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ORIGINAL ARTICLE

Loss of phospholipase Cγ1 suppresses hepatocellular carcinogenesis through blockade of STAT3-mediated cancer development

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Abstract

Phospholipase C gamma 1 (PLCγ1) plays an oncogenic role in several cancers, alongside its usual physiological roles. Despite studies aimed at identifying the effect of PLCγ1 on tumors, the pathogenic role of PLCγ1 in the tumorigenesis and development of hepatocellular carcinoma (HCC) remains unknown. To investigate the function of PLCγ1 in HCC, we generated hepatocyte-specific PLCγ1 conditional knockout ($PLC\gamma1^{f/f}$; Alb-Cre) mice and induced HCC with diethylnitrosamine (DEN). Here, we identified that hepatocyte-specific PLCγ1 deletion effectively prevented DEN-induced HCC in mice. $PLC\gamma1^{f/f}$; Alb-Cre mice showed reduced tumor burden and tumor progression, as well as a decreased incidence of HCC and less marked proliferative and inflammatory responses. We also showed that oncogenic phenotypes such as repressed apoptosis, and promoted proliferation, cell cycle progression and migration, were induced by PLCγ1. In terms of molecular mechanism, PLCγ1 regulated the activation of signal transducer and activator of transcription 3 (STAT3) signaling. Moreover, PLCγ1 expression is elevated in human HCC and correlates with a poor

prognosis in patients with HCC. Our results suggest that PLCγ1 promotes the pathogenic progression of HCC, and PLCγ1/STAT3 axis was identified as a potential therapeutic target pathway for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common type of cancer and the fourth leading cause of cancer-related deaths worldwide.[1] HCC also accounts for most primary liver cancers. The 5-year survival rate is poor compared with other cancer types, and targeted molecular therapies are difficult to implement due to a lack of biomarkers.[2] Over the past decade, chemotherapy for advanced HCC has been limited to sorafenib, and chemoresistance has been common.[3] Recently, atezolizumab plus bevacizumab and lenvatinib were approved as first-line therapy for the treatment of unresectable HCC.[4] Major risk factors of HCC include infection with hepatitis B virus or hepatitis C virus, excessive alcohol consumption, and nonalcoholic steatohepatitis.[5] However, the complex molecular mechanisms underlying the development of HCC remain poorly understood. Therefore, understanding the molecular mechanisms that promote HCC progression is essential for proper diagnosis and treatment.

Phosphoinositide-specific phospholipase C (PLC) is a membrane-associated enzyme that is essential for many cellular processes. PLC is activated by a variety of extracellular ligands to regulate signaling mediated by cellular ligands. PLC hydrolyzes *phosphatidylinositol* 4,5-*bisphosphate* to generate the second messengers, inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG).[6] IP3 increases intracellular calcium levels and DAG activates protein kinase C (PKC).[7] PLC gamma 1 (PLCγ1), which is 1 of 13 mammalian PLC subtypes, is directly activated by extracellular stimulation to mediate receptor tyrosine kinase signaling.[8] Evidence from recent studies suggests that PLCγ1 has an oncogenic function in several cancers. PLCγ1 is highly expressed in several tumors, including breast cancer, colorectal cancer, and gastric cancer.[9–11] PLCγ1 also plays a critical role in metastasis and tumor growth.[12,13]

Despite efforts to determine the role of PLCγ1 in cancer, little is known about its pathogenic function in HCC, and mechanistic studies are lacking.[14] HCC is a major cancer worldwide, so molecular research into its causes is vital. In the present study, we aimed to identify the role and molecular mechanism of PLCγ1 in HCC development. We hypothesized that PLCγ1-deficient mice would be the most useful model to investigate the function of PLCγ1 *in vivo*. However, knockout of PLCγ1 results in embryonic lethality at about embryonic day 9.[15] Thus, we generated hepatocyte-specific PLCγ1 conditional knockout mice to determine the behavior of PLCγ1 in HCC. Then, we induced HCC in both wild-type (WT) and PLCγ1$^{f/f}$; Alb-Cre mice using diethylnitrosamine (DEN) and analyzed the pathological phenotypes. Our study has revealed the oncogenic role of PLCγ1 in liver cancer, suggesting a molecular therapeutic target pathway for HCC.

METHODS

Mice

Mice were bred and housed in the Animal Research Facility under specific pathogen-free conditions at the Ulsan National Institute of Science and Technology (UNIST). They were maintained under controlled temperature, humidity, and illumination (12-h light/dark cycle) conditions. Standard chow (A03; Scientific Animal Food & Engineering) and water were provided *ad libitum*. The Institutional Animal Care and Utilization Committee approved all of the procedures in accordance with the UNIST guide for the care and use of laboratory animals (UNISTIACUC-14-011).

Generation of PLCγ1 conditional knockout mice

The *Plcg1*-targeting vector was designed to delete exons 3–5 ("Loxp-exon 3-exon 4-exon 5-Loxp-Frt-Neo-Frt").[16] E14K ES cells were electroporated with the *Plcg1*-targeting vector, and single clones were microinjected into blastocysts. The Frt-Neo cassette was removed by crossing with flippase transgenic mice, and the *Plcg1*-floxed mice were backcrossed to C57BL/6J for at least 10 generations. The *Plcg1*-floxed mice were genotyped using the following primers: forward (5′-GCA CAG ACA GAC TTG GAC-3′) and reverse (5′-GTT GCT CAA GGT GAA GGC TCT-3′). To generate the liver-specific *Plcg1* deletion, *Plcg1*-floxed mice were crossed with albumin-Cre transgenic mice (B6.Cg-Tg[Alb-Cre]21Mgn/J; Jackson Laboratory). The controls were sibling littermates.

Liver tumorigenesis

To achieve chemical-induced hepatocarcinogenesis, mice were intraperitoneally injected with a single dose of DEN (25 mg/kg, N0258-1G; Sigma-Aldrich), and the

controls were injected with saline (0.9% sodium chloride) at postnatal day 15. Male mice were humanely sacrificed at 9 months following injection, and liver tissues and blood were collected.

Serum analysis and enzyme-linked immunosorbent assay

Mouse blood was collected in heparin-coated round-bottom tubes, and serum was obtained after centrifugation at 3000 rpm for 10 min. The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were detected with a mouse AST/ALT activity assay kit (Biovision) according to the manufacturer's instructions. The serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 cytokine levels were measured using mouse TNF-α/IL-6 enzyme-linked immunosorbent assay (ELISA) kits (Abbkine) according to the manufacturer's instructions. Human HCC cell culture supernatants were collected and IL-6 levels were measured using the human IL-6 ELISA development kit (Peprotech) according to the manufacturer's instructions.

Cell lines and culture

The human HCC cell lines Hep3B and Huh7 were purchased from the ATCC. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin streptomycin solution. The cells were incubated in a humidified atmosphere under 5% CO_2 at 37°C.

Transfection of small interfering RNAs and plasmids

The small interfering RNAs (siRNAs) against PLCγ1 and negative controls were designed by and purchased from Qiagen. HCC cells were transfected with the siRNAs using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the forward protocol in the manufacturer's instructions. Cells were harvested 48 h after transfection. The pcDNA3.1-PLCγ1 plasmid was provided by UNIST. Plasmids were transfected to the HCC cell lines using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. To construct stable cell lines, pcDNA3.1-PLCγ1 was transfected into HCC cells, and cells stably expressing PLCγ1 were selected with G418.

Colony formation assay

Cells were plated in six-well plates at a density of 500 cells per well. After culturing the cells for 7–14 days, colonies were fixed with 70% ethanol and stained with 0.1% crystal violet solution.

Flow cytometry assay

For the cell cycle analysis, cells were washed with phosphate-buffered saline (PBS) and fixed in 75% ethanol at 4°C overnight. Cells were stained with propidium iodide RNase staining solution (BD Biosciences) and analyzed by flow cytometry (BD LSRFortessa; BD Biosciences). For the apoptosis analysis, 5 μl annexin V-FITC and 5 μl propidium were added to the 100-μl cell suspension and incubated for 15 min in the dark. The reaction was terminated by the addition of 400 μl of binding buffer and analyzed by flow cytometry (BD LSRFortessa).

Migration and invasion assay

A Transwell assay was performed to assessed cell migration and invasion. To analyze migration, 5×10^4 cells in serum-free medium were seeded into a Falcon insert with 8-μm pore size (Becton-Dickinson) and complete growth medium (DMEM containing 10% FBS and 1% PBS) was added to the lower chamber. For the invasion assay, the inserts were precoated with Matrigel (Corning) before the same assay was performed. After incubation at 37°C for 24–48 h, the inserts were fixed and stained using Diff-Quik (Sysmex). Cell migration was also assessed by a wound-healing assay. HCC cells were seeded into a six-well plate at a density of 1×10^6 cells per well, and wounds were made using a 200-μl pipette tip. At 48 h after wound formation, wound recovery was measured.

Human HCC tissue collection

Twelve pairs of liver tumors and adjacent noncancerous tissue specimens were collected from the Department of Pathology, Seoul National University College of Medicine (Seoul, Korea), with approval from the institutional review board (IRB No. H-2004-152-1118).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism v.8 software (GraphPad Software, Inc.). Data are presented as means ± SD or means ± SEM. Fisher's exact test was performed to analyze incidence data. Overall survival (OS) and disease-free survival (DFS) were evaluated by the Kaplan–Meier and log-rank tests. The Mann–Whitney U test or two-tailed unpaired Student's *t* test was performed to compare the

differences between two groups. p values < 0.05 were taken as statistically significant.

RESULTS

Generation of hepatocyte-specific PLCγ1 knockout mice and induction of HCC by DEN

To examine the potential role of PLCγ1 in HCC, we generated a hepatocyte-specific PLCγ1 conditional deficient mouse model using PLCγ1 floxed mice and Albumin-Cre mice (Figure S1A; see Materials and Methods). Immunoblotting analysis confirmed that PLCγ1 expression was depleted in the liver, but not in other organs (Figure S1B). In addition, messenger RNA (mRNA) expression levels of liver extract were effectively decreased in PLCγ1$^{f/f}$; Alb-Cre mice compared with WT mice (Figure S1C). To chemically induce hepatocarcinogenesis, mice were injected with a single dose of DEN at postnatal day 15. The mice were sacrificed, and liver tissues were collected 9 months after injection (Figure 1A; see Materials and Methods). PLCγ1 protein expression of the liver extract was completely abolished in only the PLCγ1$^{f/f}$; Alb-Cre mice, in both the saline-treated and DEN-treated groups (Figure S1D). To verify PLCγ1 depletion in the hepatocytes, immunostaining of the liver tissues was performed; the immunohistochemistry intensity results confirmed the absence of PLCγ1 in the PLCγ1$^{f/f}$; Alb-Cre mice groups (Figure S1E). In particular, the immunofluorescence results showed that PLCγ1 (green, anti-PLCγ1) was not expressed in hepatocytes (red, anti-Albumin) in the PLCγ1$^{f/f}$; Alb-Cre mice groups (Figure 1B). PLCγ1 was also removed from female mice (Figure S2A–C). As a first step, we generated mice in which PLCγ1 was effectively removed from the hepatocytes.

Loss of PLCγ1 suppresses tumorigenesis and the development of DEN-induced primary liver cancer in mice

To determine the oncogenic role of PLCγ1 in liver malignancy, we assessed the effect of PLCγ1 deletion on HCC *in vivo*. Remarkably, hepatocyte-specific deletion of PLCγ1 decreased tumorigenesis and the tumor burden in the liver (Figure 1C). All WT male mice developed visible hepatic tumors, but only two of the seven PLCγ1$^{f/f}$; Alb-Cre mice presented with small and few tumor nodules (Figure 1D). In the saline group, tumors did not develop in any WT or PLCγ1$^{f/f}$; Alb-Cre mice. Similarly, female mice did not develop DEN-induced HCC (Figure S2D,E). Analysis of the liver showed that PLCγ1 deletion noticeably reduced the number, volume, and size of DEN-induced liver tumors (Figure 1E–G). Next, we performed immunohistochemistry to detect proliferating cell nuclear antigen (PCNA) in the liver tissues and assess the proliferation response. Compared with liver tissues from the control mice, the tumors derived from the DEN-treated WT mice had considerably increased PCNA-positive hepatocytes. Remarkably, there were few PCNA-positive cells in the tumors derived from the DEN-treated PLCγ1$^{f/f}$; Alb-Cre mice (Figure 1H; Figure S3A). These results indicated that PLCγ1 deletion significantly reduced the number of proliferating tumor cells, suggesting that PLCγ1 mediates hepatic carcinoma growth. Furthermore, we performed Masson's trichrome (MT) staining to evaluate the formation of tumor fibrosis by detecting collagen fibers in liver tissues. MT staining revealed that the formation and progression of fibrotic areas only occurred in the DEN-treated WT mice, and not in the control mice or DEN-treated PLCγ1$^{f/f}$; Alb-Cre mice (Figure 1I; Figure S3B). Collectively, these results demonstrated that loss of PLCγ1 in hepatocytes significantly suppressed tumor formation and growth, and that PLCγ1 can be considered to play a critical role in tumorigenesis and the development of HCC.

PLCγ1 deficiency attenuates the DEN-induced inflammatory response and liver injury in mice

Histological examination using hematoxylin and eosin staining confirmed tumor formation in the liver tissues of the DEN-treated mice. In particular, the tumors from DEN-treated WT mice showed marked inflammatory responses. In these tumors, the trabecular architecture was lost, and we observed inflammatory cell infiltration and eosin-positive cytoplasmic inclusions. These features were not observed in the control or DEN-treated PLCγ1$^{f/f}$; Alb-Cre mice (Figure 2A). Tumors were not generated in female mice in any of the groups, but abnormal hepatocytes were observed in WT mice treated with DEN. Dysplastic foci include hepatocytes with atypical morphology such as multinucleated, massive nucleated, and giant cells. These preneoplastic foci did not appear in PLCγ1$^{f/f}$; Alb-Cre mice even after DEN treatment (Figure S4A). To confirm liver inflammation and injury, we measured serum concentrations of ALT and AST (hepatic enzymes) by enzyme activity analysis. Serum ALT and AST levels were significantly increased following DEN treatment but recovered to control levels following the deletion of PLCγ1 (Figure 2B,C). We also investigated TNF-α and IL-6 (hepatic inflammatory cytokines), both of which were increased in the DEN-treated WT mice, but which were reduced by knockout of PLCγ1 (Figure 2D,E). These results were confirmed by quantitative reverse-transcription polymerase chain reaction analysis of TNF-α and IL-6 transcripts in mouse

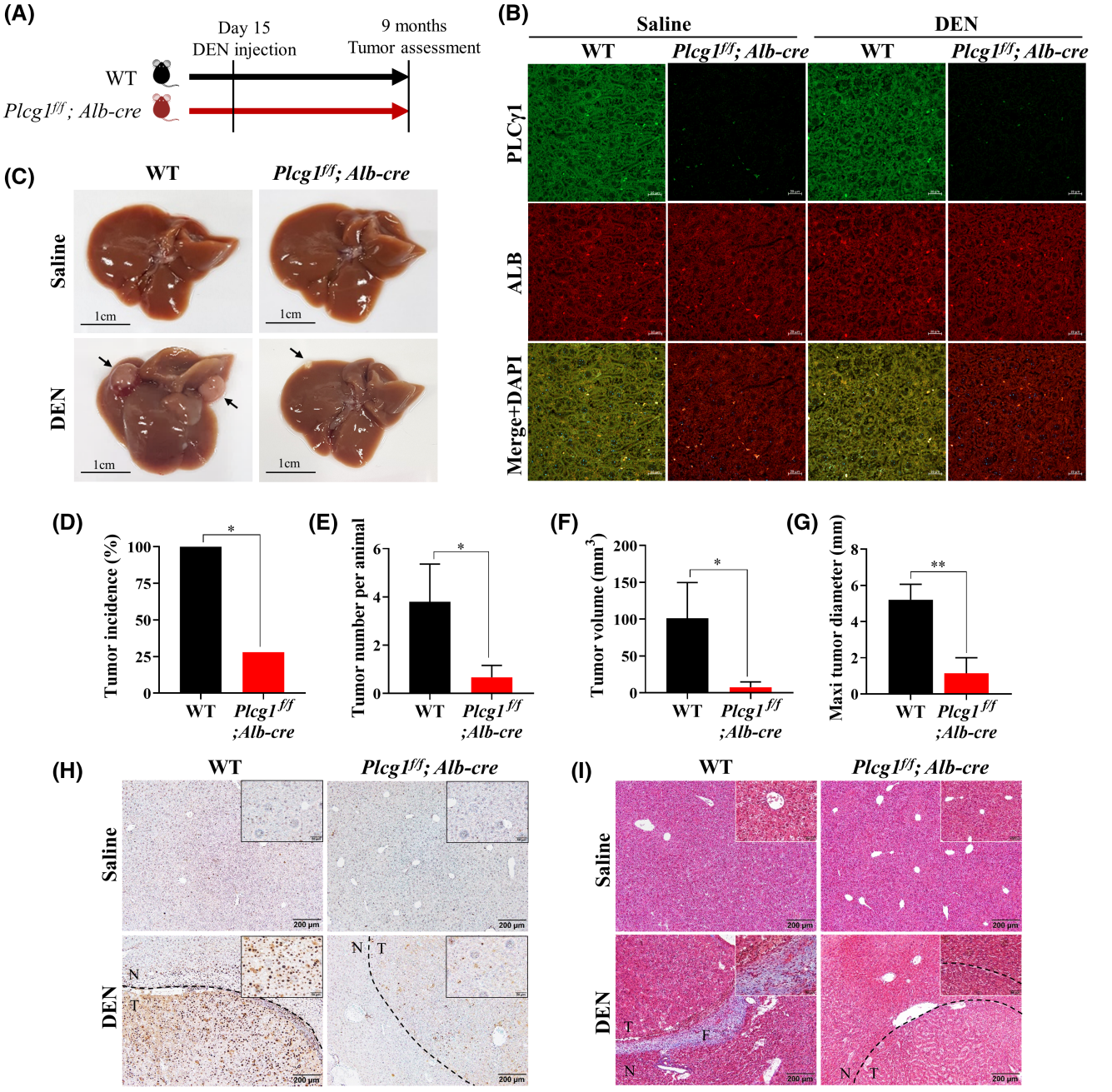

FIGURE 1 Loss of phospholipase C gamma 1 (PLCγ1) suppresses the tumorigenesis and development of diethylnitrosamine (DEN)–induced primary liver cancer in mice. (A) Scheme of experimental design for DEN-induced mice model of hepatocellular carcinoma (HCC). Wild-type (WT) or PLCγ1$^{f/f}$; Alb-Cre mice were injected with DEN (25 mg/kg) or saline (0.9% NaCl) at 2 weeks of age. At 9 months following injection, mice were sacrificed and liver was analyzed. (B) Immunofluorescence staining for PLCγ1 (green), albumin (red), and 4′,6-diamidino-2-phenylindole (DAPI; blue) were performed in liver from four groups of mice. (C) Representative images of liver from mice and arrows indicate the tumor nodules. (D) Tumor incidence (statistic was performed using Fisher's exact test). (E) Tumor number per animal. (F) Average tumor volume. (G) Average maximum tumor diameter was compared in DEN-treated WT and PLCγ1$^{f/f}$; Alb-Cre mice. Values are expressed as mean ± SEM. Statistics were performed using Student's *t* test. (E–G) *$p < 0.05$, **$p < 0.01$. (H) Representative images of immunohistochemistry for proliferating cell nuclear antigen (PCNA) in liver tissues. Dashed lines present the border between tumor region and adjacent nontumor. (I) Representative images of Masson trichrome staining in liver tissues. Blue color indicates collagen fiber. F, fibrous area; N, nontumor; T, tumor.

livers (Figure 2F,G). Interestingly, the same trend was observed in female mice, with lower absolute values (Figure S4B,C). We also confirmed the activation of nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3), transcription factors mediating inflammatory responses. Immunostaining demonstrated that both activated NF-κB (serine 536 phosphorylation) and STAT3 (tyrosine 705 phosphorylation) was prominently expressed in the hepatocytes' nuclei of DEN-treated WT mice and not in PLCγ1f/f;

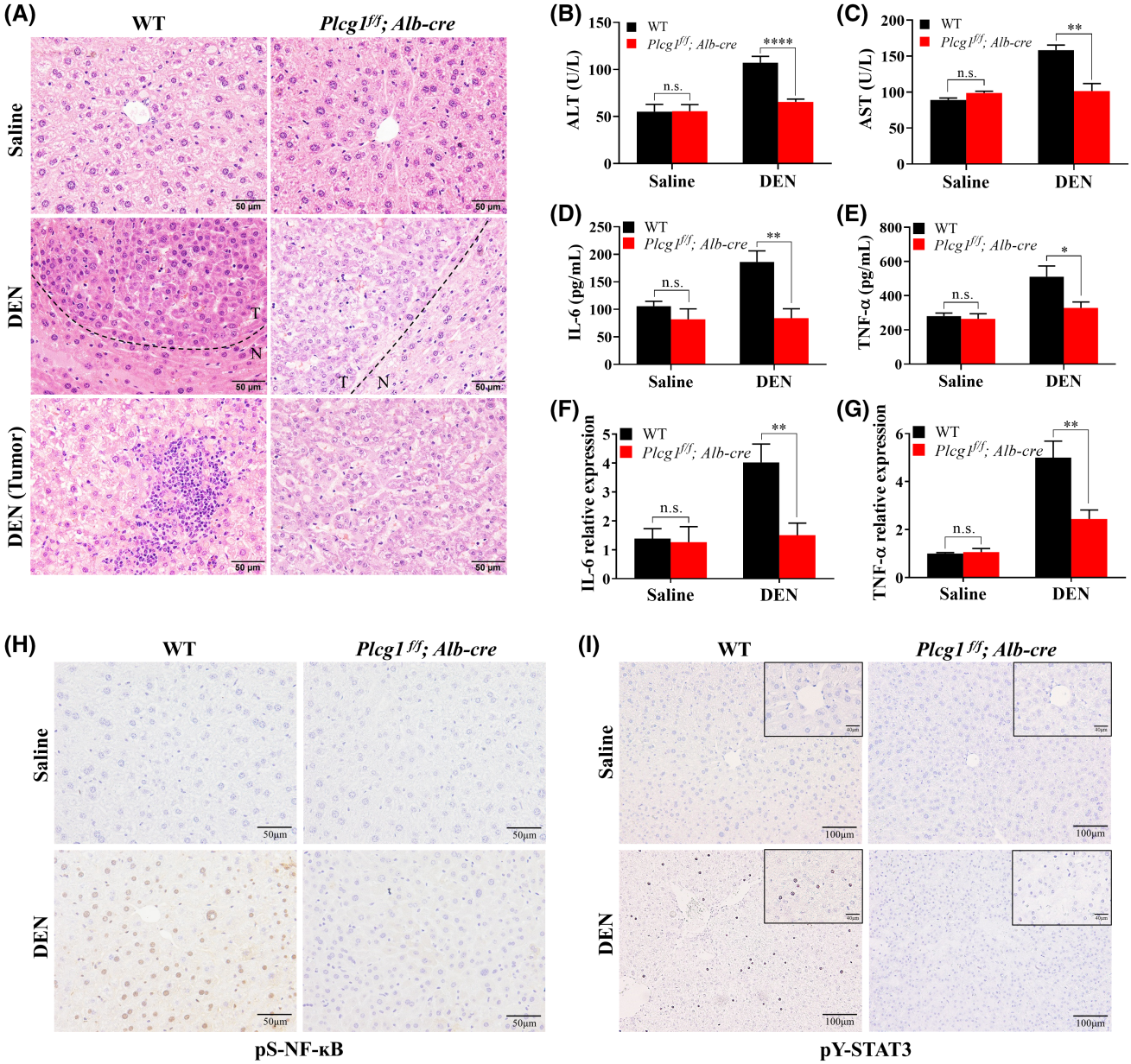

FIGURE 2 PLCγ1 deficiency attenuates DEN-induced inflammatory response and liver injury in mice. (A) Representative hematoxylin and eosin–stained images of liver tissues from mice. Tumor nodules are outlined and labeled. Tumors from WT liver showed infiltrate of inflammatory cells and eosin-positive cytoplasmic inclusions. Serum alanine aminotransferase (ALT) (B) and aspartate aminotransferase (AST) (C) levels were measured in the four groups of mice. Serum interleukin-6 (IL-6) (D) and tumor necrosis factor-α (TNF-α) (E) levels were measured by enzyme-linked immunosorbent assay (ELISA). IL-6 (F) and TNF-α (G) messenger RNA (mRNA) levels in liver extracts were measured by quantitative reverse-transcription polymerase chain reaction (PCR). Representative images of immunohistochemistry for serine 536 phosphorylation of nuclear factor kappa B (NF-κB) (H) and tyrosine 705 phosphorylation of signal transducer and activator of transcription 3 (STAT3) (I) in liver tissues from mice. Brown dots indicate positive staining in nuclei. Values are expressed as mean ± SEM. Statistics were performed using Student's *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Alb-Cre mice (Figure 3H,I). The data indicated that PLCγ1 contributes to liver inflammation and damage.

NF-κB/IL-6/STAT3 axis and HCC cell proliferation are promoted by PLCγ1

Next, we explored the mechanisms underlying the oncogenic role of PLCγ1in HCC. Based on the results of our *in vivo* experiments, we expected that PLCγ1 would regulate the NF-κB/IL-6/STAT3 axis in HCC. Crosstalk between NF-κB and STAT3 has an oncogenic role in some cancer types.[17,18] To investigate the potential oncogenic function and mechanism of PLCγ1 in human HCC, we constructed Hep3B and Huh7 cell lines stably overexpressing PLCγ1 (Figure 3A). To analyze the effects of PLCγ1 deficiency, we used specific siRNA against the PLCγ1

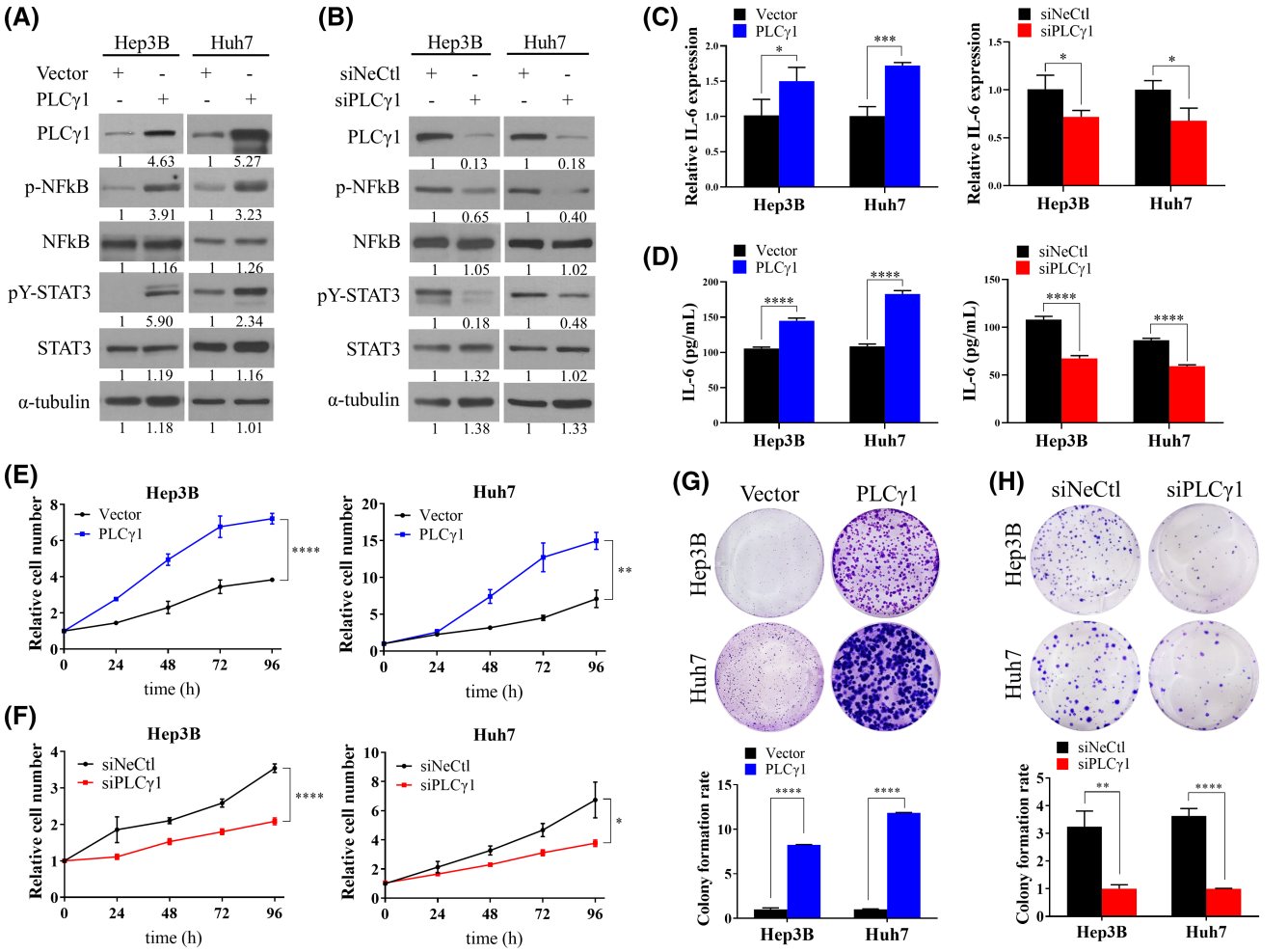

FIGURE 3 NF-κB/IL-6/STAT3 axis and HCC cell proliferation are promoted by PLCγ1. (A) Hep3B and Huh7 cells were transfected with either the empty vector or PLCγ1 expression vector and selected with G418. Overexpression of PLCγ1 and downstream signals were confirmed by western blot analysis. (B) Hep3B and Huh7 cells were either transfected with the negative control (siNeCtl) or PLCγ1 targeting (siPLCγ1) small interfering RNAs. Down-regulation of PLCγ1 and related target protein expressions were confirmed by western blot analysis. (C) IL-6 mRNA levels in cell lysates were quantified by quantitative reverse-transcription polymerase chain reaction (PCR). (D) IL-6 cytokine levels in supernatant culture medium were measured by ELISA. (E,F) Cell proliferation rates of HCC cells with different PLCγ1 levels. (G,H) Representative images of colony formation assays and quantitative analysis of colony-forming ability based on crystal violet staining. All values are expressed as mean ± SD (n = 3). Statistics were performed using Student's *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

gene transcript (Figure 3B). Immunoblotting analysis showed that PLCγ1 activates phosphorylation of both the NF-κB and STAT3 proteins (Figure 3A,B; Figure S5A–F). In addition, both intracellular IL-6 mRNA expression (Figure 3C) and IL-6 cytokine levels released into the supernatant medium were positively regulated by PLCγ1 (Figure 3D). Next, we examined the phenotypes expressed by PLCγ1 in human HCC cell lines. Growth curves demonstrated that up-regulation of PLCγ1 significantly promoted Hep3B and Huh7 cell growth (Figure 3E), but down-regulation of PLCγ1 decreased HCC cell growth compared with the negative control (Figure 3F). In the colony formation assay, PLCγ1 increased the colony-forming ability of HCC cells (Figure 3G), whereas silencing of PLCγ1 led to a decrease in the clonogenic survival of the cells (Figure 3H). These results demonstrated that PLCγ1 affects tumorigenesis by promoting HCC cell proliferation.

PLCγ1 plays a critical role in accelerating cell cycle progression and inhibiting apoptosis

Flow cytometric analyses were performed to determine whether PLCγ1 affects the cell cycle or cell death. Fluorescence-activated cell sorting (*FACS*) analysis showed that PLCγ1 contributes to the cell cycle phase. HCC cells overexpressing PLCγ1 had an increased population of S phase cells and an accompanying decrease in G1 phase cells (Figure 4A). In

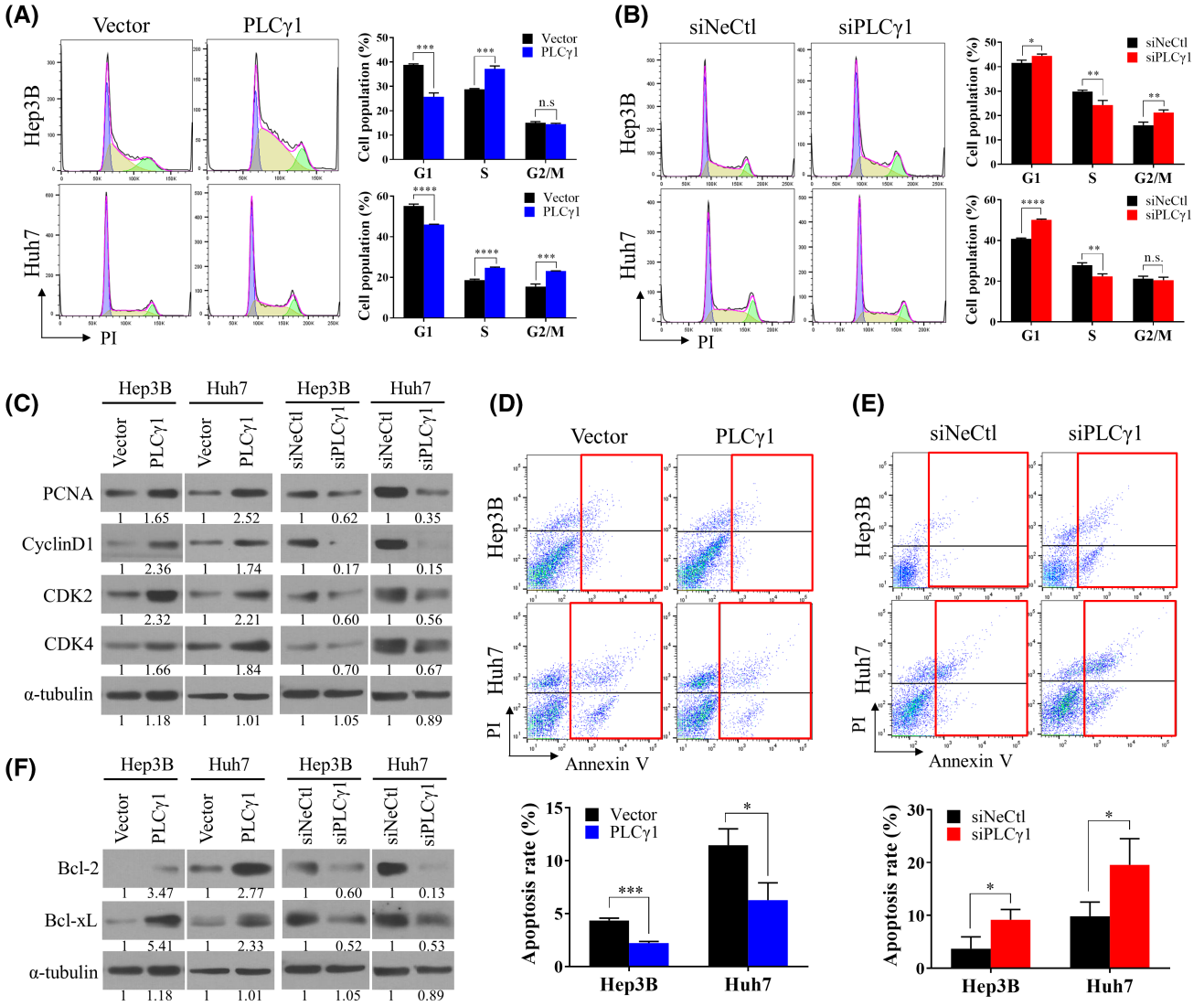

FIGURE 4 PLCγ1 plays a critical role in accelerating cell cycle progression and inhibiting apoptosis. (A,B) Cell cycle analysis in HCC cells and quantitative measurement of cell populations at each cell cycle phase. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. (C) Cell cycle–related protein-expression levels detected by western blot analysis following PLCγ1 levels. (D,E) Apoptosis assay and statistical analysis of apoptosis rates in HCC cell lines with different PLCγ1 levels. Cells were stained with annexin V–fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) and analyzed by flow cytometry. (F) Expression levels of anti-apoptotic proteins detected by western blot analysis following PLCγ1 levels. All values are expressed as mean ± SD (n = 3). Statistics were performed using Student's *t* test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. n.s., not significant.

contrast, PLCγ1 knockdown in HCC cells induced G1 arrest (Figure 4B). Consistent with these observations, PLCγ1 regulated the expression of PCNA, CyclinD1, CDK2, and CDK4, which are cell cycle-related proteins required for the G1 to S-phase transition (Figure 4C; Figure S6A–H). These results indicated that PLCγ1 accelerates cell cycle progression from G1 to the S phase. FACS analysis also showed that PLCγ1 regulates apoptosis. The percentage of apoptotic cells decreased when PLCγ1 was up-regulated (Figure 4D), whereas down-regulation of PLCγ1 induced apoptosis (Figure 4E). In addition, PLCγ1 induced expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL (Figure 4F; Figure S7A–D). Briefly, these results suggest that PLCγ1 promotes tumorigenesis by facilitating cell cycle progression and inhibiting apoptosis in HCC.

Metastatic potential is enhanced by PLCγ1-mediated epithelial–mesenchymal transition

PLCγ1 plays a critical role in promoting metastasis in several cancers as well as regulating tumor growth.[11–13] In our *in vivo* experiments, lung lesion was observed only in DEN-treated male WT mice (Figure S8A–C). In addition, the function of PLCγ1 in HCC for metastasis, a representative phenotype of malignant tumors,

has not been well elucidated. Therefore, we then explored the metastatic activity of PLCγ1 in hepatocytes. To investigate whether PLCγ1 affects tumor metastasis in HCC, we analyzed differences in cell migration and invasion based on the PLCγ1 protein levels. We performed a Transwell assay to assess cell migration, and Matrigel-coated Transwell chambers were used to assess cell invasion. A Transwell assay showed that up-regulation of PLCγ1 markedly increased the migration and invasion of Hep3B and Huh7 cells (Figure 5A). Conversely, PLCγ1 silencing effectively inhibited cell migration and invasion compared with the negative control (Figure 5B). Consistent with the Transwell assay results, a wound-healing assay showed that cell motility was increased in HCC cells overexpressing PLCγ1 (Figure 5C) but decreased by PLCγ1 knockdown (Figure 5D). In line with these results, PLCγ1 regulated the expression of Snail and matrix metalloproteinase 2 (MMP2), which are closely linked to metastasis (Figure 5E; Figure S9A–D). Furthermore, we

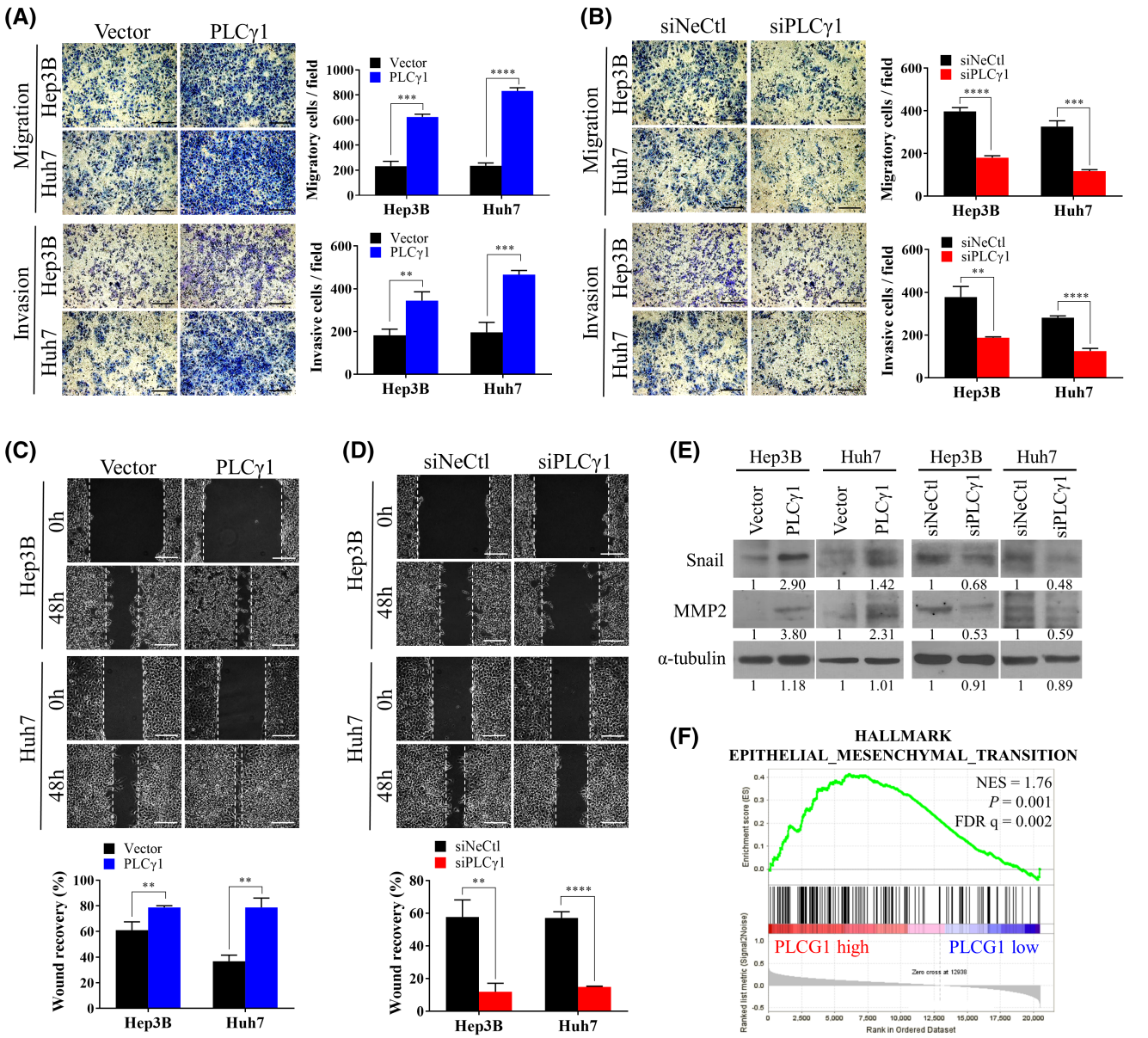

FIGURE 5 Metastatic potential is enhanced by PLCγ1-mediated epithelial–mesenchymal transition (EMT). (A,B) Representative images and statistical plots of Transwell migration assays and Transwell Matrigel-coated invasion assays. Migratory or invasive cells were stained with crystal violet. Scale bar: 200 μm. (C,D) Representative images and statistical plots of wound-healing assays in HCC cells. Scale bar: 200 μm. (E) Protein expression levels of Snail and matrix metalloproteinase 2 (MMP2) following PLCγ1 levels detected by western blot analysis. All values are expressed as mean ± SD (n = 3). Statistics were performed using Student's *t* test. $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. (F) Gene Set Enrichment Analysis (GSEA) enrichment plot of the EMT gene set positively correlated with the PLCG1-high group. Data sets derived from patients with HCC were imported from The Cancer Genome Atlas (TCGA) and divided into two groups according to the levels of PLCG1 mRNA expression. FDR, false discovery rate; NES, normalized enrichment score.

performed a Gene Set Enrichment Analysis (GSEA) on the Cancer Genome Atlas (TCGA) data sets to confirm the association between PLCγ1 and metastasis in HCC. Patients with HCC were sorted into PLCG1-high and PLCG1-low groups based on the median expression levels (Figure S10A). The PLCG1-high group showed positive correlations with the epithelial–mesenchymal transition (EMT)–related gene sets (Figure 5F). The same results were confirmed when analyzing the Genomic Spatial Event (GSE) database (Figure S10B,C). These results demonstrated the crucial role played by PLCγ1 in promoting cell metastasis in HCC.

PLCγ1 is an essential target molecule as a clinical biomarker for HCC

To ascertain the clinical relevance of PLCγ1 in HCC, PLCγ1 expression in human liver samples of patients with HCC was analyzed by immunohistochemistry. Immunostaining analysis demonstrated that the PLCγ1 and active STAT3 levels were significantly elevated in HCC tissues compared with adjacent noncancerous tissues (Figure 6A). To fully explore these results, we compared PLCγ1 expression between normal and HCC samples using the GSE databases. All of the data sets showed that PLCγ1 mRNA levels

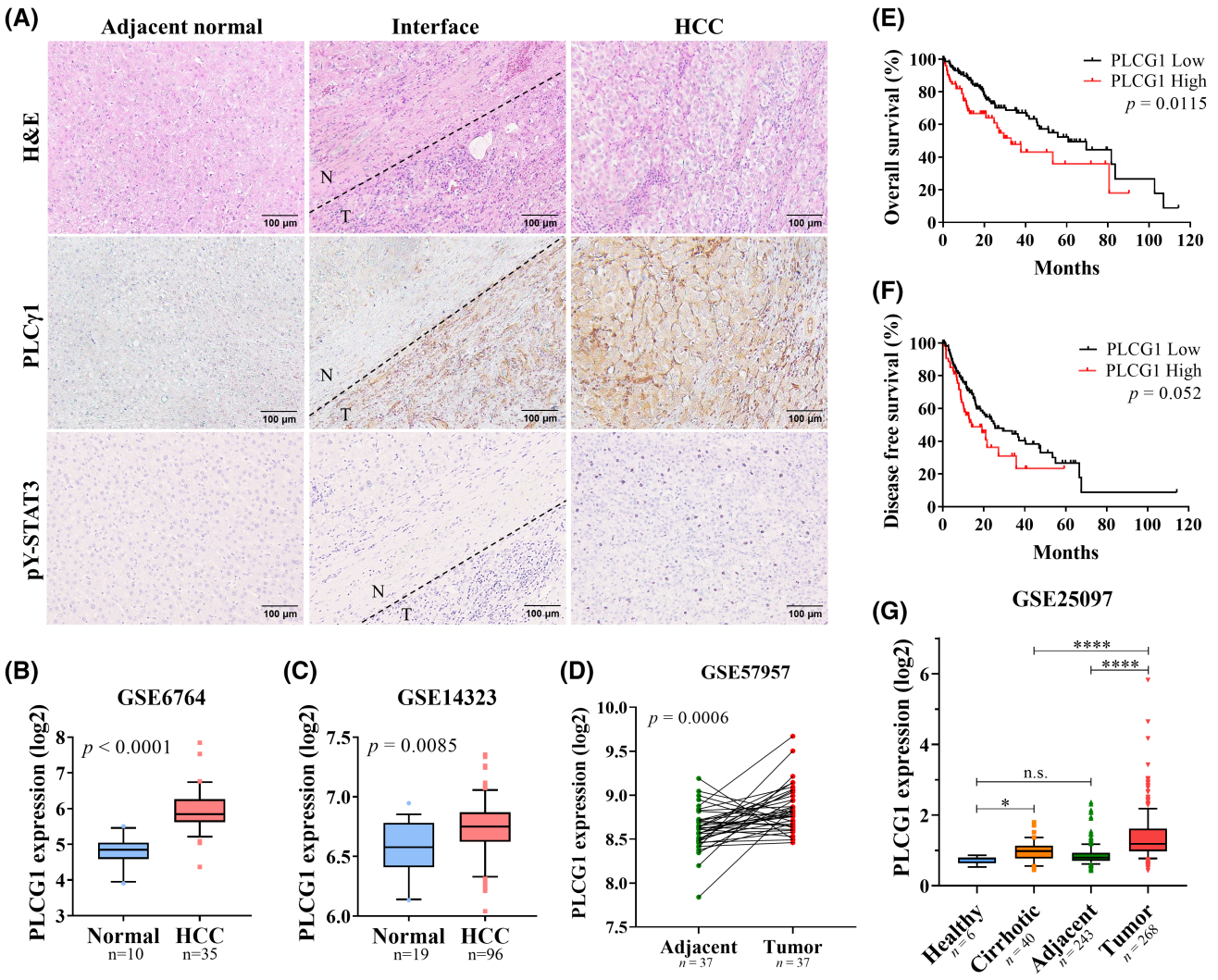

FIGURE 6 PLCγ1 expression is elevated in human HCC and correlates with poor prognosis in patients with HCC. (A) Representative images of hematoxylin and eosin (H&E) staining and immunohistochemistry for PLCγ1 and pY-STAT3 in human liver tissues that contained HCC and adjacent normal tissues. Dashed lines present the border between tumor region and adjacent nontumor. Brown color indicates positive staining. PLCγ1 mRNA levels in normal and HCC liver tissue samples from the GSE6764 (B) and GSE14313 (C) data sets. Horizontal lines represent the median values. Boxes and whiskers represent 25–75 and 10–90 percentiles, respectively. The *p* value was calculated by Mann-Whitney test. (D) PLCγ1 mRNA expression levels in 37 pairs of adjacent nontumor tissues versus tumors in patients with HCC from the GSE57957 data set. The *p* value was calculated by paired two-tailed *t* test. (E) Kaplan-Meier analysis of overall survival (E) and disease-free survival (F) of patients with HCC were performed in PLCG1-high and PLCG1-low groups. The *p* value was calculated by log-lank test. (G) PLCγ1 mRNA expression levels of healthy liver, cirrhosis, HCC, and adjacent nontumor tissues from the GSE25097 data set. The *p* value was calculated by Mann-Whitney test.

were significantly elevated in the HCC samples compared with the healthy subjects (Figure 6B,C). In addition, PLCγ1 expression was significantly increased in tumors compared with adjacent normal tissue pairs in patients with HCC (Figure 6D). Next, to determine whether PLCγ1 levels affected the prognosis of patients with HCC, survival analysis was performed using the TCGA database. Patients with HCC were classified into PLCG1-high and PLCG1-low groups based on the mean PLCG1 expression levels (Figure S10A). Both OS and DFS were lower in the PLCG1-high group compared with the PLCG1-low group (Figure 6E,F). Kaplan–Meier and log-lank analyses indicated that patients with HCC with high PLCγ1 expression had a reduced OS rate and higher recurrence rate relative to patients with low PLCγ1 expression. Moreover, the expression of PLCγ1 showed a tendency to progressively increase in pathological stage (Figure 6G). Overall, these results imply that overexpression of PLCγ1 plays a key role in the development and progression of human HCC.

DISCUSSION

PLCγ1 is oncogenic in some cancer types,[19–21] liver cancer being a major cancer type; however, the understanding of the function of PLCγ1 in liver cancer is very poor. Based on results from other studies, we expected PLCγ1 to function as an oncogene in liver cancer, such that clarification of its role is essential for the diagnosis and treatment of liver cancer. We aimed to understand the pathological role of PLCγ1 in liver cancer, and to determine the phenotype of liver cancer caused by PLCγ1.

In this study, we generated a transgenic mouse model to demonstrate the function of PLCγ1 in HCC. In Alb-Cre mice, PLCγ1 was knocked out specifically in hepatocytes, as PLCγ1 is essential for embryonic development, and examined its role in the liver.[15] Next, we used DEN to induce HCC in rodents.[22,23] DEN treatment mimics spontaneous HCC of human by causing DNA damage, the accumulation of which can stimulate the inflammatory response and hyperproliferation that support tumor development.[24,25] Our results showed that deletion of PLCγ1 led to dramatically attenuated hepatocarcinogenesis, HCC progression, and proliferative responses in tumors.

Inflammation is a key factor in various types of cancer; in particular, it is the main inducer of liver cancer.[26,27] Recent studies reinforced the notion that tumors are promoted by inflammatory signals in the surrounding tumor microenvironment.[28,29] In this study, we demonstrated that ablation of PLCγ1 markedly attenuated the histopathological inflammatory response in liver tumors of mice, and led to a decrease in the production of the inflammatory cytokines IL-6 and TNF-α. These typical liver inflammatory cytokines stimulate inflammation and autoimmune responses in many diseases.[30] Previous studies reported higher IL-6 levels in cases of HCC,[31] and the up-regulation of IL-6 increased inflammation through infiltration of immune cells.[32] Of note, ablation of PLCγ1 also suppressed both NF-κB and STAT3 activation in hepatocytes. NF-κB serves as a pivotal mediator of inflammatory responses by inducing the expression of various pro-inflammatory genes.[33] STAT3, which is frequently activated in malignant cells, regulates many genes important for cancer inflammation in the tumor microenvironment.[34] Furthermore, higher serum ALT and AST levels indicated more severe liver injury in a group of WT mice.[35]

The data presented were collected from male mice due to the estrogen-mediated inhibitory mechanism that occurs in females.[36] Tumors did not develop in females, but PLCγ1 deficiency relieved the formation of dysplastic hepatocytes and hepatic inflammatory responses. As an extension of the *in vitro* experiment, we note that metastasis occurred only in WT mice and not in PLCγ1$^{f/f}$; Alb-Cre mice, although this difference was not statistically significant.

To investigate the function of PLCγ1 fully *in vitro*, we used stable cells and silencing systems in human HCC cell lines. Based on *in vivo* experimental results, we explored proliferation, cell cycle, and apoptosis in HCC cell lines. Our results showed that PLCγ1 promotes HCC cell growth and cell cycle progression, but prevents apoptosis. In metastasis, PLCγ1 mediated the migration and invasion of HCC cells. GSEA analysis of the gene set associated with PLCγ1 showed that PLCγ1 positively correlated with genes associated with the EMT. Our data also confirmed that PLCγ1 regulates the expression of the signaling proteins associated with these phenotypes.

STAT3 is a key molecule transducing signals from a variety of factors, including IL-6.[37] It is reported that IL-6 acts as an autocrine growth factor in human prostate cancer.[38] Several studies have demonstrated that abnormal IL-6/STAT3 signaling promotes HCC progression.[39] In our previous study, it was reported that IL-6/STAT3 signaling induces cancer stemness through up-regulation of CD133 expression during liver carcinogenesis.[40] We confirmed in this study that PLCγ1-mediated STAT3 activation is involved in metastatic potential of HCC cells. Furthermore, IL-6 is an NF-κB-dependent tumor growth factor,[41] and its production is positively regulated by NF-κB activation.[42] In addition, NF-κB is activated by PKC downstream of PLCγ1.[43,44] Based on these research backgrounds, we hypothesized that PLCγ1 could regulate the activation of STAT3 via the NF-κB/IL-6/STAT3 axis. A recent study reported crosstalk between STAT3 and PLCγ1 in colorectal cancer.[45] However, studies on whether PLCγ1 can regulate the activity of STAT3 are lacking, and the relationship

between PLCγ1 and STAT3 associated with liver cancer is still unknown. In the present study, serum IL-6 and IL-6 mRNA levels in liver extracts were decreased in PLCγ1$^{f/f}$; Alb-Cre mice, in which NF-κB and STAT3 activation were also inhibited in hepatocytes. In our *in vitro* study, PLCγ1 induced phosphorylation of NF-κB and STAT3 in human HCC cell lines. In addition, both intracellular Il-6 expression and extracellularly secreted Il-6 cytokine levels were positively regulated by PLCγ1. Moreover, proteins that underwent phenotype testing, such as cyclin D1, Bcl-2, Bcl-xL, Snail and MMP2, were STAT3 downstream target genes. Thus, we suggest that PLCγ1 regulates the activation of STAT3 as a possible mechanism promoting HCC progression. Further studies are needed to determine the detailed mechanisms of PLCγ1 signaling in HCC and to understand the regulation of the pathway.

Additionally, a clinical approach using human liver tissue verified that PLCγ1 and activated STAT3 were highly expressed in liver cancer cells compared with adjacent normal liver cells. We confirmed the clinical relevance of this through bioinformatics analysis using the TCGA database. The expression of PLCγ1 was higher in patients with HCC compared to the normal group, indicating that PLCγ1 is associated with the development of HCC. The study of patients with HCC showed that both OS and DFS were poor in the PLCγ1-high expression group, suggesting that PLCγ1 is also associated with the progression and relapse of HCC. This finding supports the results observed in the current study.

CONCLUSIONS

In this study, we suggested that PLCγ1 is a key molecule in hepatocellular carcinogenesis and progression. Loss of PLCγ1 efficiently attenuated the tumor growth and inflammatory responses in the tumor microenvironment. Moreover, PLCγ1 induced representative oncogenic phenotypes and expression of corresponding factors in human HCC cells. The PLCγ1/STAT3 axis is a potential therapeutic target pathway with oncogenic function in HCC. Thus, our findings could inform strategies for the treatment and prognostic diagnosis of HCC.

AUTHOR CONTRIBUTIONS

Study design and experiments: Eun-Bi Seo. *Discussion of the results*: Sun-Ho Kwon and Yong-Jin Kwon. *Data collection*: Seul-Ki Kim, Song-Hee Lee, and Ae Jin Jeong. *Data analysis*: Hyun Mu Shin, Yong-Nyun Kim, and Stephanie Ma. *Generation of mice*: Hyun-Jun Jang and Pann-Ghill Suh. *Clinical resources*: Haeryoung Kim *Study concept*: Sang-Kyu Ye. *Data interpretation*: Yun-Han Lee. *Manuscript draft*: Eun-Bi Seo. *Manuscript review*: Sang-Kyu Ye. All authors had final approval of the submitted and published versions.

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CONFLICT OF INTEREST

Nothing to report.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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