


# Phospholipase D2 promotes disease progression of renal cell carcinoma through the induction of angiogenin

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A hallmark of clear cell renal cell carcinoma (ccRCC) is the presence of intracellular lipid droplets (LD) and it is assumed that phosphatidic acid (PA) produced by phospholipase D (PLD) plays some role in the LD formation. However, little is known about the significance of PLD in ccRCC. In this study, we examined the expression levels of PLD in ccRCC. The classical mammalian isoforms of PLD are PLD1 and PLD2, and the levels of both mRNA were higher at the primary tumor sites than in normal kidney tissues. Similarly, both PLD were significantly abundant in tumor cells as determined by analysis using immunohistochemical staining. Importantly, a higher level of PLD was significantly associated with a higher tumor stage and grade. Because PLD2 knockdown effectively suppressed the cell proliferation and invasion of ccRCC as compared with PLD1 in vitro, we examined the effect of PLD2 in vivo. Notably, shRNA-mediated knockdown of PLD2 suppressed the growth and invasion of tumors in nude mouse xenograft models. Moreover, the higher expression of PLD2 was significantly associated with poorer prognosis in 67 patients. As for genes relating to the tumor invasion of PLD2, we found that angiogenin (ANG) was positively regulated by PLD2. In fact, the expression levels of ANG were elevated in tumor tissues as compared with normal kidney and the inhibition of ANG activity with a neutralizing antibody significantly suppressed tumor invasion. Overall, we revealed for the first time that PLD2-produced PA promoted cell invasion through the expression of ANG in ccRCC cells.

## KEYWORDS

angiogenin, cancer progression, phosphatidic acid, phospholipase D2, renal cell carcinoma

## 1 | INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 3% of all adult malignancies and there has been a continuous increase of new cases during the most recent 10-year period.<sup>1,2</sup> Approximately one-quarter of patients with RCC present with locally advanced or metastatic disease at diagnosis, and approximately 20%-40% of

those with confined primary tumors will develop metastatic disease.<sup>3</sup> Although new target therapies and immunotherapies have emerged,<sup>1</sup> their efficacy is not sufficient to overcome advanced RCC.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid (PA) and choline. The classical mammalian isoforms of PLD are PLD1 and PLD2.<sup>4,5</sup> As a lipid second messenger, PA produced by PLD plays roles in numerous essential

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cellular functions, such as vesicular trafficking, exocytosis, autophagy and the regulation of cellular metabolism.<sup>6</sup> A hallmark of clear cell RCC (ccRCC), the most common histological type of RCC, is the presence of intracellular lipid droplets (LD), which consist of a neutral lipid core containing triglycerides and cholesterol-esters surrounded by a phospholipid monolayer.<sup>7</sup> Although it has been reported that PA produced by PLD promotes the formation of intracellular LD,<sup>8-10</sup> Wettersten et al<sup>11</sup> showed that LD was lost in the high grade Fuhrman 4 or spindle cell type. Interestingly, a previous report showed that the expression and the activity of PLD2 was elevated in ccRCC;<sup>12</sup> however, little is known about the roles of PLD in the tumor progression of ccRCC.

We previously reported the significant roles of PLD1 in the tumor microenvironment of a mouse melanoma lung metastasis model, showing that the administration of the PLD inhibitor led to a significant reduction of tumor angiogenesis and metastases.<sup>13</sup> Importantly, the elevated expression and the activity of PLD were associated with cancer proliferation,<sup>14</sup> survival,<sup>15</sup> invasion and metastasis<sup>15-18</sup> in a series of human cancers.<sup>19</sup>

Based on the above findings, we have examined the roles of PLD in the tumor progression of ccRCC. In this report, it was revealed that PLD2 promoted tumor growth and the invasion of ccRCC cells, and the level of PLD2 in a tumor specimen was significantly correlated with the prognoses of patients. Moreover, we clarified a new mechanism of PLD2-mediated cell invasion through the induction of angiogenin (ANG). Our results suggest that PLD2 might become a new therapeutic target in the treatment of advanced RCC.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and clear cell renal cell carcinoma samples

Samples of primary sites from 67 patients who received radical or partial nephrectomy were obtained at Tsukuba University Hospital between 2006 and 2015 under the protocols approved by the Ethics Committee of Tsukuba University. These samples were used as formalin-fixed and paraffin-embedded (FFPE) tissue sections of the primary sites. In 52 of 67 patients, frozen tissue samples of the cancer and adjacent normal region were available for the primary sites. These samples were used for extracting RNA. Among 67 patients, 16 underwent metastasectomy or biopsy of the metastatic site. These samples were used for FFPE tissue sections of the metastatic sites.

The patient characteristics of tumor stages were assigned according to the TNM staging of the Union for International Cancer Control.<sup>20</sup> Pathological grades were classified according to the 4-tiered Fuhrman grading system.<sup>21</sup> The characteristics of the patients are summarized in Table S1.

### 2.2 | Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were cut into 4- $\mu$ m-thick sections. The sections were deparaffinized and

rehydrated. For antigen retrieval, the sections were pretreated by microwave for 21 minutes in a citric acid buffer. After the antigen retrieval procedure, endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> for 25 minutes, and the slides were incubated with the primary antibody at 4°C overnight. The immunohistochemical reaction was visualized using the secondary antibody Histofine Simple Stain MAX PO (Nichirei Bioscience, Tokyo, Japan) with diaminobenzidine as the chromogen. The primary antibodies were listed in Table S2. To evaluate the protein expression of PLD1 and PLD2, the staining intensities of tumor cells were stratified into high and low subgroups. When tumor cells were strongly stained in not only cell membrane but also cytoplasm, we defined this as high expression. The other cases were defined as low expression.

### 2.3 | Cell culture

293T was purchased from RIKEN BioResource Center (Ibaraki, Japan). SKRC52 and SKRC59 cells were kindly gifted by Dr JG Old (Memorial Sloan Kettering Cancer Center, NY, USA). These cell lines were cultured in RPMI 1640 medium or DMEM supplemented with 10% FBS at 37°C and a 5% humidified CO<sub>2</sub> atmosphere.

### 2.4 | Quantitative RT-PCR

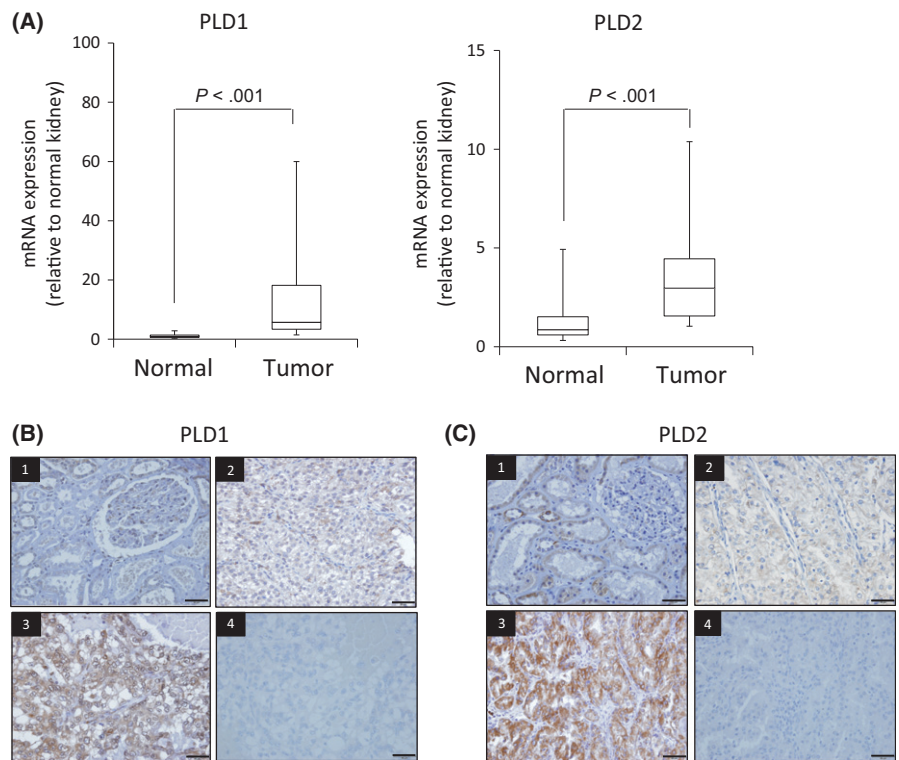
Gene expression levels were quantitatively measured using a 7500 Fast Real-Time PCR machine with Fast SYBR Green Master Mix (Applied Biosystems). Hypoxanthine phosphoribosyltransferase 1 (*HPRT*) was used as an internal control. The primer sequences were listed in Table S3.

### 2.5 | Western blot analysis

Western blot analysis was carried out as described previously.<sup>22</sup> The primary antibody concentrations used for western blot analysis are listed in Table S4. As a secondary antibody, anti-rabbit or anti-mouse immunoglobulin G, HRP-linked whole donkey Ab (GE Healthcare), was used at 1:10 000. Western blots were visualized with ImmunoStar Zeta (Wako, Tokyo, Japan) using a Fujifilm LAS-4000 imager (Fujifilm, Tokyo, Japan).  $\beta$ -actin was used as an internal control.

### 2.6 | siRNA-mediated expression knockdown experiments

SKRC52 and SKRC59 cells were transfected with 25 nmol/L of ON-TARGETplus human PLD1 SMART pool siRNA (L-009413-00-0005, Dharmacon, Thermo Fisher Scientific, Rockford, IL, USA), 25 nmol/L of ON-TARGETplus human PLD2 SMART pool siRNA (L-005064-00-0005) or 25 nmol/L of ON-TARGETplus non-targeting pool siRNA (D-001810-10-05) using Lipofectamine RNAiMax Reagent (Invitrogen). Pooled siRNA containing 4 different target sequences was used to eliminate the possibility of off-target effects.



**FIGURE 1** The abundance of PLD1 and PLD2 in corresponding normal and tumor tissues. A, The level of PLD1 or PLD2 mRNA expression in clinical specimens. *P*-value was assessed by the Wilcoxon rank sum test. B,C, Representative images of immunohistochemical staining of PLD1 (B) or PLD2 (C). (1) Normal tissue. (2) Primary tumor exhibiting low staining intensity. (3) Primary tumor exhibiting high staining intensity. (4) Negative control of (3) stained with a control antibody (rabbit IgG). Scale bar, 50  $\mu$ m

## 2.7 | Construction of plasmids

The shRNA for PLD2 characterized in previous reports<sup>23,24</sup> were subcloned into the lentiviral vector pLKO.1. The oligonucleotide sequences used in the construction of the shRNA vector are listed in Table S5.

## 2.8 | Lentivirus production and transduction

Lentiviruses were generated in 293T cells by cotransfecting 4 plasmids including the lentiviral vector (pLKO-shControl or pLKO-shPLD2), pMDLg/pRRE, pRSV-Rev and pMD2.G using Lipofectamine 2000 Reagent (Invitrogen). At 48 hours post-transfection, virus-containing supernatants were collected for infection. For viral transductions, the pLKO-shControl or pLKO-shPLD2 lentiviruses were incubated with SKRC52 and SKRC59 cells overnight at 37°C in a humidified cell culture incubator. Twenty-four hours post-infection, cells were selected in the presence of 1.5  $\mu$ g/mL puromycin.

## 2.9 | Cell proliferation and invasion assays

Cell proliferation was assessed by MTT assay using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates, and 10  $\mu$ L of WST-8 was added after 24, 48, 72 and 120 hours. The OD were measured after 1-hour incubation. Cell invasion was assayed using a BioCoat Matrigel invasion chamber (#354480, Corning, Corning, NY, USA) according to the manufacturer's protocol. A cell suspension of

$5 \times 10^5$  in 0.5 mL medium was added into each well of the upper chamber. As a chemoattractant, 3 nmol/L EGF was used. After 22 hours, the non-invading cells that remained on the upper surface of the membrane were removed by scraping. The number of invasive cells was then counted under a microscope. The assays were performed in triplicate in at least 2 independent experiments, and 5 fields were counted per transwell filter.

## 2.10 | Reagents

Neutralizing antibodies against human ANG were purchased from R&D Systems (AB-265-NA). The neutralizing antibody or control

**TABLE 1** Correlation of PLD1 protein expression and clinicopathological factors

	High PLD1 (n = 18) n (%)	Low PLD1 (n = 49) n (%)	<i>P</i> -value
Age: mean $\pm$ SD	67.4 $\pm$ 12.4	66.0 $\pm$ 10.8	.5474
Clinical stage			
1-2	4 (22.2)	29 (59.2)	.0121*
3-4	14 (77.8)	20 (40.8)	
Pathological stage			
T1-2	4 (22.2)	32 (65.3)	.0023*
T3-4	14 (77.8)	17 (34.7)	
Grade			
G1-2	3 (16.7)	35 (71.4)	.0001*
G3-4	15 (83.3)	14 (28.6)	

\*Statistically significant difference (*P* < .05).

goat IgG was added into each well of the upper chamber for cell invasion assays. The dose of the neutralizing antibody for use was determined based on the results of the cell invasion assays at each dose (Figure S1).

The recombinant human ANG was purchased from R&D Systems (AB-265-AN-050). The recombinant was diluted at 10 µg/mL in 0.1% BSA. The concentration of use was determined at 0.5 µg/mL as described previously.<sup>26</sup> The recombinant or control (0.1% BSA) were added into each well of the upper chamber for cell invasion assays.

### 2.11 | Lipid preparation

The phospholipid 1,2-dioleoyl phosphatidic acid (DOPA) was purchased from Avanti Polar Lipids (840875P, Alabaster, AL, USA). Lipid preparation was carried out as described previously.<sup>27</sup> Briefly, DOPA was prepared from powder in PBS with 0.5% BSA for a final concentration of 1 mmol/L. This solution was sonicated and extruded. Lipids were added to the cells for a final concentration of 300 nmol/L for 4 hours. Previous reports have shown that this form of PA is cell soluble.<sup>27</sup> Post-treatment cells were harvested and subjected to quantitative RT-PCR (qRT-PCR).

### 2.12 | Node mouse xenograft assays

Female 6 to 8-week-old Balb/c nude (nu/nu) mice were purchased from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan). For subcutaneous xenograft assays,  $5 \times 10^6$  cells were injected subcutaneously into the right flanks, and the tumor volumes were measured once a week. After 10 weeks, the animals were killed and the xenograft tumors were excised. Orthotopic xenograft was performed as described previously.<sup>28</sup> After the kidney was exposed,  $1 \times 10^6$  cells were injected into the renal parenchyma. The mice were killed at 12 weeks after injection. The presence of a tumor mass was finally confirmed by H&E staining. All animal studies were approved by the Animal Experiment Committee of the University of Tsukuba, and all the experiments were performed in accordance with the guidelines of the University of Tsukuba's Regulations of Animal Experiments.

### 2.13 | Statistical analysis

Data are expressed as means  $\pm$  SD. All statistical analysis was performed using JMP 10 software (SAS Institute, Cary, NC, USA). The significance of the differences between the 2 groups was assessed by Student's *t* test, Fisher's exact test and the Wilcoxon rank sum test. Survival curves were constructed using the Kaplan–Meier method, and the difference between the curves was evaluated using the log-rank test. To identify the prognostic factors for overall survival (OS), PLD2 expression and clinicopathological variables were evaluated by Cox's proportional hazard regression model. *P*-values  $< .05$  were considered statistically significant.

Additional materials and methods are detailed in Data S1.

## 3 | RESULTS

### 3.1 | Expression levels of PLD1 and PLD2 are associated with tumor progression of clear cell renal cell carcinoma

To clarify the roles of PLD1 and PLD2 in the tumor progression of ccRCC, we first examined the abundance of both phospholipases in corresponding normal and tumor tissues in all 52 cases. It was revealed that both PLD1 and PLD2 mRNA were significantly more highly expressed in tumor tissues than normal tissues (Figure 1A). In tumor specimens, both were positively stained mainly in the cytoplasm of ccRCC cells. In normal specimens, heterogeneous staining of PLD1 and PLD2 were observed in renal tubules (Figure 1B,C). The staining intensities of tumor cells were subsequently stratified into high and low subgroups, and the association between the expression of PLD proteins and clinicopathological factors was examined. It was revealed that higher PLD1 expression was significantly associated with worse clinical stage as well as higher tumor stage and Fuhrman grade (Table 1). Similarly, high PLD2 expression was significantly correlated with worse clinical stage and higher tumor grade (Table 2). Collectively, these results indicated that higher expression of PLD was related to the disease progression of ccRCC.

### 3.2 | Inhibition of PLD2 effectively suppressed cell proliferation and tumor invasion of clear cell renal cell carcinoma in vitro

To clarify the roles of PLD in the disease progression of ccRCC, we performed siRNA knockdown of PLD1 or PLD2 in 2 different *VHL*<sup>-/-</sup> ccRCC cell lines, SKRC52 and SKRC59, respectively. The effects of each siRNA were evaluated by western blot analysis, and the specific inhibition was confirmed (Figure 2A). Then, we performed MTT assays to examine the effect of the siRNA knockdown of each protein on the cell proliferation. As shown in Figure 2B, the knockdown of PLD2

**TABLE 2** Correlation of PLD2 protein expression and clinicopathological factors

	High PLD2 (n = 20) n (%)	Low PLD2 (n = 47) n (%)	<i>P</i> -value
Age: mean $\pm$ SD	68.7 $\pm$ 11.0	65.4 $\pm$ 11.2	.3511
Clinical stage			
1-2	4 (20.0)	29 (61.7)	.0029*
3-4	16 (80.0)	18 (38.3)	
Pathological stage			
T1-2	6 (30.0)	30 (63.8)	.0159*
T3-4	14 (70.0)	17 (36.2)	
Grade			
G1-2	7 (35.0)	31 (66.0)	.0304*
G3-4	13 (65.0)	16 (34.0)	

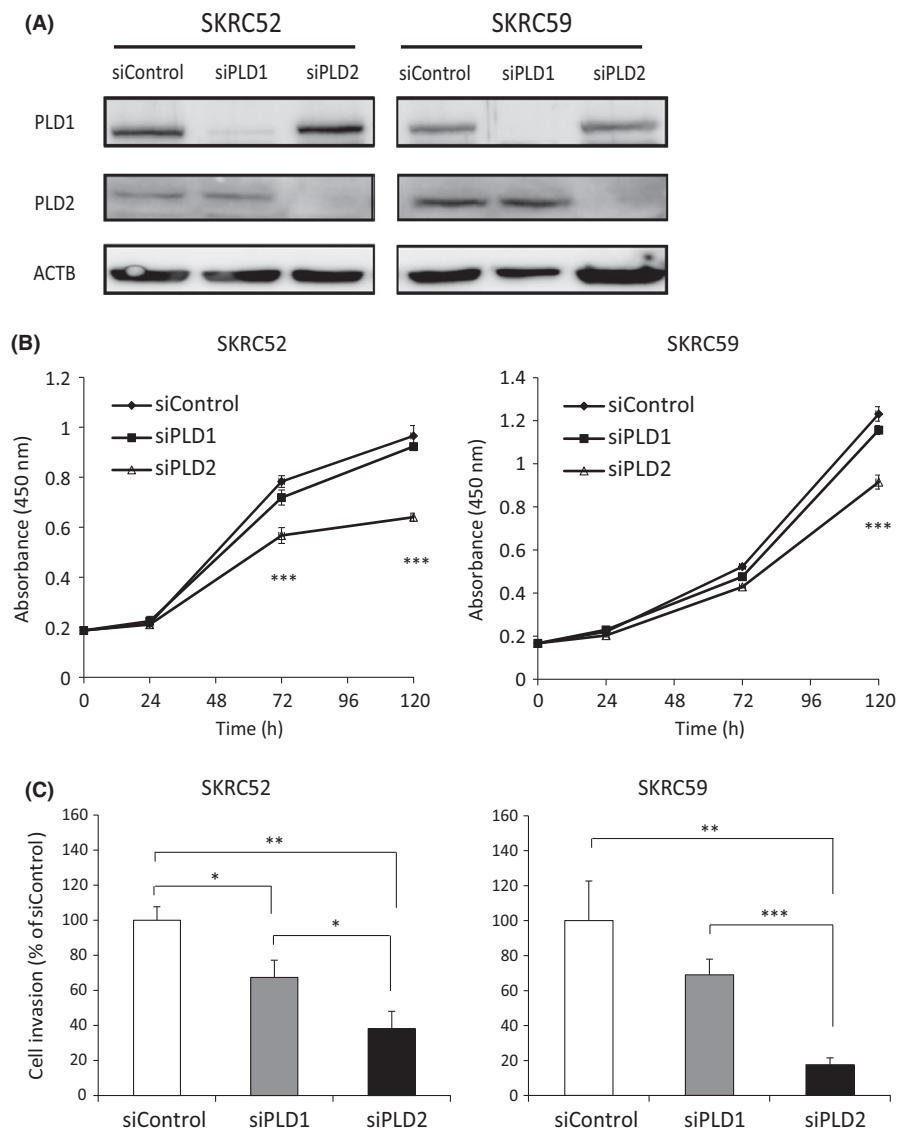
\*Statistically significant difference (*P* < .05).

significantly inhibited the cell proliferation of both cells compared to control siRNA. In contrast, no significant effect was observed in the cell proliferation of cells treated with siRNA against PLD1.

We also evaluated the effect of both proteins on the cell migration by using a Matrigel invasion assay (Figure 2C). It was revealed that knockdown of PLD2 significantly suppressed cell invasion in both cells. Notably, PLD2 knockdown more effectively suppressed tumor invasion in both cells compared with PLD1 knockdown. Then, we examined the effect of 2 different PLD inhibitors, FIPI and NFOT, on the cell proliferation and invasion of ccRCC cells. Both inhibitors possessed anti-cancer effects for breast cancer cells in recent studies.<sup>14,17</sup> FIPI acted as a dual PLD inhibitor and NFOT exhibited a specific inhibitory effect only for PLD2. Both inhibitors significantly suppressed cell proliferation and invasion compared with the control (Figures S2,S3). NFOT, the PLD2-specific inhibitor, suppressed cell invasion effectively as compared with FIPI. These results further supported the findings that PLD2 mainly promotes cell proliferation and invasion in renal cancer cells.

### 3.3 | Knockdown of PLD2 in clear cell renal cell carcinoma cells suppresses tumor growth and invasion in vivo

Next, we examined the roles of PLD2 in the tumor progression of ccRCC in vivo. For this purpose, SKRC52 cells with stably knocked-down PLD2 were established using 2 different shRNA (#1 and #2) and both successfully reduced the level of PLD2 without affecting that of PLD1 (Figure 3A). Importantly, the shRNA-mediated knocking down of PLD2 suppressed the tumor growth when the cells were implanted subcutaneously (Figure 3B). We also examined the Ki-67 index in xenograft tumors infected with scramble or PLD2 shRNA, and it was revealed that SKRC52/PLD2 shRNA cells exhibited a significantly lower Ki-67 index than did SKRC52/scramble cells (Figure 3C). These results further supported the possibility that PLD2 augmented the tumor growth in vivo. Then, we performed orthotopic xenograft assays to examine whether PLD2 also regulates the invasive ability of SKRC52 cells in vivo. Pathological examination of



**FIGURE 2** The effects of the siRNA knockdown of phospholipase D (PLD) on cell growth and invasion in clear cell renal cell carcinoma (ccRCC) cell lines. A, Western blot analysis of SKRC52 and SKRC59 transfected with control, PLD1 or PLD2 small interfering RNA. Whole-cell extracts were analyzed by immunoblotting using the indicated antibodies. B, Analysis of cell proliferation by MTT assay after transfection with siRNA. C, Analysis of cell invasion ability using a Matrigel invasion assay after transfection with siRNA. *P*-value was assessed by Student's *t* test (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001)

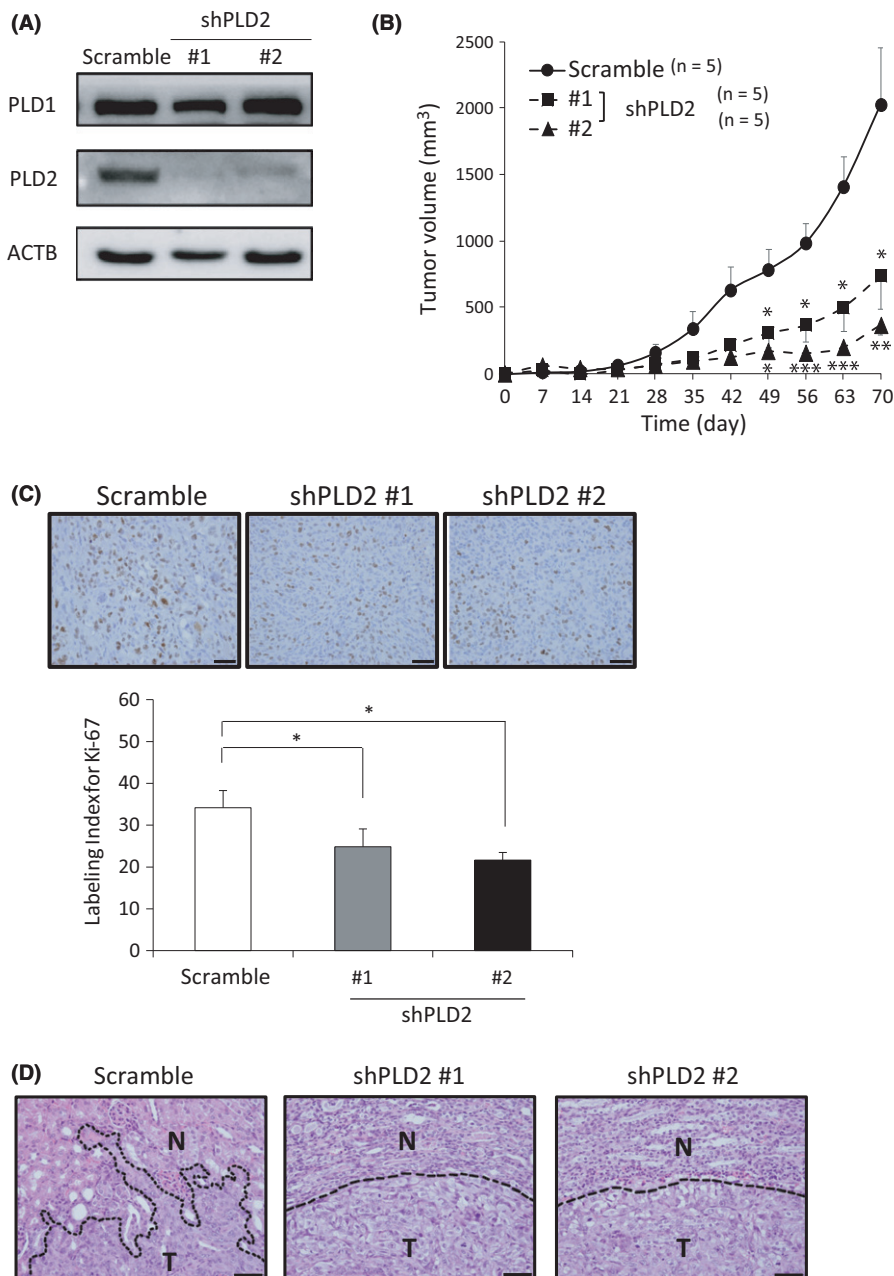


tumors implanted in an orthotopic site revealed that SKRC52/PLD2 shRNA cells hardly invaded into normal tissue, although SKRC52/scramble cells extensively infiltrated into the parenchyma of the normal kidney (Figure 3D). These results indicate that PLD2 augmented the cell invasion ability in those cells *in vivo*.

### 3.4 | Elevated expression of PLD2 is associated with poor prognosis in clear cell renal cell carcinoma

Based on the above results, we examined the association between the expression of PLD2 and the prognosis of patients to elucidate the clinical impact of this protein in ccRCC. A Kaplan-Meier analysis revealed that the high expression of PLD2 was significantly associated with a poor patient prognosis ( $P = .002$ , Figure 4A). We

also examined whether PLD2 expression would be an independent RCC prognostic factor. We employed univariate and multivariate Cox regression analyses with clinical stage, grade, microvascular invasion and PLD2 expression. Both univariate and multivariate analyses revealed that PLD2 expression was an independent prognostic marker for RCC patient OS (Table 3). Because PLD2 promoted the tumor invasion of ccRCC in mice xenograft models, we also examined the abundance of this protein in tumor cells invading microvessels. Intriguingly, abundant expression of PLD2 was observed in the site of microvascular invasion in 8 out of 10 cases (Figure 4B). Moreover, the protein expression levels of PLD2 in the metastatic sites were significantly elevated compared with the primary sites ( $P < .01$ , Figure 4C,D). Collectively, these results strongly suggested that PLD2 significantly affected the prognosis of patients



**FIGURE 3** shRNA-mediated PLD2 knockdown suppresses tumor growth and invasion in clear cell renal cell carcinoma (ccRCC) *in vivo*. A, Western analysis of PLD1 and PLD2 in SKRC52 infected with scramble or PLD2 shRNA (#1, #2). B, The sequential changes of subcutaneous xenograft tumors from SKRC52 subclones infected with scramble or PLD2 shRNA. *P*-value was assessed by Wilcoxon rank sum test (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ). The error bars represent the SE. C, Ki-67 staining of SKRC52 xenograft tumors developed from subclones infected with scramble or PLD2 shRNA. The labeling index for Ki-67 in xenograft tumors developed from SKRC52 subclones is also shown. *P*-value was assessed by the Wilcoxon rank sum test (\* $P < .05$ ). Scale bar 50  $\mu$ m. D, Orthotopic implantation of SKRC52 cells infected with scramble or PLD2 shRNA for the evaluation of invasive ability *in vivo*. Margins of tumors are indicated by a dotted line. N, normal kidney tissue; T, tumor. Scale bar, 50  $\mu$ m

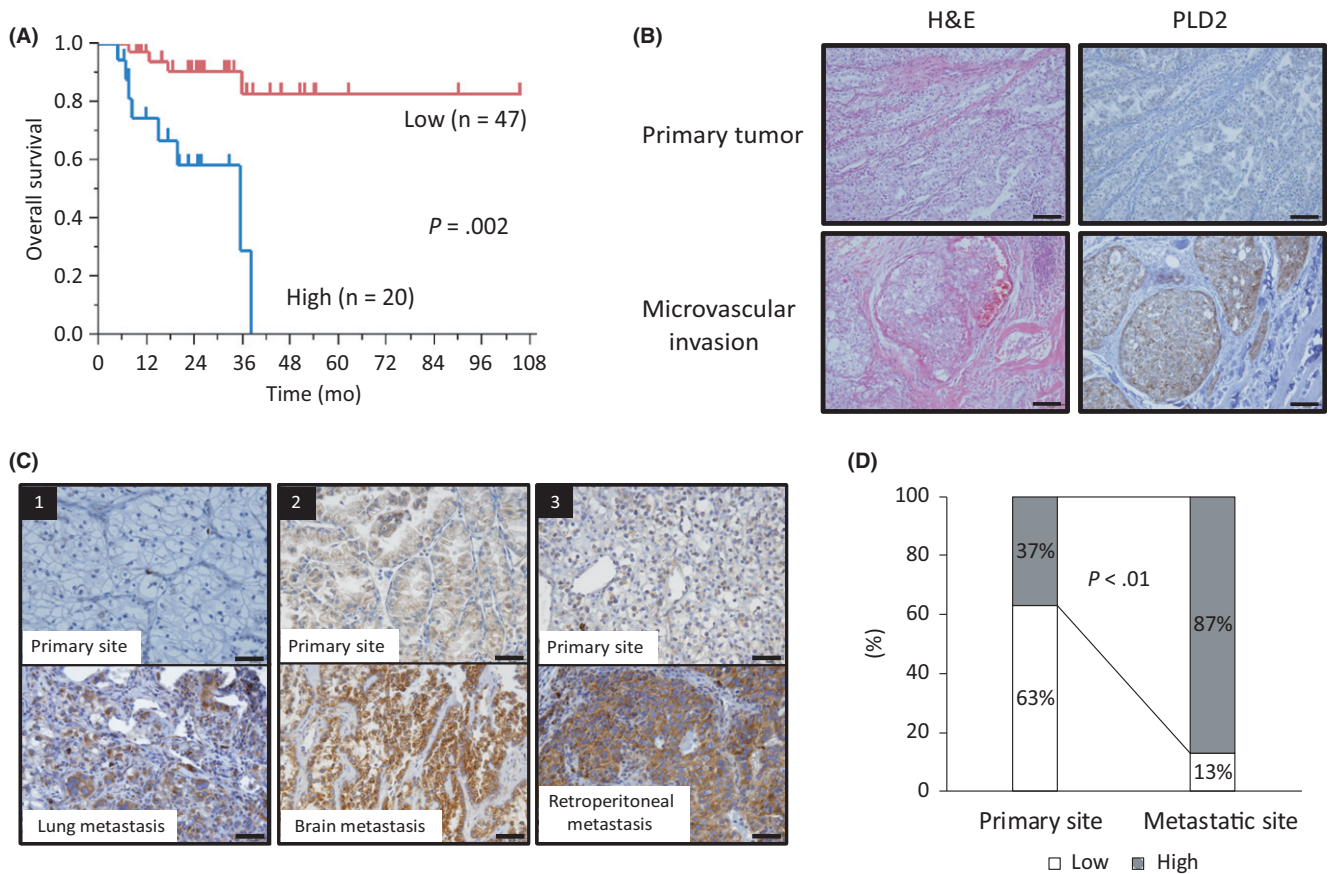
through the regulation of the invasion ability and tumor metastasis of ccRCC.

### 3.5 | Angiogenin regulated by lipase activity of PLD2 promotes cell invasion in renal cancer cells

Finally, we examined the mechanisms of PLD2 in promoting the tumor invasion and metastasis of ccRCC. Toward this end, we compared the expression profile of SKRC52/scramble and SKRC52/PLD2 shRNA cells (#1 and #2) using a qRT-PCR array. Among 168

genes involved in angiogenesis and tumor invasion, we identified 12 genes whose expression was altered at least 1.5-fold by both shRNA (Table S6). Among them, ANG was identified as a gene downregulated by 2 different PLD2 shRNA. This result was confirmed by qRT-PCR analysis using another set of primers and the cell line in SKRC59 (Figure 5A). Importantly, the mRNA levels of ANG were elevated in ccRCC as compared with normal kidney (Figure 5B).

To clarify the significance of ANG in the invasion ability and tumor metastasis of ccRCC, we examined the effects of this protein in cell invasion by using a Matrigel invasion assay. Inhibiting ANG activity

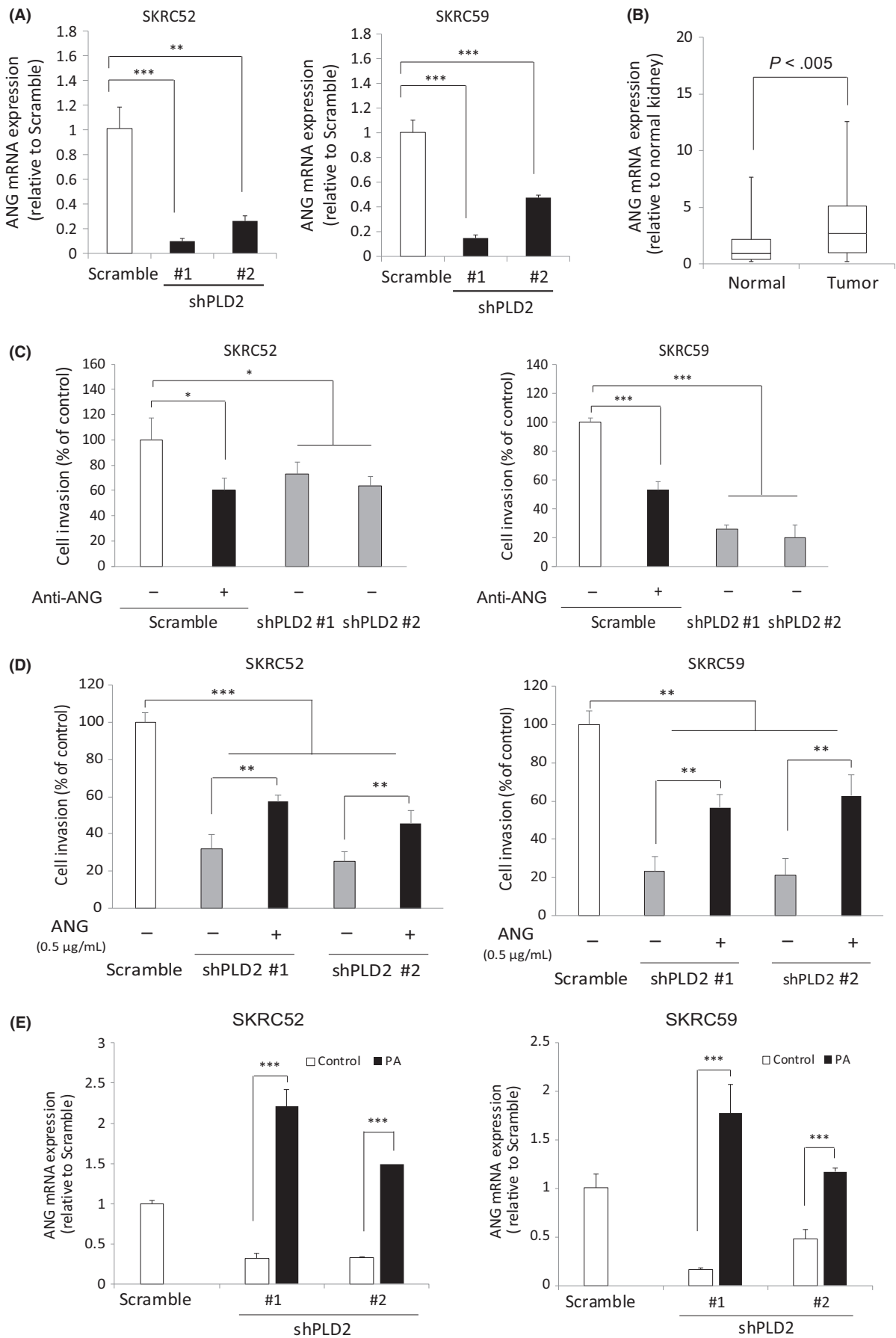


**FIGURE 4** PLD2 protein levels are correlated with poor prognosis and are elevated in the sites of metastasis in patients with clear cell renal cell carcinoma (ccRCC). A, Kaplan-Meier curves of the overall survival of 67 ccRCC patients, with regard to the PLD2 protein level of primary tumors. *P*-value was assessed by the log-rank test. B, PLD2 staining of primary tumor and microvascular invasion site in a representative patient with ccRCC. Scale bar, 200  $\mu$ m. C, Representative images comparing PLD2 immunohistochemical staining between primary and metastatic sites in patients with ccRCC. (1) Lung metastasis. (2) Brain metastasis. (3) Retroperitoneal metastasis. Scale bar, 50  $\mu$ m. D, Comparison of PLD2 protein expression between primary and metastatic sites in 16 patients with ccRCC. *P*-value was assessed by Fisher's exact test

**TABLE 3** The univariate and multivariate analysis of prognostic factors on overall survival

	Cox proportional hazard model (univariate)			Cox proportional hazard model (multivariate)		
	HR	95% CI of HR	<i>P</i>	HR	95% CI of HR	<i>P</i>
Clinical stage (1-2 vs 3-4)	17.6	3.38-323	.0001*	2.97	0.10-91.1	.4889
Grade (1-2 vs 3-4)	17.8	3.45-326	<.0001*	8.99	1.18-202	.0318*
Microvascular invasion	9.38	2.46-61.2	.0006*	1.37	0.07-8.52	.7840
PLD2 (low vs high)	11.4	2.39-30.7	.0007*	4.33	1.25-18.5	.0204*

\*Statistically significant difference ( $P < .05$ ).





**FIGURE 5** PLD2 promotes cell invasion through the regulation of ANG in SKRC52 and SKRC59 cells. A, Evaluation of the mRNA expression of ANG of SKRC52 and SKRC59 cells infected with scramble or PLD2 shRNA. *P*-value was assessed by Student's *t* test (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001). B, The mRNA expression of ANG in 52 clear cell renal cell carcinoma (ccRCC) patients. *P*-value was assessed by Wilcoxon rank sum test. C, Analysis of cell invasion ability after treatment with ANG-neutralized antibody to control IgG. *P*-value was assessed by Student's *t* test (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001). D, Analysis of cell invasion ability after treatment with recombinant human ANG protein to control (0.1%BSA). *P*-value was assessed by Student's *t* test (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001). E, The effect of PA on the mRNA expression of ANG in SKRC52 and SKRC59 infected with PLD2 shRNA. *P*-value was assessed by Student's *t* test (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001)

with a neutralizing antibody significantly suppressed cell invasion in SKRC52 and SKRC59 cells transfected with scramble shRNA (Figure 5C). Conversely, treatment with recombinant human ANG significantly promoted the cell invasion ability of both cells with shRNA mediated knockdown of PLD2 (Figure 5D). We further examined if the expression of ANG was promoted by PLD2 through the production of PA in renal cancer cells. Although ANG mRNA was downregulated by the knockdown of PLD2, the reduced expression was augmented by the treatment with PA in the same cells (Figure 5E).

Collectively, these results demonstrated that PLD2-produced PA promotes cell invasion in ccRCC cells, at least in part through regulating ANG.

## 4 | DISCUSSION

Phospholipase D expression and activity have been previously reported to be elevated in various human cancers such as colorectal, gastric, breast and kidney cancers when compared with adjacent non-cancer tissues.<sup>12,19,29</sup> The elevated expression of PLD2 was correlated with poor prognosis in colorectal cancer.<sup>29</sup> Moreover, previous studies demonstrated that PA produced by PLD promoted cancer proliferation, invasion and metastasis by various activated cell signaling pathways such as AKT and ERK signaling.<sup>6,14-18</sup>

In the present study, we showed that the elevated expression of PLD2 was associated with poor prognosis, and that PLD2 ablation and PLD2 small-molecule inhibitors suppressed the cell proliferation and invasion of renal cancer cells *in vitro*. We also revealed that PLD2 knockdown inhibited tumor growth and invasion using human renal cancer subcutaneous and orthotopic xenograft models. In keeping with previous reports, we confirmed that PLD2 regulated the phosphorylation of AKT and ERK in human renal cancer cells (Figure S4). These findings suggest that AKT and ERK signaling activated by PLD2-produced PA might contribute to promoting tumor progression in ccRCC.

In the present study, we focused on ANG as the downstream target of PLD2 in genes related to angiogenesis and tumor invasion based on the results of PCR-array analysis. ANG, a 14-kDa member of the pancreatic ribonuclease superfamily, was originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells.<sup>30</sup> ANG is a multifunctional secreted protein, which has been previously reported to play important roles in proliferation, invasion and metastasis coupled with the mediation of angiogenesis in several types of human cancer.<sup>31-34</sup> Yoshioka et al<sup>35</sup> showed that the protein levels of ANG were elevated in human cancers including

ccRCC by immunohistochemical analysis. Previous studies further showed that the levels of serum ANG in patients with ccRCC were elevated in comparison with healthy controls.<sup>36,37</sup> However, the regulation and potential roles of ANG remain to be elucidated in ccRCC. In the present study, we revealed for the first time that the production of PA by PLD2 regulated the expression of ANG, and that ANG promoted cell invasion in renal cancer cells.

As shown in Table S6, we also identified collagen type VIII alpha-1 chain (COL8A1) as a gene downregulated by 2 different PLD2 shRNA. This result was confirmed by qRT-PCR analysis using another set of primers in 2 different ccRCC cell lines (Figure S5). COL8A1 is one of the 2 alpha chains of collagen type VIII, classified as a non-fibrillar short-chain collagen. Zhao et al<sup>38</sup> demonstrated that siRNA-targeted COL8A1 inhibits proliferation and invasion in hepatocarcinoma cells. Because mRNA of COL8A1 were elevated in ccRCC specimens as compared with normal kidney (Figure S6), we speculate that COL8A1 is also related with ccRCC progression through promoting cell proliferation and invasion.

Previous reports demonstrated that not only PLD2 but also PLD1 promoted tumor invasion in several cancers.<sup>6,18,39</sup> Because high PLD1 expression was associated with higher tumor stage and worse clinical stage in patients with ccRCC (Table 1), we examined the roles of PLD1 in the regulation of ANG. In fact, it was revealed that the expression of ANG was suppressed by the knockdown of PLD1 (Figure S7). We also examined the abundance of this protein in the metastatic sites and compared it with that in the primary sites. However, the protein expression levels of PLD1 in the metastatic sites were not significantly elevated compared with the primary sites in our series of RCC samples (Figure S8). Collectively, PLD2 might play main roles in the tumor metastasis of ccRCC among 2 different PLD.

In the present targeted therapies for metastatic RCC, tyrosine kinase inhibitors (TKI) suppress the downstream targets of hypoxia-inducible factor (HIF) pathways such as VEGF and PDGF receptors. Although the tumor volume is decreased by TKI, there are few curable patients. As shown in the present study, PLD2 promoted a variety of cancer progression-related signaling in renal cancer cells. In addition, the expression of PLD2 was higher in microvascular invasion and in the metastatic sites of patients with ccRCC. Therefore, PLD2 is a new potential target of treatment for metastatic RCC. The development of targeting PLD isoenzymes with small molecule inhibitors has steadily proceeded from 2000s since PLD family members were implicated in a variety of human diseases such as viral infections, neurodegeneration and cancer.<sup>40</sup> Although there remain some problems such as off-target activity and PLD isoform selectivity in

PLD inhibitors, new efficacious PLD2 inhibitors for metastatic RCC might emerge in future.

In conclusion, we revealed that the elevated expression of PLD2 was associated with a poor prognosis and promoted tumor growth and invasion in ccRCC. We also demonstrated the new finding that PLD2-PA-ANG signaling promotes cell invasion in renal cancer cells. Our results provide the first evidence that targeting PLD2 is a good candidate for future therapeutic and clinical applications against metastatic RCC.

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## CONFLICT OF INTEREST

Eijiro Nakamura received research funding from Dainippon Sumitomo Pharma. The other authors have no conflicts of interest to declare.

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## SUPPORTING INFORMATION

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

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