the phosphorylation activation of STAT3 and inhibits the expression of CCND1 and MMP9, therefore exerting anti-tumor effects on ovarian cancer. These findings suggest that PDLIM4 might be an attractive therapeutic target for ovarian cancer.

Declaration of competing interest

There is no conflict of interest.

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