NEOPLASIA
www.neoplasia.com

Volume 19 Number 3 March 2017 pp. 154–164 154

# PLCE1 Promotes Esophageal Cancer Cell Progression by Maintaining the Transcriptional Activity of Snail[1]

Shicong Zhai[*,†,2], Cui Liu[‡,2], Lichen Zhang[§], Jian Zhu[§,¶], Jiqiang Guo[¶], Jinghang Zhang[#], Zhijun Chen[**], Wenping Zhou[††], Tingmin Chang[‡‡], Siguang Xu[§§], Yijun Qi[¶¶], Ting Zhuang[§,¶], Na Yu[†], Weilong Wang[†], Hui Wang[##], Sifan Yu[¶,***] and Xiumin Li[*,†]

[*]Center for Cancer Research, Xinxiang Medical University, Xinxiang, Henan, China; [†]Department of Gastroenterology, the Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China; [‡]School of Nursing, Xinxiang Medical University, Henan, China; [§]School of Laboratory Medicine, Xinxiang Medical University; [¶]Research Center for Immunology, Xinxiang Medical University, Henan, China; [#]Department of Pathology, the First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan, China; [**]Department of Thoracic Surgery, the First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan, China; [††]Lymphoma Institute, Zhengzhou, Henan Cancer Hospital, Zhengzhou, Henan, China; [‡‡]Department of Gastroenterology, the First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan, China; [§§]Institute of Lung and Molecular Therapy, Xinxiang Medical University, China; [¶¶]Key Laboratory of Cellular and Molecular Immunology, College of Medicine, Henan University, Kaifeng, Henan, China; [##]Ontario Cancer Institute, Campbell Family Institute for Breast Cancer Research, University of Toronto, Toronto, Canada; [***]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education) Department of Renal cancer and Melanoma, Peking University School of Oncology, Beijing Cancer Hospital and Institute, Beijing, China

## Abstract

Esophageal cancer is among the most deadly malignant diseases. However, the genetic factors contributing to its occurrence are poorly understood. Multiple studies with large clinic-based cohorts revealed that variations of the phospholipase C epsilon (PLCE1) gene were associated with esophageal cancer susceptibility. However, the causative role of PLCE1 in esophageal cancer is not clear. We inactivated the functional alleles of PLCE1 by CRISPR/Cas9 genome editing technology. The resultant PLCE1 inactivated cells were analyzed both *in vitro* and *in vivo*. Our results showed that loss of PLCE1 dramatically decreased the invasion and proliferation capacity of esophageal carcinoma cells *in vitro*. Moreover, such PLCE1 inactivated tumor grafts exhibited significantly decreased tumor size in mice. We found that PLCE1 was required to maintain protein level of snail a key transcription factor responsible for invasion. Our further transcriptomic data revealed that deficient cells were

Address all correspondence to: Xiumin Li, Department of Gastroenterology, the Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China.
E-mail: lxm3029981@126.com

[1]Grant Support: This study is supported by the National High Technology Research and Development Program of China (2012AA02A201-1, http://program.most.gov.cn), and the Science and Technology Development Plan of Henan Province (112102310283, 122102310198, http://www.hnkjt.gov.cn).

[2]Shicong Z. and Cui L. contribute equally in the study.

Received 14 November 2016; Revised 7 December 2016; Accepted 12 December 2016



1476-5586
http://dx.doi.org/10.1016/j.neo.2016.12.007

significantly decreased in expression of genes enriched as targets of Snail. Strikingly, recovery of Snail protein at least partially rescued the invasion and proliferation capacity in PLCE1 inactivated cells. In ESCC clinical specimens, PLCE1 was correlated with tumor stage ($P < .0001$). Interestingly, PLCE1 expression was positively correlated Snail by immunohistochemistry in such specimens ($P < .0001$). Therefore, our functional experiments showed the essential roles of PLCE1 in esophageal carcinoma cells and provided evidences that targeting PLCE1 and its downstream molecules could be effective therapies for esophageal cancer.

*Neoplasia (2017) 19, 154–164*

## Introduction

Esophageal cancer is one of the least studied but deadliest cancers with increasing incidence and mortality.[1] The major subtype of esophageal cancer is esophageal squamous-cell carcinoma and another type esophageal adenocarcinoma is more frequent in the developed countries.[2,3] There are approximately 450,000 diagnosed new cases with esophageal carcinoma each year ranking eighth in the most frequently diagnosed cancers worldwide, and the incidences of ESCC have inter-area variations with high incidence in certain areas such as northern China.[3] Environmental factors as smoking, heavy alcohol use are known to increase the risk of esophageal cancer, while the genetic factors contributing to this malignant disease are very poorly understood.[4,5] Early epidemiological studies showed that the genetic background contributed significantly to the susceptibility to esophageal squamous-cell carcinoma, and more recent findings from large clinical cohorts and genome wide association studies revealed the association of PLCE1 with susceptibility to ESCC.[6–10] However the functional role of PLCE1 in esophageal cancer remains unknown. Determining the specific roles of PLCE1 in esophageal cancer is decisive to develop therapeutics, and it is particularly necessary to elucidate the function of PLCE1 as it is the only gene confirmed in human population for its association with ESCC.

PLCE1 gene belongs to the phospholipase C family that catalyzes the polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP2) and generate secondary messengers including 1,4,5 triphosphate and diacylglycerol.[11] A large diversity of biological processes is under the regulation of phospholipase C family.[12] However, the detailed biological function of PLCE1 such as promoting or inhibiting ESCC remains to be unambiguously elucidated. In this study, we used CRISPR/Cas9 tool to genetically inactivate PLCE1 gene in ESCC cell line through targeting two independent esophageal carcinoma cells lines which resulted in multiple clones of cells absent in PLCE1 protein. With the PLCE1 deficient esophageal cancer model and ESCC clinical specimens, we identified the PLCE1 function in cancer proliferation and aggression and confirmed the potential to target this molecule in ECSS treatment.

## Materials and Methods

### *Plasmid Construction*

Expression vector px330 (Addgene#42230) expressing human codon-optimized version of *S. pyogenes* Cas9 protein and chimeric gRNA was digested with BbsI and purified.[24] A pair of oligonucleotides 5′-CACCGCAATATCGCAGTACATCAC-3′ and 5′-AAACGTGATGTACTGCGATATTGC-3′ was used for targeting exon2 of human PLCE1. Another pair of oligonucleotides 5′-CACCGAGTTCAGCTGGGATCAGCA-3 and 5′-AAACTGCTGATCCCAGCTGAACTC-3′ were used for targeting exon3 of human PLCE1. The oligonucleotides were annealed, phosphorylated and ligated to the linearized vector. The resulting CRISPR vectors were confirmed by sequencing and referred to px330-sgRNA2 and px330-sgRNA3, respectively.

### *Cell Line Authentication*

Two human esophageal cancer cell lines EC9706 and Eca109 used in this study were both acquired from China Infrastructure of Cell Line Resources (http://www.cellresource.cn). Cell line authentication utilizing Short Tandem Repeat (STR) profiling were performed with PowerPlex® 21 System (Promega, USA) which allowed for detection of 21 loci, including D1S1656, D2S1338, D3S1358, D5S818, D6S1043, D7S820, D8S1179, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, Amelogenin, CSF1PO, FGA, Penta D, Penta E, TH01, TPOX and vWA (Supplementary Figure 1, *A* and *B*). The PCR amplification of DNA templates from EC9706 or Eca109 cells were analyzed by capillary electrophoresis with genetic analyzer 3130 (ABI, USA) and allele call was performed by GeneMapper software V3.2.1. and 7 out of the 21 markers were used to compare with the accessible database archived by ATCC (https://www.atcc.org/str) and China Infrastructure of Cell Line Resources. Both EC9706 and Eca109 cell lines in our study were not contaminated by the other STR recorded cells, as no matches were found according to ATCC threshold of 50% allele similarity. STR profiling of our colony of Eca109 cells was found to be 100% consistent with the STR data of the Eca109 from China Infrastructure of Cell Line Resources. EC9706 cell STR profiling data was not accessible in public databases including ATCC.

### *Cell Culture and Transfection*

Esophageal carcinoma cell line EC9706 and Eca109 were cultured in RPMI 1640 (HyClone™) supplemented with 10% fetal bovine serum (CELLect™ Gold, US Origin) and penicillin streptomycin (Beyotime, China). Cells were maintained at 37 °C and 5% CO2 in a humidified incubator. EC9706 cells were plated in 24-well plates 24 hours before transfection with 70–90% confluency. Cells in each well were transfected with 5 μg of plasmid px330-sgRNA2 and px330-sgRNA3, respectively, using Lipofectamine® 3000 Transfection Reagent (ThermoFisher L3000–001) according to the manufacturer's protocol.

### *Isolation of Gene-Knockout Cells*

EC9706 and Eca109 cells transfected with plasmid px330-sgRNA2 or px330-sgRNA3 were incubated for 48 hours

and then cloned by limiting dilution as previously described.[13] Isolated single clones were analyzed using fluorescent PCR and Sanger sequencing with the primers listed below: 5′-TGGTG TCTGTAGAATGTTCAGGT-3′ in pair with 5′-TACCTTTCAG CATCATTCGTCCA-3′, and 5′-AAACCAACCCTACCAC CACC-3′ in pair with 5′-GCCCAAGTCCCGTGTTAAGA-3′.

### *Migration and Invasion Assay*

Cell migration and invasion assay were performed using modified two-chamber plates as described.[14] For the migration assay, $7.5 \times 10^4$ EC9706 and control cells were seeded in serum-free medium in the upper chamber. For the invasion assay, $1 \times 10^5$ mutant EC9706 and control cells were added to the top chamber coated with matrigel (Biocoat, USA). To stimulate migration or invasion, complete medium was added to the bottom wells. After 24 h incubation at 37 °C for migration assay and 72 h for invasion assay, cells in the upper chamber were carefully removed and the cells that had passed through the membrane were fixed and stained with Crystal Violet Staining Solution. Cellular quantification was analyzed in five fields with X100 magnification under microscope. In the wound-healing assay, $2 \times 10^5$ cells/well was cultured in 24-well plates. After scratching the monolayer with the pipette tips, cells were washed with PBS, cultured in serum-free RPMI 1640, and photographed at 0 h, 24 h and 48 h as similarly described in literature.[14]

### *Flow Cytometry*

To measure the Mean fluorescent intensity (MFI) of cytoplasm proteins, control and PLCE1 inactivated mutant cells were fixed in 4% paraformaldehyde for 20 min on ice, washed with FACS buffer (1 × PBS, 2% bovine serum, 2 mM EDTA), and permeablized with 90% methyl alcohol. Cells were stained with rabbit anti-human primary antibodies (Supplementary Figure 3), and MFI was measured by flow cytometry (Becton Dickinson) of the secondary Allophycocyanin (APC) conjugated goat anti-rabbit antibody (Life Technologies). FACS data concerning histogram overlays was analyzed by Flowjo version 10.1.

### *Immunoblotting Analysis*

Cells were harvested and washed twice with PBS. Cell lysis was performed and insoluble debris was pelleted by centrifugation under condition recommended by the reagent supplier (Beyotime, China). The supernatants were collected and protein concentrations was determined by kit (Beyotime, China), and was further adjusted according to intensity of beta-actin. Proteins were separated by electrophoresis on SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred to PVDF membrane. The antibodies used in this study were listed in Supplementary Table 1. Membranes were then washed and incubated with secondary antibodies Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG. Fluorescent signals were visualized with ECL system. (Amersham Imager 600, USA).

### *Subcutaneous Xenograft Mouse Tumor Model*

4–5 week old BALB/c nude female mice (Vital River Laboratory Animal Technology Co. Ltd. Beijing, China) were used for all experiments. The experiments were performed in accordance with the animal care and experiment regulations. Control cells and mutant cells ($4 \times 10^6$ cells suspended in 0.2 ml of PBS) were injected into the left flank region of mice (n = 5, each group). Tumor growth was measured using Vernier Caliper every 4 days until the end of the experiment. The mice were euthanized on day 30, and subcutaneous tumors were surgically excised, weighed, photographed, and stored in liquid nitrogen for further experiments.

### *Real-Time Quantitative PCR*

Total RNA was extracted using the RNeasy Plus kit (Qiagen), and cDNA templates were synthesized using reverse transcription kit (CWBIO, China). Real-time qPCR was performed using SYBY Select Master Mix (Life technologies, USA). The sequence of the primers for qPCR was listed in Supplementary Table 2. The relative gene expression was measured with reference to GAPDH as housekeeping gene, using $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ method.

### *WST-1 Cell Proliferation Assay*

EC9706 cells were seeded into each well of 96-well plates. 1 h before reading the plate, WST-1 reagent (Roche, USA) was added to each well. The absorbance of the samples was measured against a blank background control using a microplate reader at 450 nm.

### *RNA Sequencing Analysis*

The global gene expression analysis was based on RNA sequencing platform from BGI (Beijing Genomic Institute). The significant changed genes, which had fold change> 2 and P value <.01, were sent into the David online platform for pathway analysis (https://david.ncifcrf.gov/tools.jsp). The RNA sequence data are deposited in the Gene Expression Omnibus (GEO) database under the accession number SRP070098 in which "KO" refers to PLCE1 inactivated EC907 cells, and "WT" refers to EC9706 control cells. PLCE1 knockout RNA sequence data are overlapped with public available Snail ChIP sequence data.[15,16] RNA sequence data of esophageal tumors are acquired from TCGA database. By applying Z-score> 1, 35 samples with PLCE1 high and 33 samples with PLCE1 low are used for correlation analysis between PLCE1 expression and Snail target genes (https://tcga-data.nci.nih.gov/tcga/).

### *Construction of Lentiviral Based Overexpression Vector and Cell Infection*

The code sequence of human SNAI gene (Gene ID: 6615) was synthesized by GENEWIZ company, and then cloned into the PMD18-T vector by BamHI/EcoRI that we called PMD18-T-SNAI. The SNAI gene was digesting from PMD18-T- SNAI vector using BamHI/EcoRI, and then was cloned into the FUA-EF1-EGFP-(P2A)-Puro lentiviral based overexpression vector digested by BamHI/EcoRI and confirmed by DNA sequencing. For the production of lentivirus, 10-cm dish 293 T cells was transfected with 12ug vector containing our interesting SNAI gene followed by the EGFP-sorting, puromycine selection marker and 8ug packing vector pSPAX2 and 4ug envelope vector pMD2G. After 48 hours of the transfection, virus -containing supernatant derived from the 293 T cells was filter though the 0.45um filter and concentrated with PEG600 method. Target cells (probable $1 \times 10^5$) were incubated with the virus-containing supernatant media for 4 hours to overnight. After infection cells was replaced with 2 ml fresh medium. Two days after infection, we use 2ug/ml puromycine to select the positive cells.

### *Clinical Tumor Samples*

One hundred and one formalin-fixed paraffin-embedded esophageal tumor samples were collected from the Department of Thoracic

Surgery in the First Affiliated Hospital of Xinxiang Medical University, and all of the patients were diagnosed in the year 2015. All the esophageal tumors samples were obtained by surgery. The invasive depth, lymph node metastasis status and differentiation status of each sample was examined by pathological specialists. This study was reviewed and approved by the Ethical Board at the First Affiliated Hospital of Xinxiang Medical University with written informed consent from all the patients.

### *Immunohistochemistry*

PLCE1 and Snail expression were evaluated by immunohistochemistry. In general, slides with 4 microns were fixed by formalin and embedded by paraffin. Sodium citrate was used for antigen retrieval. The slides were incubated by PLCE1 (Santa Cruz Biotechnology, sc-28,402) and Snail antibody (Cell Signaling Technology, 3879). The Polymer-HRP method was used for detection. No staining was used for negative controls.

### *Statistical Analysis*

Data are shown as the mean ± SEM. Difference were considered significant for $P < .05$ and the degree of significance is indicated as follows: *, $P < .05$; **, $P < .01$; ***, $P < .001$. All data were analyzed with GraphPad Prism software (version 6.0). Statistical significance was determined with a Mann–Whitney *U* test.

## Results

### *Genetic Inactivation of PLCE1 by CRISPR/Cas9 Genome Editing Tool in ESCC Cell Lines*

In order to identify the functional role of PLCE1 in ESCC cells, we performed genetic inactivation of the gene in EC9706 cells derived from esophageal squamous cell carcinoma as used in previous studies.[17] CRISPR/Cas9 system was facilitated to generate PLCE1 knockout in the ESCC cells. Based on coding conservation among different transcripts archived in the Ensemble Genomes database, we designed two single-guide RNAs (sgRNAs) targeting conserved exons, exon2 and exon3 respectively of the ENST00000371380 transcript (Supplementary Figure 1*C*), using an expression system as described previously.[18] Following the limiting dilution of genetically manipulated cells, fluorescent PCRs were used to screen for monoclonal mutant cells.[18] The clones harboring Indel mutations giving rise to PCR product length polymorphisms were sequenced for validation of frame shift (Figure 1*A*, Supplementary Figure 1*D*). We selected the clones that had undergone dual allelic inactivation for further analyses of mRNA and protein levels, and those validated for absence of protein production were selected for functional studies. We found significant decrease in mRNA level and complete absence of protein expression in the selected mutant cells (Figure 1*B* and *E*). An independent esophageal squamous cell carcinoma cell line Eca109 was genetically engineered in an identical manner (Supplementary Figures 1*E* and 2*A*). In the Eca109 cells, we identified in single clone harboring three mutant alleles of PLCE1, indicating that copy number variation of this gene occurred in this cell line (Supplementary Figure 1*E*).

### *PLCE1 Depletion Inhibits Migration Capability in Esophageal Cancer Cells*

As metastasis is an essential determinant for prognosis of cancers, we first investigated the consequence of genetic inactivation of PLCE1 on metastatic phenotype by wound healing assay. The wound gap in a monolayer of both control and mutant cells were created by scratching, and the healing of this gap by cell migration towards the center of the gap was monitored and quantitated at different time points. Our results showed that the wound healing capacity was significantly crippled in PLCE1 inactivated EC9706 cells (Figure 2*A* and *B*). For the migration assay, following an incubation period of 24 hours in the serum free medium, the cells migrating through the permeable filter were stained, and we found that mutant cells had significant decrease in the number of migrating cells (Figure 2*C* and *D*). Such decrease in cell migration was also validated in the independent PLCE1 inactivated Eca109 cells (Supplementary Figure 2, *B* and *C*). Another *in vitro* assay for the study of cell invasion through basement membrane was performed using the Matrigel Invasion Chambers. The PLCE1 deprived cells significantly decreased their invasion ability through the basement membrane, when placed in culture medium without serum for 24 hours (Figure 2, *E* and *F*). Our results indicated that PLCE1 deficient cell showed decreased ability both in cell migration and invasion.

### *PLCE1 Inactivation Inhibits Proliferation of ESCC Cells In Vitro and In Vivo*

We first observed significant declination in the tumor graft weight between the PLCE1 mutant and control groups, and further analyzed the cell proliferation at two time points, in every 24 hours post the cell seeding in culture medium. The results from three independent experiments showed that there was a significant decrease of proliferation in both PLCE1 inactivated EC9706 and Eca109 cells (Figure 2*G*, Supplementary Figure 2*D*). Then we studied the tumor graft growth kinetics in xenograft animal models. Sizes of the tumor grafts from mutant and control cells were analyzed for their growth kinetics for 30 days. An equivalent number of cells were injected subcutaneously and the tumor volume was measured on a weekly basis. Starting from the second week, PLCE1 deficient grafts were significantly smaller than the control ones in volume. Such difference continued and enlarged by the time when we harvested the tumor tissues at the end of the fourth week (Figure 2, *H* and *I*). The weight of tumor xenografts originating from the PLCE1 deficient cells was less than half of those from control cells (Figure 2*J*).

### *PLCE1 Inactivation Decreases Expression of The Genes Involving in Cell Proliferation and Invasion*

Inactivation of PLCE1 caused significant change in cell migration and invasion, and also resulted in retarded proliferation in ESCC cells. We aimed to obtain a more global view on how the PLCE1 deficiency affects esophageal cancer cells, especially how it changes the tumor associated gene expression profiles. We investigated the transcriptomic differences between the mutant and control cells by genome wide RNA sequencing. The three mutant cell samples were collected from three PLCE1 inactivated clones, with three equivalently prepared control samples. 172 genes were significantly down-regulated in the mutant group with Log Fold change ≤−2.0, and such genes were significantly enriched in multiple tumor associated pathways (Figure 3*A*), including top enriched pathways of cell adhesion. In addition, cell proliferation and secretion, wound healing pathways are also affected at the genomic level in PLCE1 inactivated ESCC cells. Further analysis reveals that PLCE1 inactivation significantly affects gene expression in epithelial–mesenchymal transition (EMT) and cell adhesion (Figure 3*B*). We selected representative genes that were deferentially expressed in RNA

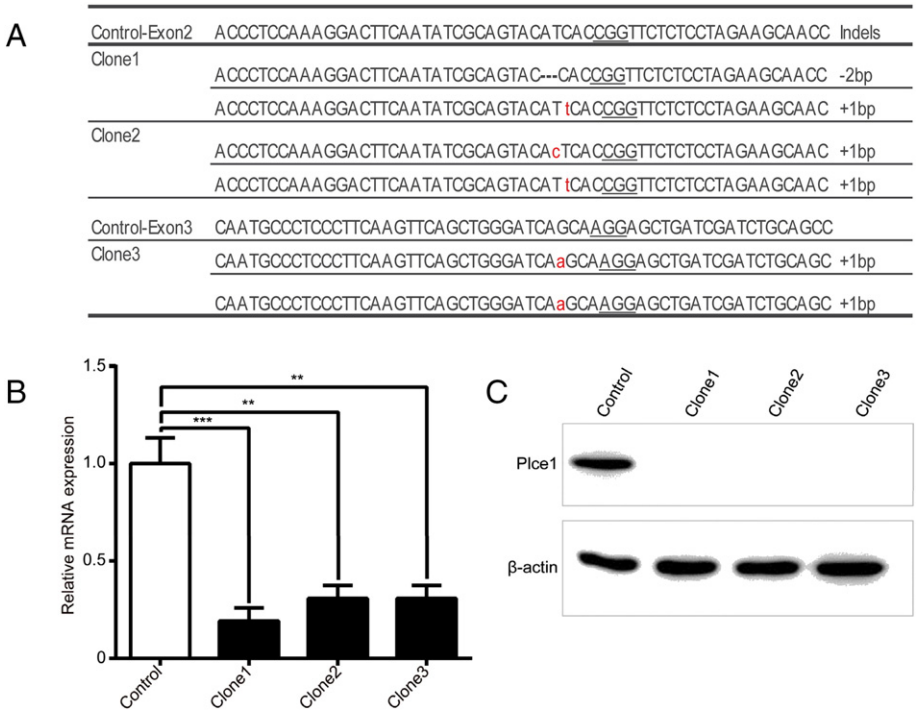


**Figure 1.** Genetic inactivation of PLCE1 in ESCC cells. A: Sequence data of PLCE1 exon after PLCE1 genomic edition. Three clones were selected by length polymorphisms of PCR product and validated by sequencing. Both Clone1 and Clone2 were generated from targeting exon2 that produced Clone1 with 2-bp deletion and 1-bp insertion, and 1-bp insertion for two alleles of Clone2 at different positions. Clone3 derived from an independent targeting guide RNA carried an identical1-bp insertion in two alleles. B: PLCE1 mRNA level of EC9706 control cells and PLCE1 inactivated mutant cells. Data presented were from two experiments with triplicates of cDNA obtained from each clone in quantitative PCR. Statistical significance was determined with a Mann–Whitney *U* test. **, $P < .01$; ***, $P < .001$. C: Representative immuno-blotting result of PLCE1 protein level in EC9706 control cells and mutants.

sequencing data and were enriched in the pathway analysis. Real-time PCR were applied to validate such 13 representative genes with reported implication in carcinogenesis. The cDNA templates were prepared from three independent clones of mutant cells, and un-engineered control cells. The results showed that the representative genes promoting cell proliferation and invasion were significantly decreased consistent with the RNA sequencing data (Figure 3, *C* and *D*). Our experiments indicated that the inactivation of PLCE1 in ESCC caused transcriptomic inhibition of cell proliferation and invasion.

### *PLCE1 Inactivation Decreases Snail Protein and Regulates Its Transcriptional Function in Esophageal Cancer Cells*

Based on the alteration of migratory phenotype observed in the mutant cells and results of transcriptomic analysis, we screened the activity of EMT a critical process for cancer cell invasion driven by Snail. We analyzed EMT markers specifically the protein expressions in both PLCE1 inactivated mutant cells and control cells, by intracellular staining and flow cytometric analysis with EMT antibody sampler kit. Six out of nine markers were significantly decreased in the mutant cells, while E-Cadherin, N-Cadherin and Claudin-1 were not changed in protein expression (Figure 4, *A* and *B*, Supplementary Figure 3, *A* and *B*). The markers showed obvious change in expression level between the mutant and control cells were further analyzed by immunoblotting as a less dramatic variation would not be differentiated due to the sensitivity gap of these two methods. Among these markers Snail and ZO-1 were found significantly decreased in mutant cells, which was shown in both FACS and immunoblotting analyses of three different mutant clones involving both EC9706 and Eca109 cells (Figure 4*C*, Supplementary Figure 2*E*). We further analyzed the distribution of Snail through nuclear-cytoplasmic separation. The experiments showed that cytoplasmic and nuclear distribution of Snail was dramatically decreased in ESCC cells (Figure 4*D*).

Snail is a driving transcription factor medicating cell invasion and possible metastasis.[19] To visualize Snail distribution in the nucleus, confocal cell staining was performed and we found that PLCE1 depletion decreases Snail signal in both cytosol and nuclear (Figure 4*E*). We infer that Snail transcriptional function could be crippled under PLCE1 depletion. By analyzing the RNAseq data together with public available snail ChIP-sequencing data, we observed that 51 Snail target genes are significantly down regulated (Figure 4*F*). All these data indicate that PLCE1 could be a critical regulator for Snail transcriptional function in esophageal cancer cell lines.

### *Snail is Responsible for The Decrease of Invasive and Proliferative Capacity in PLCE1 Inactivated ESCC Cells*

Both Snail and its target genes in transcriptomic experiments were found to be regulated by PLCE1, therefore we sought to confirm whether Snail played a critical role in PLCE1 mediated carcinogenesis by lentiviral expression of Snail in PLCE1 inactivated cells. We transfected the PLCE1 inactivated mutant cells with Snail over-expression vector or empty vector both of which were engineered with GFP expressing and puromycin selection cassettes. In

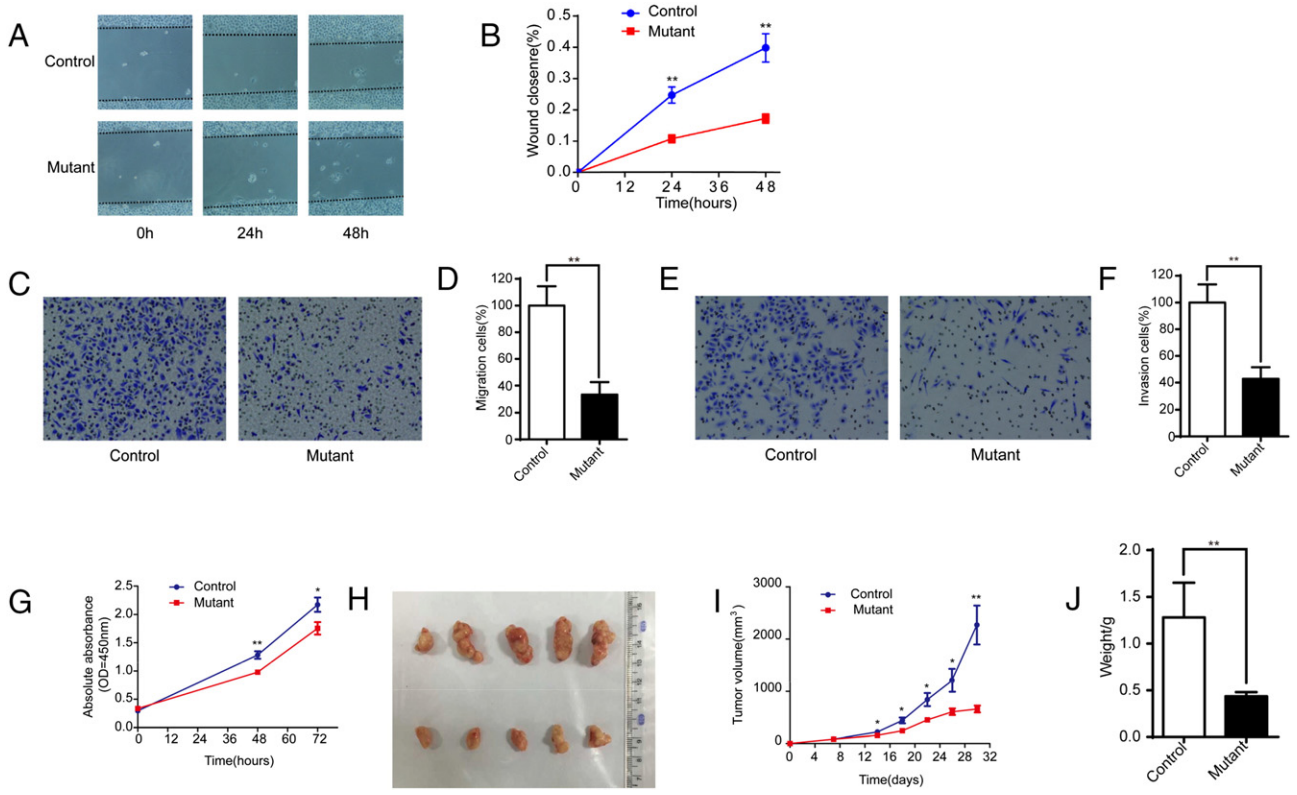


**Figure 2.** PLCE1 inactivation decreases the migration, invasion and proliferation in esophageal cancer cells. A and B: The migration capacity of PLCE1 deficient cells was measured by a wound-healing assay. The experiments were performed with three independent clones in triplicate. The equal amount of cells was seeded into the plates. Representative photographs were taken at 0 h, 24 h, 48 h, 72 h and 96 h post-wound (×40). The quantification of wound healing was shown in Figure 2B. Statistical significance was determined with a Mann–Whitney *U* test. **, $P < .01$. C and D: Analysis of the number of migratory cells using the trans-well assay. C: Representative photographs of stained cells attached to the bottom membrane of a trans-well (×100). D: Data are presented as the mean (±SEM) number of cells migrating to the underside of the trans-well filter. Statistical significance was determined with a Mann–Whitney *U* test. **, $P < .01$. E and F: Analysis of invasion using a matrigel-coated transwell assay. E: Representative photographs of stained cells (×100). F: Quantification of invasive cells, presented as the mean (±SEM) number of cells. The experiments were performed with three independent clones of cells in triplicate, and an equivalent number of cells were put in control and mutant cells. Statistical significance was determined with a Mann–Whitney *U* test. **, $P < .01$. G: The WST-1 assay for cell proliferation difference between the control and mutant cells. The cells were counted and seeded into 96 well plates with 5000 cells in each well. The 450 nm absorbance was measured at indicated time point. Statistical significance was determined with a Mann–Whitney *U* test. *, $P < .05$; **, $P < .01$. H: The xenograft experiment for cell proliferation difference between the control and mutant cells. $4 \times 10^6$ PLCE1 inactivated cells and control cells were transplanted to Balb/c-Nude mice. Tumor growth was compared between the control and mutant group. After 30 days of engraftment the tumor was harvested and compared for tumor weight. Tumor grafts were obtained from 5 mice transplanted with control cells, and 5 mice with mutant cells involving three clones. I: The growth curve of xenograft tumors from the control and mutant cells. Tumor volumes were measured for each mouse. Tumor grafts were obtained from 5 mice transplanted with control cells, and 5 mice with mutant cells involving three clones. Statistical significance was determined with a Mann–Whitney *U* test. *, $P < .05$; **, $P < .01$. J: The weight of the mutant grafts at the time when the tumor was taken from the eutharized animals. Statistical significance was determined with a Mann–Whitney *U* test. **, $P < .01$.

comparison to EC9706 control cells and PLCE1 inactivated mutant cells, the transfected cells were GFP positive as shown in the FACS data (Figure 5*A*). It was notable that intracellular staining showed decrease of Snail in PLCE1 inactivated mutant cells, which was completely rescued by transfection of Snail over-expression lentiviral vector (Figure 5, *A* and *B*). The PLCE1 inactivated mutant cells re-expressing Snail *via* lentiviral vector were analyzed in parallel with the Snail deficient PLCE1 inactivated cells. Strikingly, we found that re-expression of Snail sufficed to rescue the proliferative and invasive capacity of PLCE1 inactivated cells. In the wound healing assay, by 48 h the PLCE1 inactivated cells transfected with Snail over-expression vector reached complete closure which was even faster than the EC9706 control cells indicating critical role of Snail in compensating PLCE1 deficiency (Figure 5, *C* and *D*). We also observed that expression of Snail rescued completely the invasiveness and proliferation of PLCE1 inactivated cells (Figure 5, *E, F* and *G*, Supplementary Figure 4, *A* and *B*). These results suggest that PLCE1 has multiple roles in malignant potency of ESCC cells, and all these roles are tightly linked to Snail.

### *PLCE1 Expression in Human Esophageal Cancer Specimens Significantly Correlates With Snail Activity and Tumor Invasivesness*

As determined in ESCC cells that PLCE1 inactivation regulated invasion and proliferation by restraining Snail, we further explored to

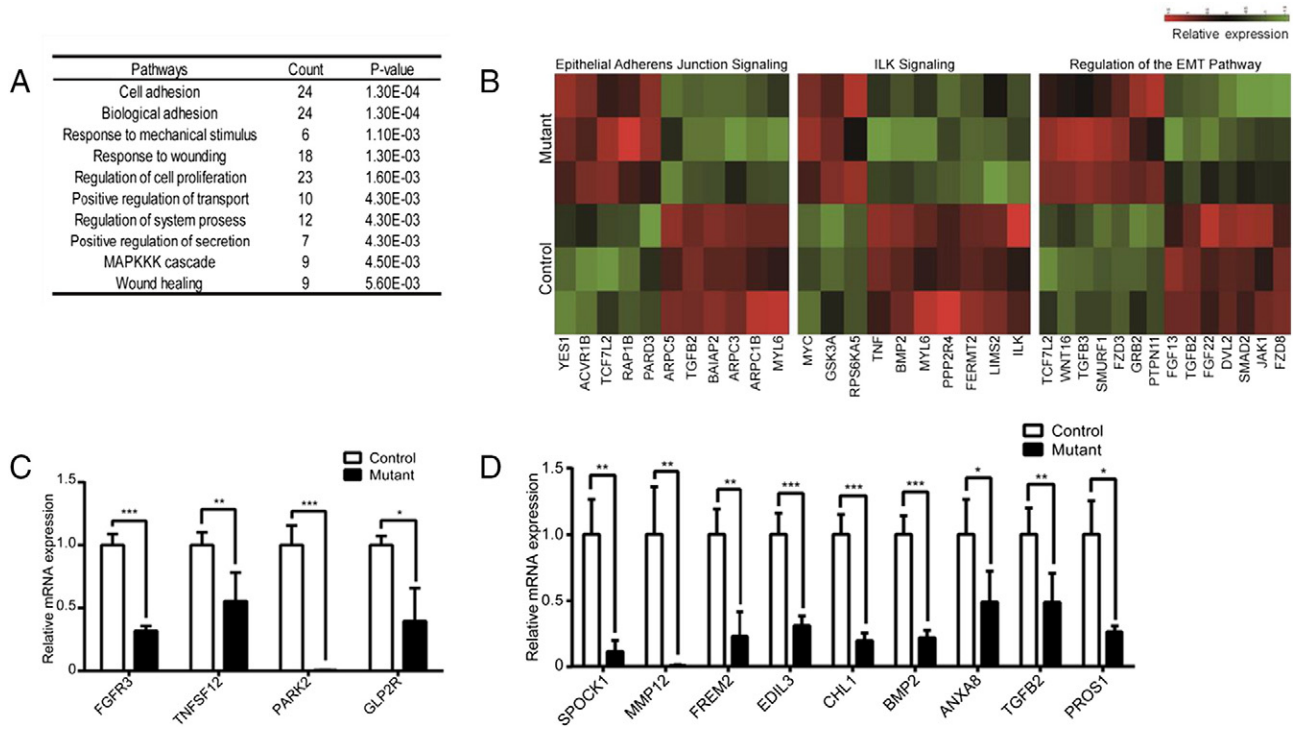


**Figure 3.** Whole genome expression analysis reveals that PLCE1 inactivation impairs invasion and proliferation pathways. A: The top ten pathway affected by PLCE1 depletion in esophageal cancer cells. The control and mutant cells were subject to lysis and RNA extraction with triplicates. The samples underwent RNA-sequence analysis and the differentially expressed genes with *P* value <.05 and fold change >2 were selected for David platform online pathway analysis. B: The heat map result of cell migration related pathway: Epithelial adhesion junction pathway, integrin linked kinase pathway and EMT pathway. C: Real-time PCR results of cell invasion genes, which showed significant decrease in RNA sequence data of PLCE1 deficient cells. Statistical significance was determined with a Mann–Whitney *U* test. *, $P < .05$; **, $P < .01$; ***, $P < .001$. D: Real time PCR results of proliferation related genes, which showed significant decrease in RNA sequence data of PLCE1 deficient cells. Statistical significance was determined with a Mann-Whitney test. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

delineate the clinical relevance of PLCE1 expression on tumor invasion and Snail signaling. First, by analyzing the esophageal tumor samples from TGCA database (The Cancer Genomic Atlas), we observed that 32 Snail target genes were significantly decreased in PLCE1 low expression group (Figure 6*A*). We further analyzed one hundred and one esophageal squamous carcinoma samples for the expression of PLCE1 and Snail by immunohistochemistry (Figure 6*B*). We did the Chi-square test for the correlation between PLCE1 and the clinical characteristics, including invasion depth, lymph node metastasis and pathological grade. PLCE1 was found to correlate with late tumor stage. Interestingly, PLCE1 expression was positively correlated with Snail expression, which reflected our observation in basic study that PLCE1 controlled Snail protein level in ESCC cell lines (Table 1).

## Discussion

Esophageal cancer is a malignant disorder with rapidly increasing incidence worldwide.[20] Esophageal squamous carcinoma has special geographic distributions, and more than half cases are in China, the knowledge of genetic factors contributing to it remains extremely limited.[21] In recent years, a gene named PLCE1 was found to be associated with ESCC in independent cohorts using Chinese patient subjects and normal controls.[9] A more recent meta-analysis based on various independent population genetics studies confirmed association of PLCE1 with esophageal cancer.[6–9] It therefore remains extremely intriguing to understand how exactly this gene functions in contribution to the ESCC carcinogenesis. In our study, we designed experiments to elucidate the role of PLCE1 in ESCC by thorough inactivation of the gene using CRISPR/Cas9 system. CRISPR/Cas9 system is an emerging genetic engineering tool that elucidates function of a given gene in an unambiguous manner especially when independent targets are designed to rule out off-target effect.[22] In this study, we used two independent esophageal squamous carcinoma derived cell lines and targeted 2 loci which led to frame-shift and absence of protein production. It is important to note that knockdown models using RNA silencing generally cannot guarantee constitutive loss of a specific protein, which might generate inconsistent results.[22] Loss of PLCE1 is not lethal both in ESCC cell lines and in the mouse, therefore complete inactivation using CRISPR/Cas9 tool is necessary and desirable for elucidation of its function. The experiments were performed using EC9706 cells, and validation of critical phenotype was also done with the Eca109 cell line.

PLCE1 might be involved in various physiological activities that could be under redundant regulation by the other phospholipases.[23] In humans, mutations in PLCE1 were found to be responsible for a recessive nephrotic syndrome with diffuse mesangial sclerosis or focal segmental glomerulosclerosis.[24–26] However, little is known about the function of PLCE1 in carcinogenesis, even though it is statistically correlated with esophageal cancer and gastric cancer.[10,27] Our

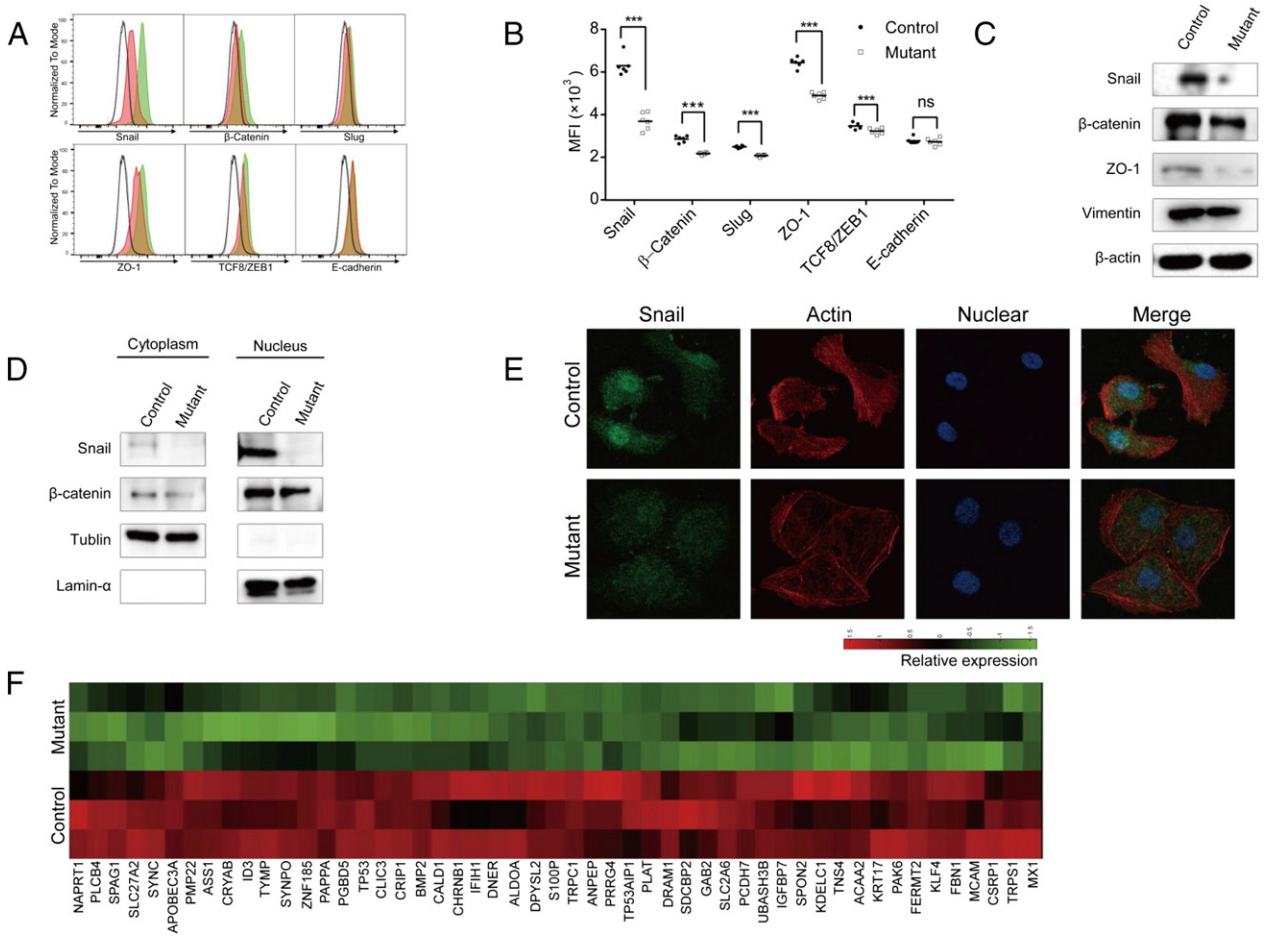


**Figure 4.** PLCE1 inactivation in esophageal cancer cells decreases snail protein level and impairs its transcriptional activity. A: Comparison of EMT marker expression in protein level between control and mutant cells. Log fluorescence intensity for each protein was compared using histogram overlay between stained control (Green) and PLCE1 inactivated cells (Red), with the negative from EC9706 un-engineered cells (solid black trace) and PLCE1 inactivated cells (dashed black trace). Representative data from 2 independent experiments involving 6 individual assays for each protein. B: Mean fluorescence intensity (MFI) for each protein stained in Figure 4A was compared between control and mutant cells. Statistical significance was determined with a Mann–Whitney *U* test. ***, $P < .001$. C: The EMT related proteins were subject to western blotting analysis, including snail, β-catenin, ZO-1 and Vimentin. Snail and ZO-1 were among the most dramatically reduced proteins in PLCE1 deficient cells according to flow cytometry analysis. β-actin was applied as the internal control. D: PLCE1 depletion decreased distribution of Snail in the nucleus. Subcellular Protein Fractionation Kit (Thermo Scientifc, 78,840) was applied for extraction of cytoplasmic and nuclear proteins from esophageal cancer cell. Tublin and Lamin-α is used to identify the cytoplasmic and nuclear fraction. E: Immuno-staining of Snail in the control and PLCE1 deficient cells. Intracellular localization of Snail and Actin were stained in green and red respectively. DAPI was used to stain for the nuclei (blue). F: The heat map result showing that 43 Snail target genes were significantly decreased in PLCE1 inactivated ESCC cell line by RNA sequence data.

findings from the cell migration and invasion experiments showed that inactivation of PLCE1 significantly inhibited cell migration and invasion. The proliferation of PLCE1 inactivated mutant cells was also inhibited. *In vivo* experiments with PLCE1 inactivated xenografts showed dramatically decreased growth rate of tumor cells. Therefore, our results confirmed that PLCE1 could drive invasiveness and tumor growth of ECSS.

The findings in cell migration and invasiveness led us to analyze the EMT process driven by an essential transcription factor Snail which induces cell migration and has been extensively studied and well documented for its role in cancer progression.[28–32] Strikingly, Snail was not only decreased in total protein in the PLCE1 inactivated ESCC cells, but it was almost undetectable in the nucleus as shown in the immunoblotting and imaging experiments. We therefore postulate such inhibition of EMT and its driving transcription factor could explain the phenotypic alteration in migration and invasion assays in which the mutant cells were strongly affected *in vitro*. In our study, it was difficult to make assessment *in vivo* whether PLCE1 inactivation could impair the metastasis of tumor grafts, as subcutaneous tumor graft of both mutant and control cells did not embark on metastasis in the mouse model. Nevertheless, in three independent assays including the trans-well migration and invasion assay, and the wound-healing assay, we observed significantly impaired migration and invasion capacity of the mutant cells. These finding indicates that PLCE1 could be a promising therapeutic target to block cancer metastasis.

By unbiased genome wide RNA sequencing, we observed PLCE1 depletion significantly affect several cell behaviors including

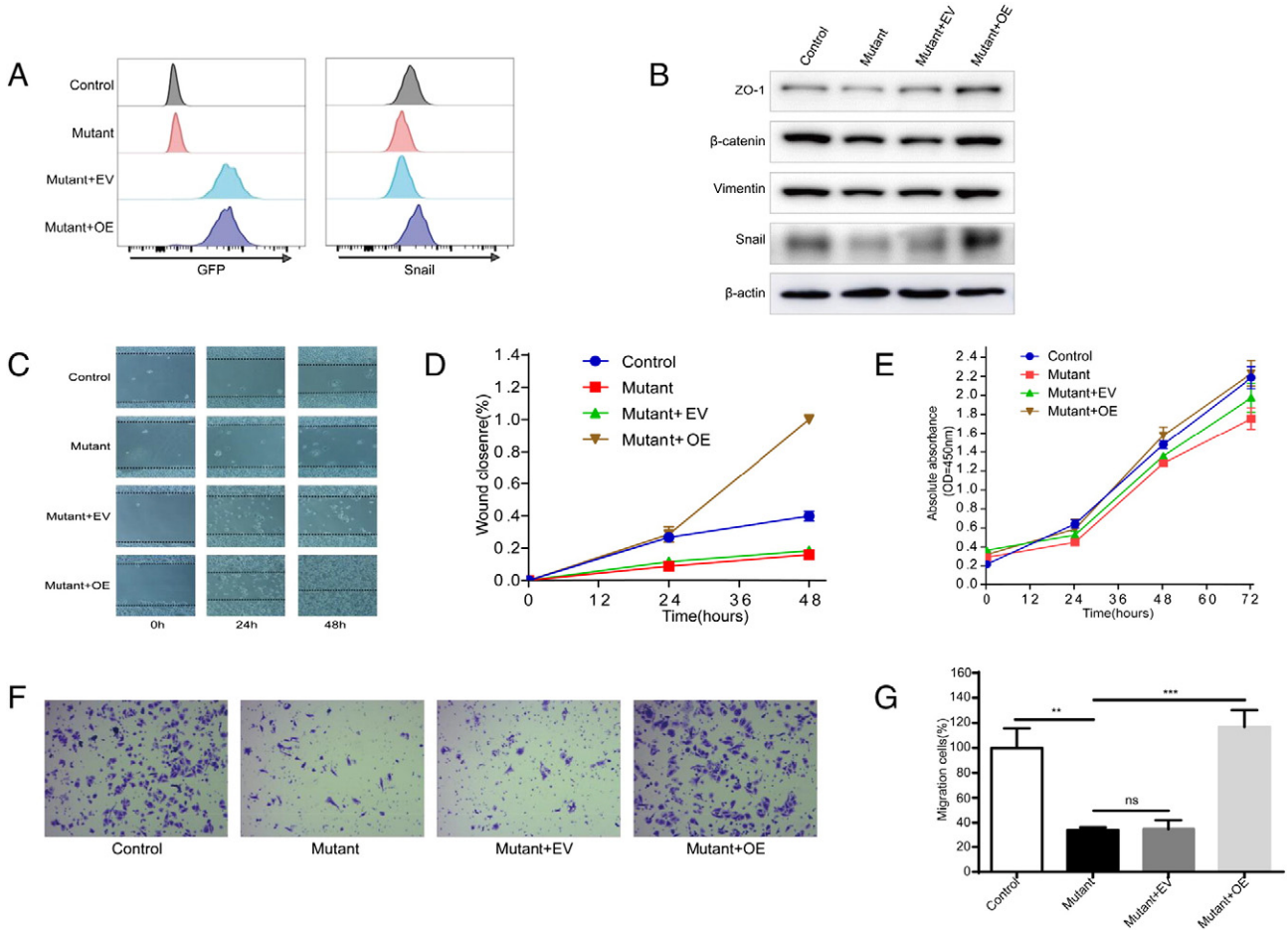


**Figure 5.** Snail overexpression rescues the inhibition of cell migration and proliferation caused by PLCE1 depletion in ESCC cells. A: FACS validation of Snail overexpression in ESCC cells. The left panel showing GFP expression in EC9706 control cells (Control), PLCE1 inactivated mutant cells (Mutant), PLCE1 inactivated mutant cells transfected with empty vector (Mutant + EV), and PLCE1 inactivated mutant cells transfected with Snail overexpression vector (Mutant + OE). The transfected cells were GFP positive. The right panel showed Snail expression in the same samples as the left panel. PLCE1 inactivated mutant cells transfected with Snail overexpression vector (Mutant + OE) recovered Snail expression. B: Western blot showed that Snail overexpression could recover the Snail protein which was decreased by PLCE1 inactivation, and cells transfected empty vector were used as control (Mutant + EV versus Mutant + OE). C and D: Snail overexpression in PLCE1 inactivated cells could rescue the wound healing inhibition caused by PLCE1 knockout (Mutant + EV versus Mutant + OE). The quantification of wound-healing was shown in Figure 5D. E: The WST-1 assay showing that Snail overexpression in PLCE1 depletion cells could rescue the proliferation inhibition caused by PLCE1 knockout (Mutant + EV versus Mutant + OE). F and G: The trans-well assay showing that Snail overexpression in PLCE1 depletion cells could rescue the migration inhibition caused by PLCE1 knockout (Mutant + EV versus Mutant + OE). F: Representative photographs of stained cells attached to the bottom membrane of a trans-well (×100). G: Data are presented as the mean (±SEM) number of cells migrating to the underside of the trans-well filter. Statistical significance was determined with a Mann–Whitney *U* test. **, $P < .01$; ***, $P < .001$.

migration and cell cycle progression. By the signaling pathway enrichment analysis, quite a few migration-related pathways regulated by PLCE1 were found, besides EMT mediated by Snail and Slug. For example, Rho GTP kinases signaling were also significantly changed, which were proved to be necessary for cell mobility by exerting its kinase activity and interacting with myosin/integrin the essential molecules for cell migration.[33,34] Thus, we infer that Snail signaling alteration in PLCE1 inactivated cells is critical for the phenotype change, but there could be other pathways involved. The possible regulation of PLCE1 on Rho GTP kinase is still valuable to be elucidated.

In this study, we focused on dissecting the cancer inhibiting mechanism of PLCE1 inactivation in ESCC cells. As evinced by protein expression experiments and the transcriptomic data, PLCE1 regulates both Snail and its target genes in ESCC cells, and correlation between expression of PLCE1 and Snail target genes were also observed in esophageal cancer specimens. We further overexpressed Snail in PLCE1 inactivated ESCC cells, expression of which was comparable to EC9706 control cells. Surprisingly, recovered Snail protein was sufficient to completely rescue the decreased capacity of ESCC cell invasion and proliferation. Repressed Snail expression using gene silence approach was reported to induce mesenchymal to epithelial transition, inhibition of invasion and xenograft growth, and increase of E-cadherin expression.[35–37] In addition E-cadherin was found hyper-methylated in esophageal adenocarcinoma specimens.[38] In our study, PLCE1 inactivation significantly reduced Snail expression, which was accompanied by down-regulation of mesenchymal markers, however we did not find change of E-cadherin.

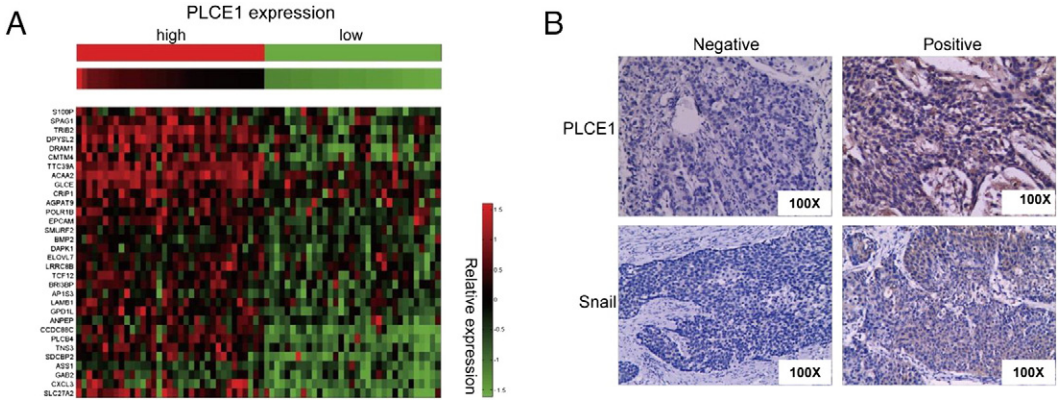


**Figure 6.** PLCE1 expression correlates with invasion depth and Snail protein level in ESCC clinical samples. A: The heat map result showing that 32 Snail target genes were significantly decreased in the PLCE1 lower esophageal tumor specimens (35 PLCE1 high specimens versus 33 PLCE1 low specimens). B: Examples of positive/negative PLCE1 and Snail staining in ESCC tumor samples were shown by 100× magnification.

There are a few studies showing that the hyper-methylation is observed in esophageal tumor samples and esophageal cancer cell lines.[38] Over-expression of Snail in PLCE1 inactivated cells also did not change E-cadherin level (data not shown). Our results suggest that Snail is responsible for PLCE1 mediated invasiveness but does not require modulation of E-cadherin in ESCC cells.

In patient specimens, PLCE1 expression was found in the invasive carcinoma but not in the carcinoma *in situ* samples. Snail expression in the human ESCC samples significantly correlated with the PLCE1 protein level, and expression of Snail target genes in human esophageal cancer samples were also correlated with the PLCE1 abundance. As our study validated PLCE1 as an oncogene in esophageal cancer cells and proved the essential role of Snail as a downstream molecule, together with the data from clinical samples, we conclude that PLCE1 could constitute a valuable diagnostic tool and drug target for esophageal cancers. It still remains intriguing to decipher whether PLCE1 functions as a single member of the phospholipase family of formidable complexity in ESCC or it exerts a critical role in the carcinogenesis redundantly.

## Conflict of Interest

The authors declare no conflict of interest.

**Table 1.** The Correlation Between PLCE1 and the Clinical Characteristics in Esophageal Squamous Carcinoma Samples

| Clinical and molecular characteristics (Cases, %) | | PLCE1 expression | | |
|---|---|---|---|---|
| | | Positive | Negative | *P* value |
| Tumor stage | T1 | 6 (8%) | 15 (56%) | <.0001 |
| | T2 | 13 (21%) | 5 (16%) | |
| | T3 | 45 (71%) | 7 (26%) | |
| Pathological grade | Low | 7 (12%) | 3 (9%) | 0.94 |
| | Medium | 21 (34%) | 11 (34%) | |
| | High | 33 (54%) | 18 (56%) | |
| Lymph node metastasis | Positive | 14 (20%) | 7 (22%) | 1.00 |
| | Negative | 55 (80%) | 25 (78%) | |
| Invasive depth | Sub-mucosa | 16 (50%) | 10 (15%) | <0.0001 |
| | Muscularis propria | 6 (19%) | 13 (19%) | |
| | Adventitia | 10 (31%) | 45 (66%) | |
| | Nearby stracture | 0 (0%) | 0 (0%) | |
| Snail expression | Positive | 44 (88%) | 23 (49%) | <0.0001 |
| | Negative | 6 (12%) | 24 (51%) | |

## Acknowledgements

This work was supported by the National High Technology Research and Development Program of China (2012AA02A201-1, http://program.most.gov.cn) and the Science and Technology Development Plan of Henan Province (112102310283;122102310198, http://www.hnkjt.gov.cn).

## Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.12.007.

## References

[1] Zhang Y (2013). Epidemiology of esophageal cancer. *World J Gastroenterol* **19**, 5598–5606.

[2] Castro C, Bosetti C, Malvezzi M, Bertuccio P, Levi F, Negri E, La Vecchia C, and Lunet N (2014). Patterns and trends in esophageal cancer mortality and incidence in Europe (1980-2011) and predictions to 2015. *Ann Oncol* **25**, 283–290.

[3] Lin Y, Totsuka Y, He Y, Kikuchi S, Qiao Y, Ueda J, Wei W, Inoue M, and Tanaka H (2013). Epidemiology of esophageal cancer in Japan and China. *J Epidemiol* **23**, 233–242.

[4] Pennathur A, Gibson MK, Jobe BA, and Luketich JD (2013). Oesophageal carcinoma. *Lancet* **381**, 400–412.

[5] Spechler SJ (2013). Barrett esophagus and risk of esophageal cancer: a clinical review. *JAMA* **310**, 627–636.

[6] Chen YZ, Cui XB, Hu JM, Zhang WJ, Li SG, Yang L, Shen XH, Liu CX, Pan QF, and Yu SY, et al (2013). Overexpression of PLCE1 in Kazakh esophageal squamous cell carcinoma: implications in cancer metastasis and aggressiveness. *APMIS* **121**, 908–918.

[7] Dura P, Bregitha CV, te Morsche RH, Roelofs HM, Kristinsson JO, Wobbes T, Witteman BJ, Tan AC, Drenth JP, and Peters WH (2013). GWAS-uncovered SNPs in PLCE1 and RFT2 genes are not implicated in Dutch esophageal adenocarcinoma and squamous cell carcinoma etiology. *Eur J Cancer Prev* **22**, 417–419.

[8] Ma H, Wang LE, Liu Z, Sturgis EM, and Wei Q (2011). Association between novel PLCE1 variants identified in published esophageal cancer genome-wide association studies and risk of squamous cell carcinoma of the head and neck. *BMC Cancer* **11**, 258.

[9] Wang LD, Zhou FY, Li XM, Sun LD, Song X, Jin Y, Li JM, Kong GQ, Qi H, and Cui J, et al (2010). Genome-wide association study of esophageal squamous cell carcinoma in Chinese subjects identifies susceptibility loci at PLCE1 and C20orf54. *Nat Genet* **42**, 759–763.

[10] Wang M, Zhang R, He J, Qiu L, Li J, Wang Y, Sun M, Yang Y, Wang J, and Yang J, et al (2012). Potentially functional variants of PLCE1 identified by

GWASs contribute to gastric adenocarcinoma susceptibility in an eastern Chinese population. *PLoS One* **7**, e31932.

[11] Henry J, Guillotte A, Luberto C, and Del Poeta M (2011). Characterization of inositol phospho-sphingolipid-phospholipase C 1 (Isc1) in *Cryptococcus neoformans* reveals unique biochemical features. *FEBS Lett* **585**, 635–640.

[12] Lopez I, Mak EC, Ding J, Hamm HE, and Lomasney JW (2001). A novel bifunctional phospholipase c that is regulated by Galpha 12 and stimulates the Ras/mitogen-activated protein kinase pathway. *J Biol Chem* **276**, 2758–2765.

[13] Lee HJ, Kim E, and Kim JS (2010). Targeted chromosomal deletions in human cells using zinc finger nucleases. *Genome Res* **20**, 81–89.

[14] Di J, Huang H, Qu D, Tang J, Cao W, Lu Z, Cheng Q, Yang J, Bai J, and Zhang Y, et al (2015). Rap2B promotes proliferation, migration, and invasion of human breast cancer through calcium-related ERK1/2 signaling pathway. *Sci Rep* **5**, 12363.

[15] Zeitlinger J, Zinzen RP, Stark A, Kellis M, Zhang H, Young RA, and Levine M (2007). Whole-genome ChIP-chip analysis of Dorsal, Twist, and Snail suggests integration of diverse patterning processes in the Drosophila embryo. *Genes Dev* **21**, 385–390.

[16] Ye X, Tam WL, Shibue T, Kaygusuz Y, Reinhardt F, Ng Eaton E, and Weinberg RA (2015). Distinct EMT programs control normal mammary stem cells and tumour-initiating cells. *Nature* **525**, 256–260.

[17] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, and Marraffini LA, et al (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823.

[18] Foley JE, Maeder ML, Pearlberg J, Joung JK, Peterson RT, and Yeh JR (2009). Targeted mutagenesis in zebrafish using customized zinc-finger nucleases. *Nat Protoc* **4**, 1855–1867.

[19] Yang J and Weinberg RA (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* **14**, 818–829.

[20] Napier KJ, Scheerer M, and Misra S (2014). Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. *World J Gastrointest Oncol* **6**, 112–120.

[21] Zhang HZ, Jin GF, and Shen HB (2012). Epidemiologic differences in esophageal cancer between Asian and Western populations. *Chin J Cancer* **31**, 281–286.

[22] Jackson AL and Linsley PS (2004). Noise amidst the silence: off-target effects of siRNAs? *Trends Genet* **20**, 521–524.

[23] Kalwa H, Storch U, Demleitner J, Fiedler S, Mayer T, Kannler M, Fahlbusch M, Barth H, Smrcka A, and Hildebrandt F, et al (2015). Phospholipase C epsilon (PLCepsilon) induced TRPC6 activation: a common but redundant mechanism in primary podocytes. *J Cell Physiol* **230**, 1389–1399.

[24] Boyer O, Benoit G, Gribouval O, Nevo F, Pawtowski A, Bilge I, Bircan Z, Deschenes G, Guay-Woodford LM, and Hall M, et al (2010). Mutational analysis of the PLCE1 gene in steroid resistant nephrotic syndrome. *J Med Genet* **47**, 445–452.

[25] Hinkes B, Wiggins RC, Gbadegesin R, Vlangos CN, Seelow D, Nurnberg G, Garg P, Verma R, Chaib H, and Hoskins BE, et al (2006). Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet* **38**, 1397–1405.

[26] Jefferson JA and Shankland SJ (2007). Familial nephrotic syndrome: PLCE1 enters the fray. *Nephrol Dial Transplant* **22**, 1849–1852.

[27] Cui XB, Chen YZ, Pang XL, Liu W, Hu JM, Li SG, Yang L, Zhang WJ, Liu CX, and Cao YW, et al (2013). Multiple polymorphisms within the PLCE1 are associated with esophageal cancer via promoting the gene expression in a Chinese Kazakh population. *Gene* **530**, 315–322.

[28] Hsu DS, Lan HY, Huang CH, Tai SK, Chang SY, Tsai TL, Chang CC, Tzeng CH, Wu KJ, and Kao JY, et al (2010). Regulation of excision repair cross-complementation group 1 by Snail contributes to cisplatin resistance in head and neck cancer. *Clin Cancer Res* **16**, 4561–4571.

[29] Bruyere F, Namdarian B, Corcoran NM, Pedersen J, Ockrim J, Voelzke BB, Mete U, Costello AJ, and Hovens CM (2010). Snail expression is an independent predictor of tumor recurrence in superficial bladder cancers. *Urol Oncol* **28**, 591–596.

[30] Hung JJ, Yang MH, Hsu HS, Hsu WH, Liu JS, and Wu KJ (2009). Prognostic significance of hypoxia-inducible factor-1alpha, TWIST1 and Snail expression in resectable non-small cell lung cancer. *Thorax* **64**, 1082–1089.

[31] Peinado H, Olmeda D, and Cano A (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* **7**, 415–428.

[32] Waldmann J, Feldmann G, Slater EP, Langer P, Buchholz M, Ramaswamy A, Saeger W, Rothmund M, and Fendrich V (2008). Expression of the zinc-finger transcription factor Snail in adrenocortical carcinoma is associated with decreased survival. *Br J Cancer* **99**, 1900–1907.

[33] Li J, Wang J, Jiao H, Liao J, and Xu X (2010). Cytokinesis and cancer: Polo loves ROCK'n' Rho(A). *J Genet Genomics* **37**, 159–172.

[34] Mardilovich K, Olson MF, and Baugh M (2012). Targeting Rho GTPase signaling for cancer therapy. *Future Oncol* **8**, 165–177.

[35] Galvan JA, Zlobec I, Wartenberg M, Lugli A, Gloor B, Perren A, and Karamitopoulou E (2015). Expression of E-cadherin repressors SNAIL, ZEB1 and ZEB2 by tumour and stromal cells influences tumour-budding phenotype and suggests heterogeneity of stromal cells in pancreatic cancer. *Br J Cancer* **112**, 1944–1950.

[36] Montserrat N, Gallardo A, Escuin D, Catasus L, Prat J, Gutierrez-Avigno FJ, Peiro G, Barnadas A, and Lerma E (2011). Repression of E-cadherin by SNAIL, ZEB1, and TWIST in invasive ductal carcinomas of the breast: a cooperative effort? *Hum Pathol* **42**, 103–110.

[37] Natsugoe S, Uchikado Y, Okumura H, Matsumoto M, Setoyama T, Tamotsu K, Kita Y, Sakamoto A, Owaki T, and Ishigami S, et al (2007). Snail plays a key role in E-cadherin-preserved esophageal squamous cell carcinoma. *Oncol Rep* **17**, 517–523.

[38] Corn PG, Heath EI, Heitmiller R, Fogt F, Forastiere AA, Herman JG, and Wu TT (2001). Frequent hypermethylation of the 5' CpG island of E-cadherin in esophageal adenocarcinoma. *Clin Cancer Res* **7**, 2765–2769.