

Origin recognition complex 1 regulates phospholipase C δ 1 to inhibit cell proliferation, migration and epithelial-mesenchymal transition in lung adenocarcinoma

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Abstract. As a common pulmonary malignant disease, lung adenocarcinoma exhibits high mortality and morbidity rate. Phospholipase C δ 1 (PLCD1), an enzyme involved in the homeostasis of energy metabolism, is downregulated in lung adenocarcinoma. According to GEPIA, origin recognition complex 1 (ORC1) is a highly expressed gene in lung adenocarcinoma and is negatively associated with PLCD1. To the best of our knowledge, the present study was the first to investigate the role of ORC1 in regulating PLCD1 in lung adenocarcinoma. According to TCGA database, low expression of PLCD1 was correlated with the low overall survival rate of patients suffering from lung adenocarcinoma. The protein and mRNA expression levels of PLCD1 and ORC1 were detected in A549 cells by western blot analysis and reverse transcription-quantitative PCR, respectively. Cell proliferation, invasion and migration were analyzed by MTT, colony formation, Transwell and wound healing assay. Immunofluorescence staining was adopted to estimate the content of Ki67 and western blot was applied for the evaluation of PLCD1, MMP2, MMP9, E-cadherin, N-cadherin, vimentin, Snail and ORC. The binding interaction between ORC1 and PLCD1 was analyzed using chromatin immunoprecipitation and luciferase reporter enzyme gene assays. The results indicated that PLCD1 was lowly expressed in lung adenocarcinoma cells in comparison with that in 16HBE. When PLCD1 was overexpressed in cancer cells, cell proliferation, invasion and migration were significantly inhibited. However, in the presence of both ORC1 and

PLCD1 overexpression, the suppressive effects of PLCD1 overexpression alone on cell proliferation, invasion, migration and EMT were attenuated. In conclusion, ORC1 was indicated to inhibit PLCD1, thus regulating the proliferation, migration and EMT processes of lung adenocarcinoma cells, which suggested that ORC1 might be a target for the treatment of lung adenocarcinoma.

Introduction

Lung cancer is a common malignancy worldwide and a major cause of cancer-associated mortality (1,2). It was estimated that lung cancer contributed to 2.1 million new diagnoses and 1.8 million deaths worldwide (3). According to different histological subtypes, lung cancer is classified into lung adenocarcinoma, lung squamous carcinoma, large cell lung cancer and small cell lung cancer (4). Of these, lung adenocarcinoma accounts for >40% of lung cancer cases and is the most prevalent histological subtype (5,6). Thus, mechanistic studies are expected to be useful for the prevention and treatment of lung adenocarcinoma.

Phospholipase C (PLC) is a critical enzyme in the phosphatidylinositol metabolic system and is involved in various physiological processes, such as cytoskeletal transformation, tissue differentiation and tumorigenesis (7,8). PLC δ 1 (PLCD1), which is a member of the PLC δ subgroup, is considered to be the basic isoform of the PLC family (9). PLCD1 is involved in energy metabolism, calcium homeostasis and intracellular motility (10,11). PLCD1 is frequently absent in a variety of cancer types, including lung cancer (12-14). However, to date, limited studies on PLCD1 in lung adenocarcinoma have been reported. Origin recognition complex 1 (ORC1) is an origin recognition complex gene that is regulated during the cell division cycle and has an essential role in the initiation of DNA replication (15). The ORC1 gene has been indicated to be weakly expressed in quiescent cells, but it was able to be upregulated by cell growth signals (16).

The present study was designed to discuss the role of ORC1 in lung adenocarcinoma as well as its relationship with PLCD1, aiming to find possible targeted-therapy for the prevention and treatment of lung adenocarcinoma.

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Materials and methods

Cell culture and transfection. The human bronchial epithelial cell line 16HBE and the human lung cancer cell lines A549, PC9, H1975 and H3255 were purchased from BeNa Culture Collection. Cells were cultured in RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd.) containing 10% FBS and 1% penicillin/streptomycin (both purchased from Beijing Solarbio Science & Technology Co., Ltd.), and were maintained at 37°C in the presence of 5% CO₂. PLCD1 overexpression plasmid (oe-PLCD1), ORC1 overexpression plasmid (pcDNA3.1-ORC1) and the corresponding pcDNA3.1 empty vectors used as negative controls (NCs; oe-NC and pcDNA3.1-NC) were obtained from Shanghai GenePharma Co., Ltd. The sequences of the small hairpin (sh)RNAs used in the present study were as follows: ORC1 (sh-ORC1-1; target sequence, 5'-AGCCTGGTGCACAGGAAATAT-3'; forward, 5'-CCGGAGCCTGGTGCACAGGAAATATCTCGAGATATTTCTGTGCACCAGGCTTTTTTG-3' reverse, 5'-AAT TCAAAAAGCCTGGTGCACAGGAAATATCTCGA GATATTTCTGTGCACCAGGCT-3'; sh-ORC1-2; target sequence, 5'-CTATTTGCGGAGTTGAATAAA-3'; forward, 5'-CCGGCTATTTGCGGAGTTGAATAAACTCGAGTT TATTCAACTCCGCAAATAGTTTTTG-3' and reverse, 5'-AAT TCAAAAAGCCTGGTGCACAGGAAATATCTCGA GATATTTCTGTGCACCAGGCT-3'; and NC (sh-NC; forward, 5'-CACCGCAATTTTTTTTTTTGATTACGAAT GAATCAAAAAAAAAAATGC-3' and reverse, 5'-AAA AGCAATTTTTTTTTTTGATTCATTCGTGAATCAAAA AAAAAAAAAATGC-3'). The above shRNAs were obtained from Shanghai GenePharma, Co., Ltd. A total of 50 nM shRNAs against ORC1 (sh-ORC1) were transfected into A549 cells at 37°C for 24 h with Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) transfection reagent according to the manufacturer's instructions. After 24 h, the cells were adopted for follow up experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from A549 cells with TRIzol® reagent (Thermo Fisher Scientific, Inc.). cDNA was synthesized using a PrimeScript™ RT Reagent kit (Takara Bio, Inc.). The iTaq Universal SYBR Green kit (Bio-Rad Laboratories, Inc.) was then utilized to perform RT-qPCR according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation at 95°C for 8 min; denaturation at 95°C for 25 sec, annealing at 60°C for 30 sec; extension at 72°C for 30 sec; and final extension at 72°C for 10 min. GAPDH was used as the internal reference gene. Relative expression levels were measured using the 2^{-ΔΔC_q} method (17). The following primer pairs were used: PLCD1 forward, 5'-ACCAGCGCAATACATAGACC-3' and reverse, 5'-GCCTGAGTGGTGGATGATCTT-3'; ORC1 forward, 5'-ACTACCCACAAGGCTGAAGA-3' and reverse, 5'-AGTGCAGTTTTTCGATCCAACA-3'; and GAPDH forward, 5'-ACAACCTTGGTATCGTGGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'.

Western blot analysis. Total protein was collected from A549 cells with RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was determined using the BCA method. Proteins (30 μg) were separated via

10% SDS-PAGE and transferred to PVDF membranes, which were then blocked with 5% skimmed milk for 1 h at room temperature and next incubated with primary antibodies against PLCD1 (cat. no. ab134936; 1:1,000 dilution; Abcam), MMP2 (cat. no. ab92536; 1:1,000 dilution; Abcam), MMP9 (cat. no. ab76003; 1:1,000 dilution; Abcam), E-cadherin (cat. no. ab40772; 1:10,000 dilution; Abcam), N-cadherin (cat. no. ab76011; 1:5,000 dilution; Abcam), vimentin (cat. no. ab92547; 1:1,000 dilution; Abcam), Snail (cat. no. ab216347; 1:1,000 dilution; Abcam), ORC1 (cat. no. ab85830; 1:2,000 dilution; Abcam) or GAPDH (cat. no. ab9485; 1:2,500 dilution; Abcam) overnight at 4°C. The samples were then incubated with a horseradish peroxidase-conjugated antibody (cat. no. ab109489; 1:1,000 dilution; Abcam) for 2 h at room temperature. Finally, protein signals were visualized with enhanced chemiluminescence reagent (cat. no. P0018S; Beyotime) and semi-quantitatively analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Cell proliferation assay. Cell proliferation was analyzed using the MTT method. Transfected A549 cells were inoculated in 96-well culture plates (5x10³ cells/well). Upon cell deposition, the cells were incubated for 24, 48 and 72 h. Subsequently, 10 mg/ml MTT (Beyotime Institute of Biotechnology) was added to the cell culture plates. After incubation at 37°C for 4 h, DMSO was used to dissolve the formazan and the absorbance was measured at 570 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Inc.).

Colony formation assay. A549 cells were inoculated in 6-well plates (1x10³ cells/well) and incubated at 37°C for 12 days until colonies had formed, which were observed under a light microscope (magnification, x100; Olympus Corporation). Subsequently, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and stained with 0.5% crystal violet at room temperature for 10 min. Cell colonies (≥50 cells) were visualized under a light microscope (Olympus Corporation).

Immunofluorescence analysis. Transfected cells were inoculated into 24-well plates (5x10⁴ cells/well). Once the cells were 70-80% confluent, they were fixed with 3.7% paraformaldehyde for 10 min at room temperature, followed by permeabilization with 0.05% Triton X-100 for 15 min at room temperature. Cells were incubated sequentially with 5% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature, a primary antibody against Ki67 (cat. no. ab243878; 1:100 dilution; Abcam) overnight at 4°C and a DyLight® 488-conjugated secondary antibody (cat. no. ab98571; 1:100 dilution; Abcam) for 2 h at 37°C. Finally, nuclei were stained with DAPI for 10 min at room temperature and cells were imaged under a fluorescence microscope (magnification, x200; Olympus Corporation).

Cell invasion assay. The cell invasion capacity was examined using a Transwell assay. Transwell chambers (8-μm pore size) were pre-treated with Matrigel® (Corning, Inc.) at 37°C for 30 min. Transfected cells (1x10⁴ cells/well) were inoculated into serum-free DMEM (Shanghai Yimiao Chemical Technology Co., Ltd.) which was placed in the upper Transwell

chamber. Complete RPMI-1640 medium containing 5% FBS was added to the bottom chamber and cells were incubated at 37°C for 12 h prior to being fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then stained with 0.1% crystal violet at room temperature for 15 min. Next, cells were counted under a light microscope (Olympus Corporation).

Cell migration assay. A wound-healing assay was used to determine the migratory capacity of cells. Transfected cells were inoculated in 6-well culture plates (2×10^5 cells/well). Once cell confluency reached 80-90%, the cells were scratched with a 200- μ l sterile pipette tip. To remove cell debris, A549 cells were washed with PBS three times. After continued culture for 0 or 24 h in the presence of serum-free medium, images of the wound areas were acquired with a light microscope (Thermo Fisher Scientific, Inc.) to measure the migration distance.

Database analysis. The GEPIA database (Ver.2, <http://gepia2.cancer-pku.cn>) is a newly developed interactive web tool used for the analysis of RNA sequencing expression data of tumors and normal samples from The Cancer Genome Atlas (TCGA) database and the Genotype-Tissue Expression project using a standard processing pipeline. The expression levels of ORC1 and PLCD1 in lung adenocarcinoma tissues were determined and an association analysis was performed using the GEPIA database. Survival rates were analyzed using the TCGA database (tcga.org/). The Human Transcription Factor DataBase (TFDB, Ver.3.0) website (http://bioinfo.life.hust.edu.cn/HumanTFDB#!/tfbs_predict) was employed to predict the binding site between ORC1 and the PLCD1 promoter according to default parameters.

Luciferase reporter gene assay. The interaction of ORC1 with the PLCD1 promoter in cells was confirmed with a luciferase reporter gene assay. PLCD1 wild-type (WT) and mutant (MUT) sequences obtained from Shanghai GenePharma Co., Ltd. were cloned into the pGL3 luciferase vector (Promega Corporation) and were named PLCD1-WT and PLCD1-MUT, respectively. A total of 0.1 μ g PLCD1-WT and PLCD1-MUT were transfected into pcDNA3.1-negative control (NC) or pcDNA3.1-ORC1-transfected cells, respectively, using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Following transfection at 37°C for 48 h, the relative luciferase signals were analyzed with a Luciferase Reporter Gene Assay Kit (cat. no. 11401ES60; Yeasen) according to the manufacturer's protocol and the firefly luciferase activity was normalized to that of *Renilla* luciferase.

Chromatin immunoprecipitation (ChIP). The ChIP Assay Kit (cat. no. P2078; Beyotime) was used according to the manufacturer's protocol. In brief, cells were incubated with 1% formaldehyde at 37°C for 10 min to crosslink DNA with proteins, followed by the addition of glycine to interrupt the crosslinking. The samples were centrifuged at 10,000 \times g for 10 min at 4°C to separate the insoluble material. Next, samples were incubated overnight at 4°C with an anti-ORC1 antibody (cat. no. ab85830; 2 μ g/mg; Abcam) or with IgG (cat. no. ab6715; 2 μ g/mg; Abcam). The precipitated immune complexes were washed with elution buffer. The complexes

were incubated at 65°C overnight to reverse the crosslinks and then treated with proteinase K (part of the kit) at 45°C for 1.5 h. The purified DNA (Shanghai Xiangshu Industrial Co., Ltd.) was subjected to PCR amplification as aforementioned and its product was run on a 1% DNA agarose gel and visualized with ethidium bromide. Analysis was performed semi-quantitatively using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Statistical analysis. GraphPad Prism software (version 8.0; GraphPad Software, Inc.) was used for all statistical analyses. Unpaired Student's t-test was used to compare 2 groups, while one-way ANOVA followed by Tukey's post-hoc test was used to compare >2 groups. The two-stage procedure and the Neyman's smooth test were used for survival analysis (18). Correlation analysis was performed by Pearson's test. Values are expressed as the mean \pm standard deviation. Each experiment was conducted ≥ 3 times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Low expression of PLCD1 in lung adenocarcinoma tissues and cells. The expression of PLCD1 in tissues, as analyzed by the GEPIA database, was low in lung adenocarcinoma compared with that in normal tissues and was associated with low overall survival in patients with lung adenocarcinoma (Fig. 1A and C). PLCD1 expression was not associated with disease stage (Fig. 1B). The expression of PLCD1 in bronchial epithelial and lung cancer cells was detected by RT-qPCR and western blot analysis (Fig. 1D and E). The results indicated that PLCD1 expression was significantly reduced in lung cancer cells compared with that in bronchial epithelial cells. Of note, PLCD1 had the lowest expression in A549 cells; therefore, subsequent studies were performed in A549 cells.

Overexpression of PLCD1 inhibits lung adenocarcinoma cell proliferation. To investigate the role of PLCD1 in lung cancer, PLCD1 overexpression was performed via cell transfection and the overexpression efficacy was confirmed using RT-qPCR and western blot analysis (Fig. 2A and B). The results of the cell proliferation assay revealed that PLCD1 overexpression significantly inhibited the proliferation of lung cancer cells compared with that of the oe-NC group (Fig. 2C). In addition, in the colony formation assay, markedly slower growth of PLCD1-overexpressing cells compared with that of the oe-NC group was observed (Fig. 2D). Similarly, detection of Ki67 expression levels in cells using immunofluorescence revealed that Ki67 expression was markedly reduced in the oe-PLCD1 group (Fig. 2E).

Overexpression of PLCD1 inhibits lung adenocarcinoma cell invasion, migration and the epithelial-mesenchymal transition (EMT) process. Transwell and wound-healing assays suggested that cell invasion and migration were significantly inhibited in the oe-PLCD1 group compared with the oe-NC group (Fig. 3A and B). The results of the western blot analysis indicated that the protein expression levels of MMP2 and MMP9 were significantly reduced in the oe-PLCD1 group compared with those in the oe-NC group (Fig. 3C). Western

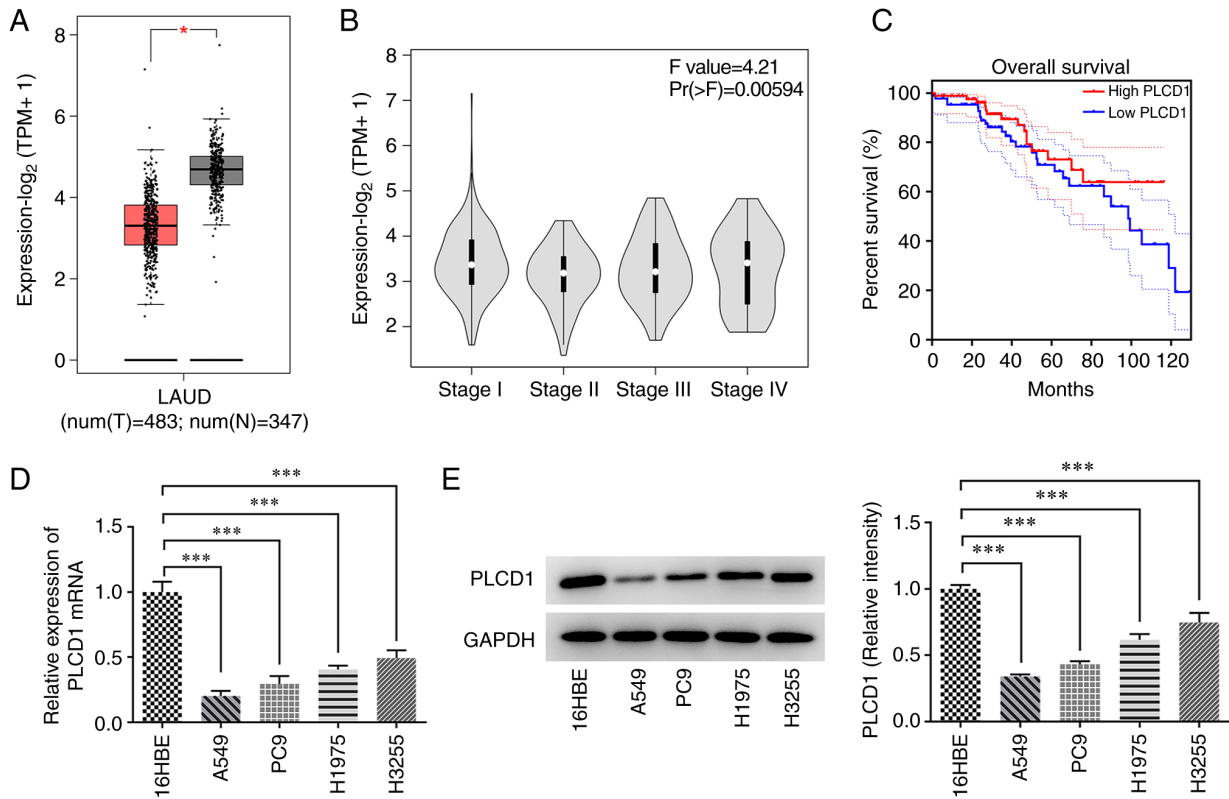


Figure 1. Low expression of PLCD1 in LUAD tissues and cells. (A) PLCD1 expression was downregulated in LUAD in the GEPIA database analysis. (B) PLCD1 expression was associated with LUAD stage in the GEPIA database analysis. (C) Low expression of PLCD1 was associated with low overall survival in patients with LUAD. (D) mRNA and (E) protein expression levels of PLCD1 in lung cancer cell lines and a human bronchial epithelial cell line. * $P < 0.05$ and *** $P < 0.001$. PLCD1, phospholipase C δ 1; LUAD, lung adenocarcinoma; TPM, transcripts per million; num, number; T, tumor; N, normal.

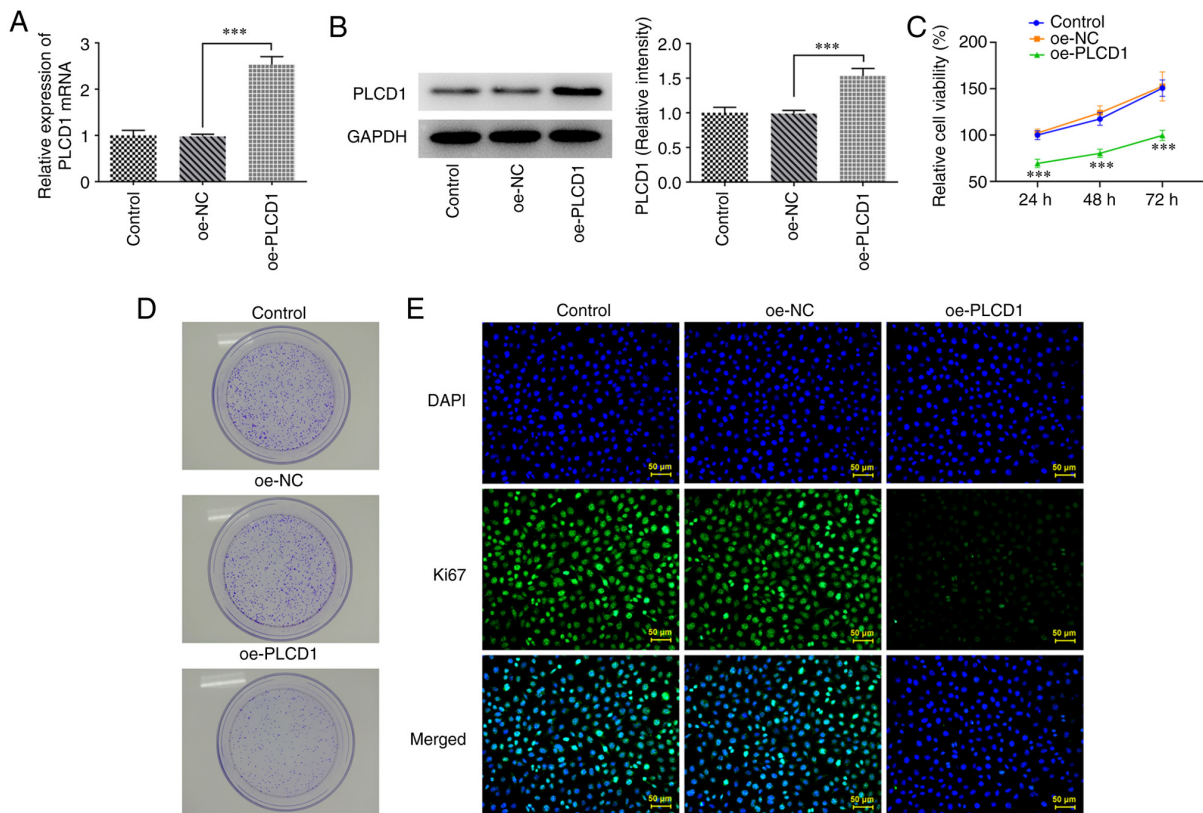


Figure 2. Overexpression of PLCD1 inhibits lung adenocarcinoma cell proliferation. (A) mRNA and (B) protein expression levels of PLCD1 in transfected cells. (C) Cell proliferation levels in the different transfection groups. (D) Results of the colony formation assay in different groups. (E) Ki67 expression levels in cells determined by immunofluorescence (scale bar, 50 μ m). *** $P < 0.001$ vs. oe-NC. PLCD1, phospholipase C δ 1; oe, overexpression; NC, negative control.

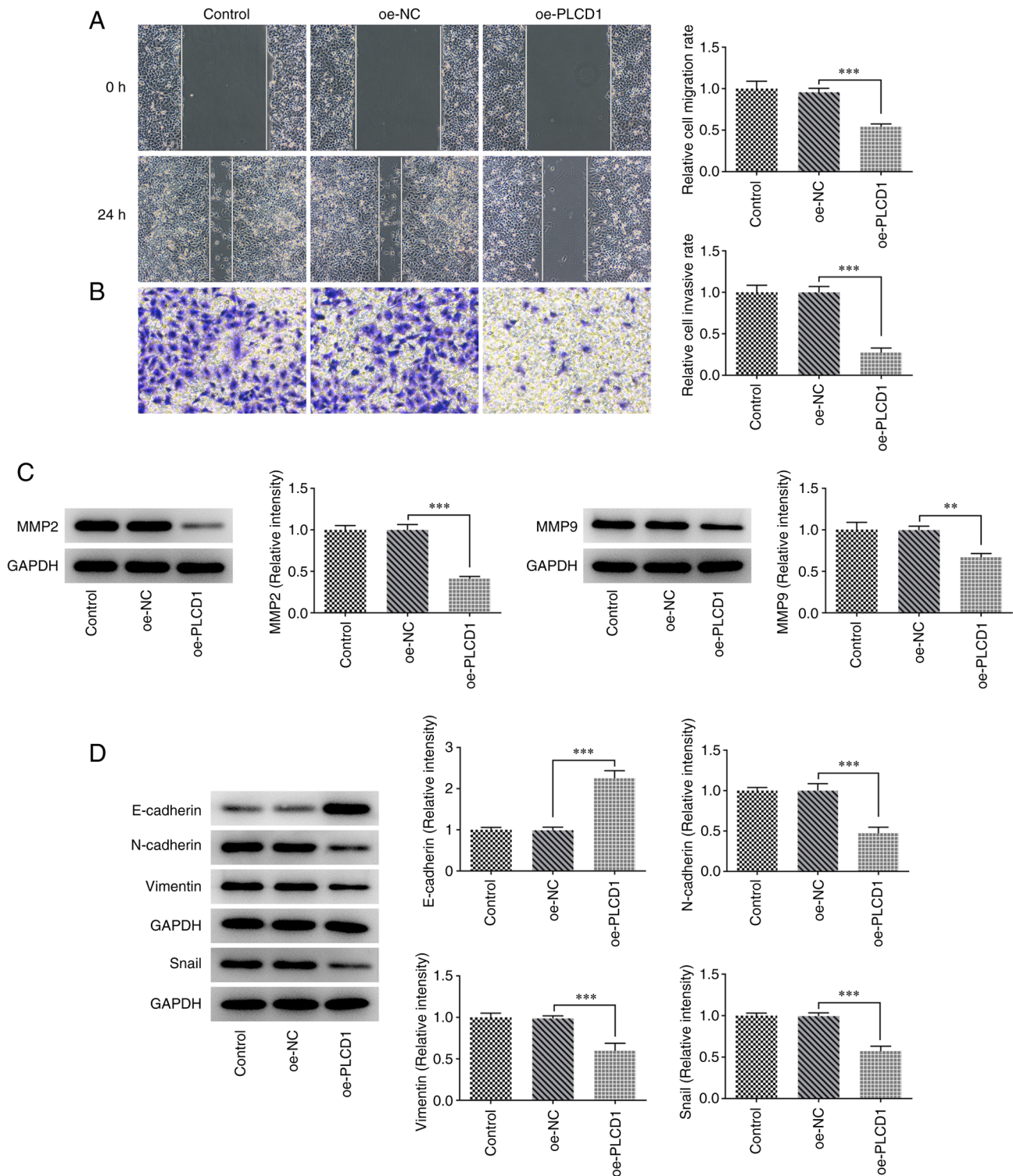


Figure 3. Overexpression of PLCD1 inhibits lung adenocarcinoma cell invasion, migration and EMT process. (A) Cell migration was detected using a wound-healing assay (magnification, x100). (B) Cell invasion was detected using a Transwell assay (magnification, x100). (C) The protein expression levels of MMP2 and MMP9 were detected via western blot analysis. (D) The EMT-related protein expression levels, including E-cadherin, N-cadherin, vimentin and Snail, were detected via western blot analysis. ** $P < 0.01$ and *** $P < 0.001$. PLCD1, phospholipase C δ 1; EMT, epithelial to mesenchymal transition; oe, overexpression; NC, negative control.

blotting was also employed for determining EMT-related proteins, and it was observed that compared with that in the oe-NC group, E-cadherin protein expression was significantly increased in the oe-PLCD1 group, while the protein expression levels of N-cadherin, vimentin and Snail were significantly decreased in the oe-PLCD1 group (Fig. 3D).

High expression of ORC1 is observed in lung adenocarcinoma tissues and is negatively regulated by PLCD1. Analysis of the GEPIA database indicated that ORC1 was highly expressed in lung adenocarcinoma compared with that in normal tissue, and ORC1 expression was associated with the pathological stage of lung adenocarcinoma (Fig. 4A and B). Compared with

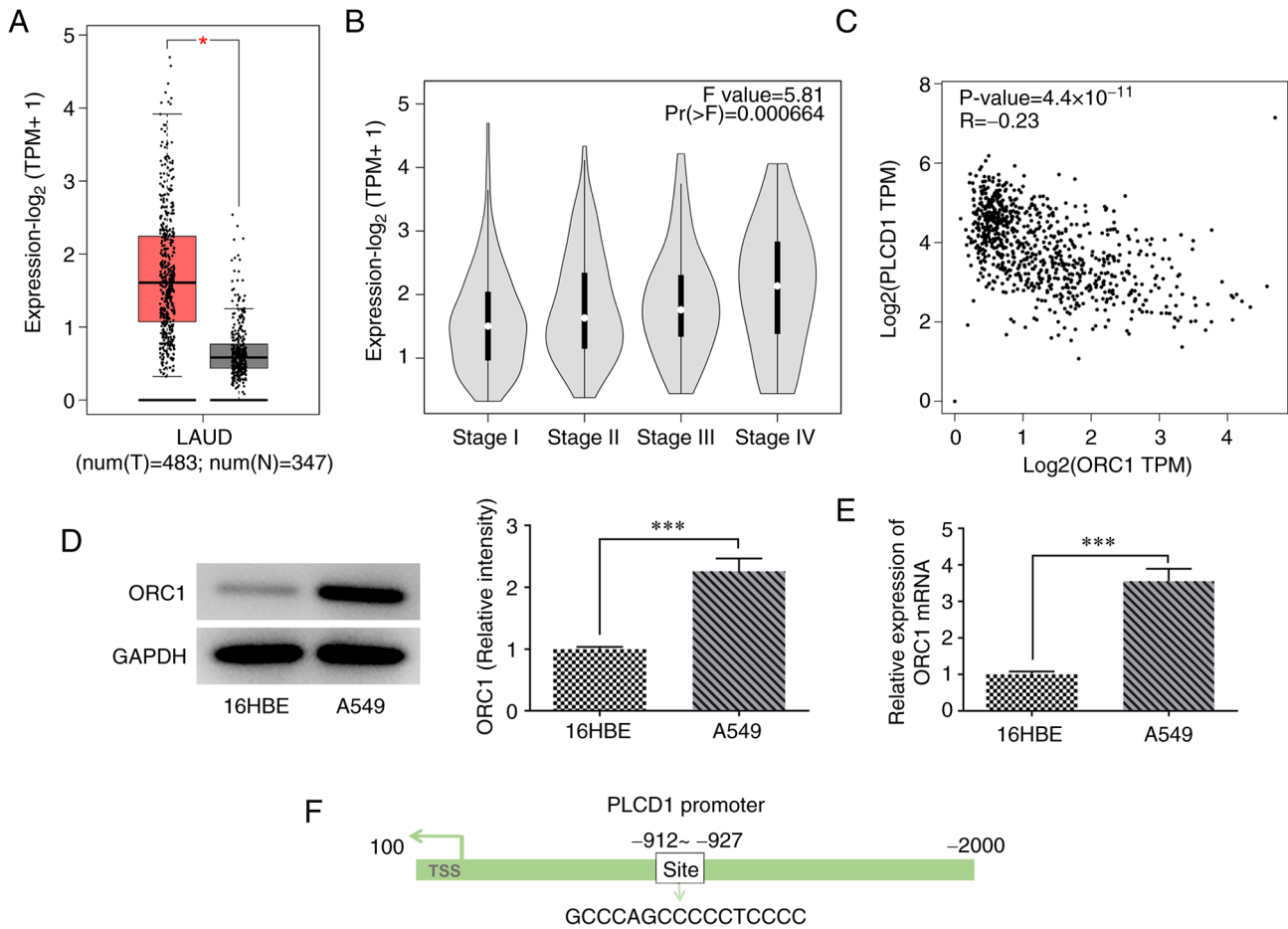


Figure 4. High expression of ORC1 in LUAD tissues and negative regulation of PLCD1. According to GEPIA database, ORC1 expression was (A) upregulated in LUAD, (B) associated with LUAD stage and (C) negatively correlated with PLCD1 expression in LUAD. The non-log scale was used for calculation and the log-scale axis for visualization. (D) Protein and (E) mRNA expression levels of ORC1 in cells. (F) Binding site of ORC1 to PLCD1 promoter. * $P < 0.05$ and ** $P < 0.001$. ORC1, origin recognition complex 1; PLCD1, phospholipase C δ 1; LUAD, lung adenocarcinoma; TPM, transcripts per million; num, number; T, tumor; N, normal; TSS, transcription start site.

Stage I, ORC1 expression was higher in stage II, III and IV, especially in Stage IV, indicating that higher expression of ORC1 was associated with more advanced disease stages. Of note, ORC1 and PLCD1 were indicated to be negatively correlated according to the data of the GEPIA database (Fig. 4C). In addition, ORC1 mRNA and protein expression levels were significantly higher in A549 lung cancer cells than in 16HBE bronchial epithelial cells (Fig. 4D and E). Furthermore, the binding site of ORC1 with the PLCD1 promoter was predicted using the HumanTFDB website (Fig. 4F).

To further investigate the association between ORC1 and PLCD1, ORC1-overexpressing cells (pcDNA3.1-ORC1) and cells with ORC1 knockdown (sh-ORC1) were established through transfection and the expression levels of ORC1 and PLCD1 in such cells were detected using western blot and RT-qPCR analyses. The results revealed that ORC1 overexpressing and knockdown cells were successfully established, since they exhibited significantly increased or decreased ORC1 expression compared with the corresponding NC, respectively, at the protein and mRNA levels (Fig. 5A and B). The protein and mRNA expression levels of PLCD1 were significantly decreased in the ORC1 overexpression compared with those in the NC group (pcDNA3.1-ORC1 vs. pcDNA3.1-NC), while the levels of PLCD1 were significantly increased in

the ORC1 RNA interference group (sh-ORC1-2 vs. sh-NC; Fig. 5C and D). ORC1 overexpression greatly reduced the activity of PLCD1. PLCD1 was enriched in anti-ORC1, suggesting that ORC1 was able to directly interact with the PLCD1 promoter (Fig. 5E and F).

ORC1 overexpression partially attenuates the inhibitory effects of PLCD1 overexpression on the proliferation, invasion, migration and EMT of lung adenocarcinoma cells. PLCD1 and ORC1 overexpression plasmids were simultaneously transfected into A549 cells and the cells were then evaluated. In terms of cell proliferation, PLCD1 overexpression inhibited cell proliferation compared with that in the oe-NC group, while ORC1 overexpression reduced the inhibitory effects of PLCD1 overexpression on cell proliferation compared with the oe-PLCD1 group (Fig. 6A). The same trend was observed in the colony formation assays, with slower cell growth noticed in the oe-PLCD1 group compared with that in the oe-NC group and faster cell growth in the oe-PLCD1 + pcDNA3.1-ORC1 group compared with that in the oe-PLCD1 group (Fig. 6B).

Immunofluorescence staining revealed that Ki67 expression was markedly diminished in the oe-PLCD1 group compared with that in the oe-NC group, whereas Ki67 expression was markedly increased in the oe-PLCD1 + pcDNA3.1-ORC1

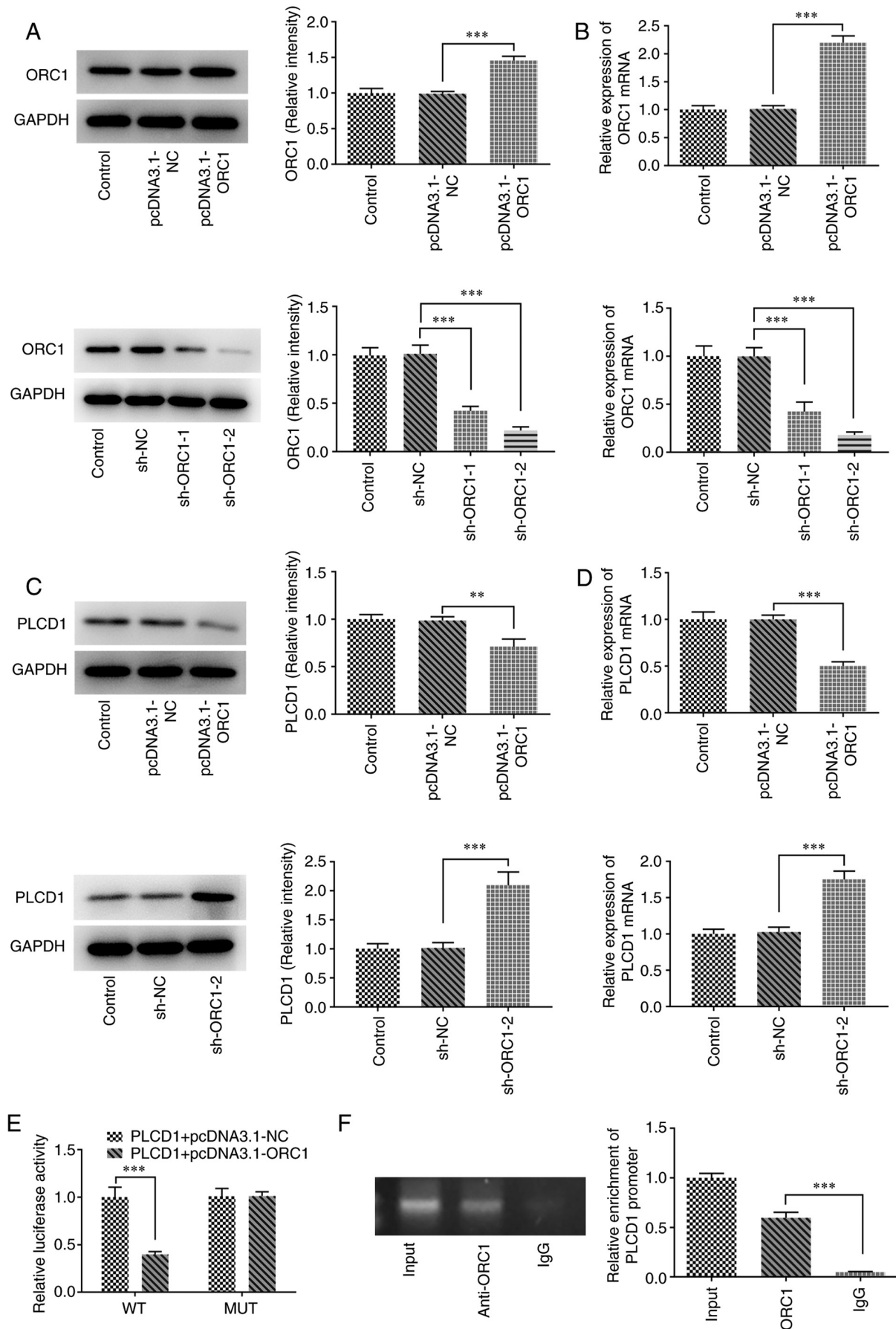


Figure 5. ORC1 is negatively associated with PLCD1 expression in lung adenocarcinoma cells. (A) Protein and (B) mRNA expression levels of ORC1 in transfected cells. (C) Protein and (D) mRNA expression levels of PLCD1 in transfected cells. (E) ORC1 affected the PLCD1 promoter activity in the luciferase reporter gene assay. (F) Binding of ORC1 to the PLCD1 promoter was confirmed using chromatin immunoprecipitation. **P<0.01 and ***P<0.001. ORC1, origin recognition complex 1; PLCD1, phospholipase Cδ1; WT, wild-type; MUT, mutant; NC, negative control; sh, short hairpin RNA.

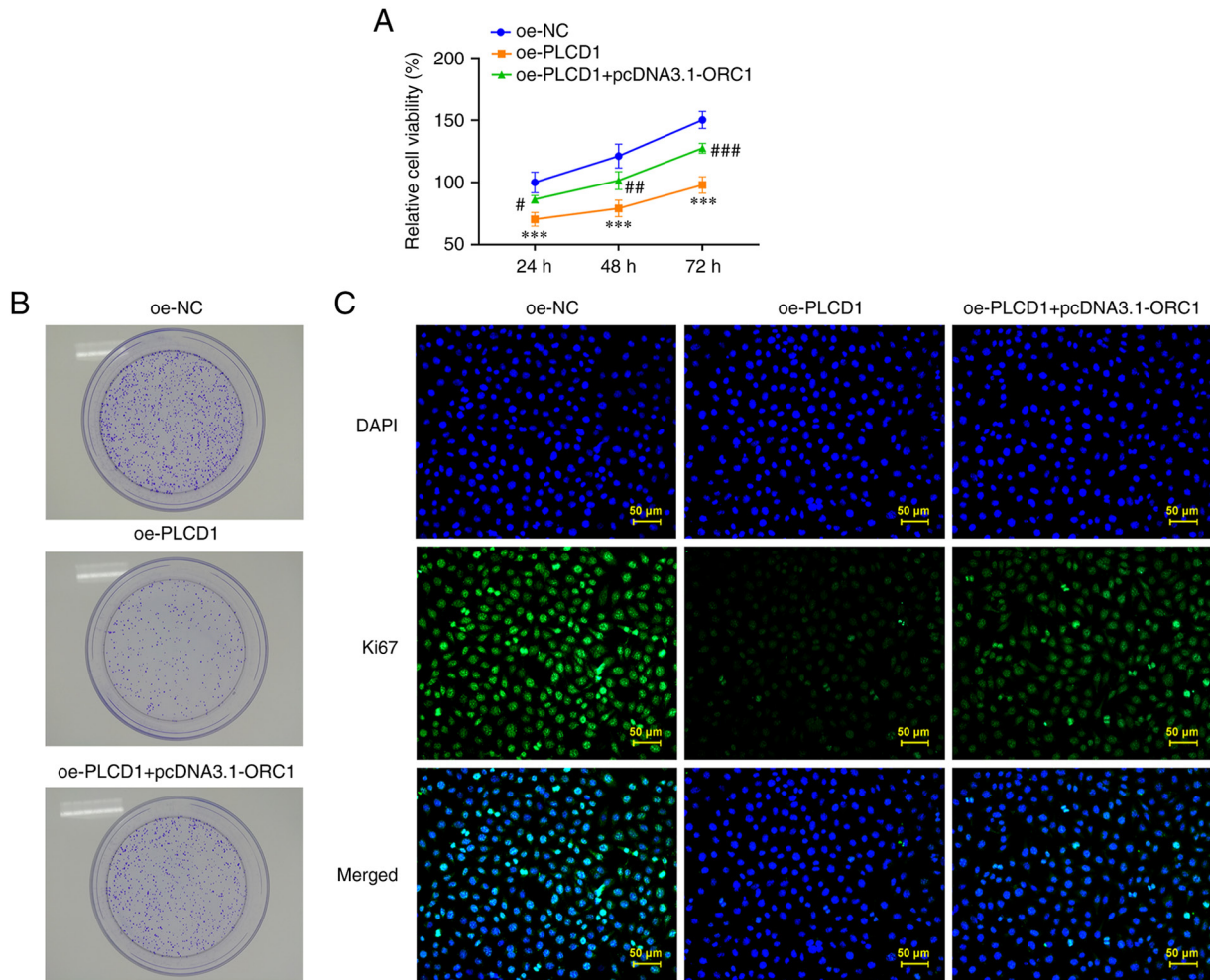


Figure 6. ORC1 overexpression attenuates the inhibitory effect of PLCD1 overexpression on lung adenocarcinoma cell proliferation. (A) Results of cell proliferation assay. (B) Results of colony formation assay in different groups. (C) Ki67 expression level in cells determined via immunofluorescence (scale bar, 50 μ m). *** P <0.001 vs. oe-NC; # P <0.05, ## P <0.01 and ### P <0.001 vs. oe-PLCD1. ORC1, origin recognition complex 1; PLCD1, phospholipase C δ 1; oe, overexpression; NC, negative control.

group compared with that in the oe-PLCD1 group (Fig. 6C). Of note, ORC1 overexpression attenuated the inhibitory effects of PLCD1 overexpression on the invasion and migration of lung cancer cells. The results suggested that cell migration and invasion were promoted by ORC1 overexpression compared with that observed in the oe-PLCD1 group (Fig. 7A and B).

Analysis of the expression of MMPs revealed that ORC1 overexpression significantly reversed the inhibitory effects of PLCD1 overexpression on MMP2 and MMP9 (oe-PLCD1 + pcDNA3.1-ORC1 vs. oe-PLCD1) (Fig. 7C). In addition, analysis of the expression levels of EMT-related proteins indicated that ORC1 overexpression significantly reversed the effects of PLCD1 overexpression on the expression levels of EMT-related proteins (Fig. 7D). Taken together, these results suggested that ORC1 exerts its functions in lung adenocarcinoma by inhibiting PLCD1 expression.

Discussion

Lung cancer is the most common cancer worldwide and the majority of patients with lung cancer are in advanced stages of the disease at the time of diagnosis (19). PLCD1 is involved

in energy metabolism, calcium homeostasis and intracellular motility (10). However, PLCD1 is frequently downregulated in multiple types of cancer, such as esophageal squamous cell carcinoma (20) and breast cancer (10). In a previous report, the genome near the homozygous deletion of chromosome 3p in lung cancer cell lines was sequenced and the gene encoding PLCD1 was observed to be close to deletion region (13). Furthermore, low PLCD1 expression in lung adenocarcinoma was indicated in the GEPIA database analysis (data not shown) and this low PLCD1 expression was associated with poor prognosis in patients with lung adenocarcinoma. These results suggest that PLCD1 may have the potential to affect the progression of lung cancer.

In the present study, the expression of PLCD1 was analyzed in clinical patients using the GEPIA database, as well as in bronchial epithelial and lung adenocarcinoma epithelial cells using RT-qPCR and western blot analysis. Consistent with the results of PLCD1 expression in clinical samples from the GEPIA database, the PLCD1 mRNA and protein expression levels were significantly decreased in the aforementioned four lung adenocarcinoma cell lines compared with those in bronchial epithelial cells.

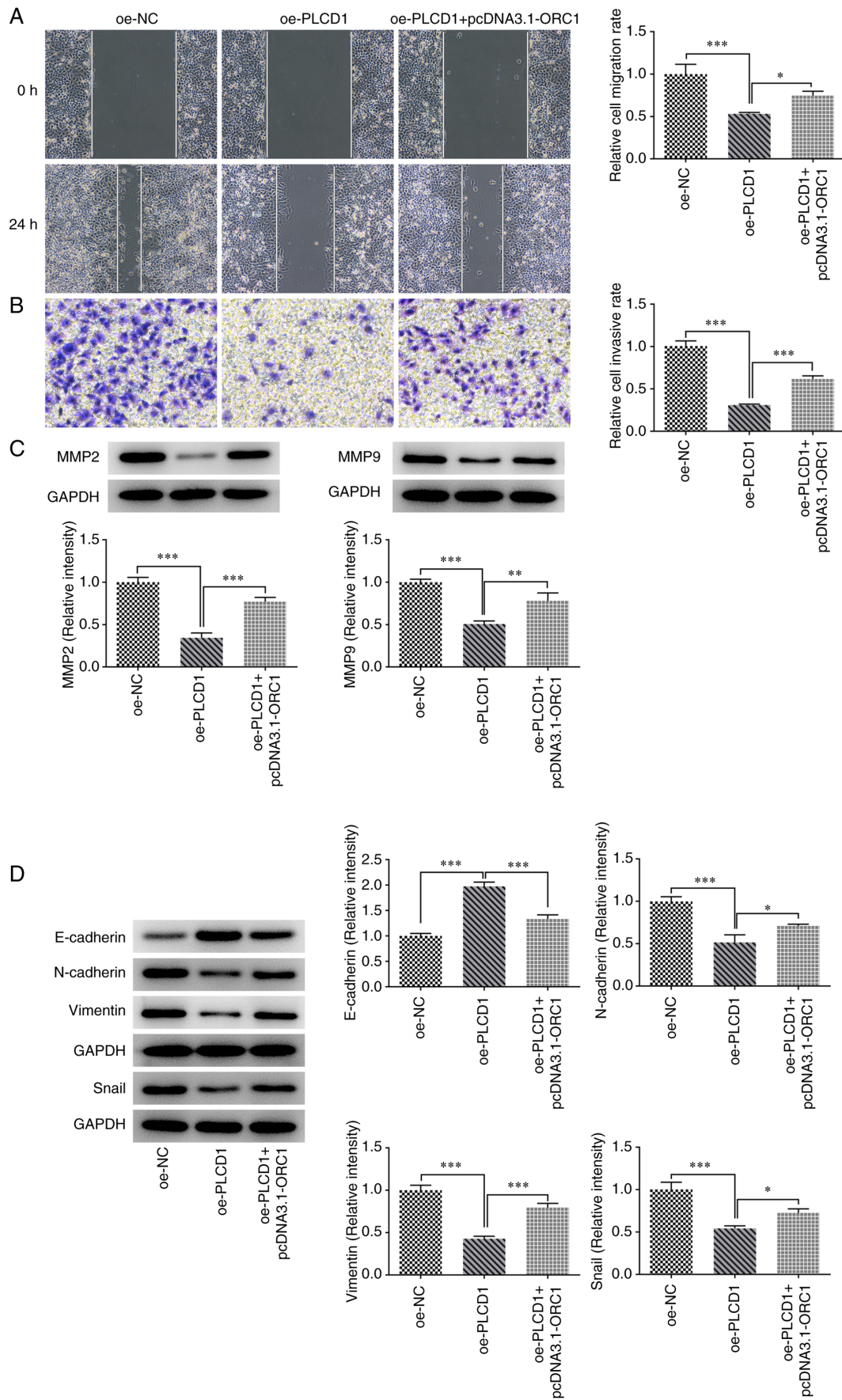


Figure 7. ORC1 overexpression partially reduces the inhibitory effect of PLCD1 overexpression on invasion, migration and EMT of lung adenocarcinoma cells. (A) Cell migration was detected using a wound-healing assay (magnification, x100). (B) Cell invasion was detected using a Transwell assay (magnification, x100). (C) The protein expression levels of MMP2 and MMP9 were detected by western blot analysis. (D) EMT-related protein expression levels, including E-cadherin, N-cadherin, vimentin and Snail, were detected by western blot analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. ORC1, origin recognition complex 1; PLCD1, phospholipase C δ 1; EMT, epithelial to mesenchymal transition; oe, overexpression; NC, negative control.

A previous study has also demonstrated low expression of PLCD1 in colorectal cancer cells and indicated that restoring PLCD1 expression inhibited colorectal cancer cell proliferation and induced apoptosis; furthermore, restoration of PLCD1 inhibited cell proliferation, metastasis and tumorigenicity in colorectal cancer (21). In esophageal squamous carcinoma cells, PLCD1 was indicated to inhibit cell proliferation, invasion and migration by suppressing the Wnt/ β -catenin signaling pathway (22). In pancreatic and breast cancer tumors, PLCD1 expression also exerted similar regulatory effects (23-25). Thus, it was hypothesized that the activation of PLCD1 contributed to tumor suppression.

In the present study, the proliferation of lung cancer cells was examined using MTT assay, colony formation assay and immunofluorescence staining, and the results indicated that the proliferation of cells overexpressing PLCD1 was significantly inhibited compared with that in the control group. In addition, the results of the cell invasion, migration and EMT assays revealed that overexpression of PLCD1 had inhibitory effects on lung cancer cells.

Of note, there are limited studies on the regulatory factors located upstream of the PLCD1 gene. ORC1 is one of the ORCs involved in the first step of DNA replication (26). Since ORC1 is synthesized in the G₁ phase and is degraded in the S phase of the cell cycle, it has been previously speculated that ORC1 expression may be closely associated with cell proliferation and the cell cycle, and it may be involved in biological processes, such as cell proliferation, apoptosis, invasion and migration (26,27). In a study on glioma, overexpression of ORC1 promoted cancer cell proliferation, while downregulation of ORC1 inhibited the activation of the ERK/JNK signaling pathway, thereby suppressing glioma invasion and migration (28).

In the present study, ORC1 was demonstrated to bind to the PLCD1 promoter using ChIP and luciferase reporter gene assays. The binding site of ORC1 to the PLCD1 promoter was predicted using the HumanTFDB website. The results indicated that ORC1 overexpression was able to significantly reduce PLCD1 expression. To further demonstrate the association between these two genes, ORC1- and PLCD1-overexpressing cells were established. However, this does not necessarily indicate that ORC1 protein has a direct effect on the PLCD1 overexpression plasmid. The downregulation of PLCD1 expression in cells transfected with ORC1 is different to the overexpression of PLCD1 caused by the transfected plasmids. Compared with the changes of PLCD1 with different treatment, it could be speculated that the effect of endogenous ORC1 on PLCD1 expression in cells is transient, whereas the effect of transfection with the pcDNA3.1-ORC1 plasmid on PLCD1 expression is stable (29). In summary, PLCD1 expression in cancer cells was increased after transfection with oe-PLCD1 plasmid. However, overexpression of ORC1 caused an upregulation of ORC1 expression, thereby suppressing PLCD1 expression, which subsequently reduced the overall PLCD1 expression and ultimately affected the cellular activities. These results demonstrated that the inhibitory effects of PLCD1 overexpression on lung adenocarcinoma cell proliferation, migration and EMT were attenuated following overexpression of both ORC1 and PLCD1 compared with those achieved by overexpression

of PLCD1 alone. These findings indicated that ORC1 and PLCD1 have a negative regulatory relationship.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate the association between low PLCD1 expression in lung adenocarcinoma and ORC1, which may downregulate PLCD1 and thus promote the proliferation, migration and EMT process of lung adenocarcinoma cells. However, the absence of animal models is a limitation of the present study. In future studies, cells will be analyzed, and xenograft mice models will be established to confirm the potential use of PLCD1 as a target marker for the diagnosis and treatment of lung adenocarcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YJ, QQ, JT and XQ conceived and designed the study, and acquired and interpreted the data. YJ and QQ were major contributors in writing the manuscript. YJ and XQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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