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ORIGINAL ARTICLE

Lung adenocarcinoma–derived vWF promotes tumor metastasis by regulating PHKG1-mediated glycogen metabolism

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Abstract

Tumor metastasis is a series of complicated biological events. Hematogenous metastasis mediated by von Willebrand factor (vWF) is critical in tumor metastasis. However, the source of vWF and its role in tumor metastasis are controversial, and the further mechanism involved in mediating tumor metastasis is still unclear. In this study, we first demonstrated that lung adenocarcinoma cells could express vWF de novo and promotes tumor metastasis. Through the analysis of transcriptome sequencing, the metastasis promotion effect of vWF may be related to phosphorylase kinase subunit G1 (PHKG1), a catalytic subtype of phosphorylase kinase (PhK). PHKG1 was highly expressed in lung adenocarcinoma patients and led to poor prognosis. Further experiments found that lung adenocarcinoma–derived vWF induced the upregulation of PHKG1 through the PI3K/AKT pathway to promote glycogenolysis. Glycogen was funneled into glycolysis, leading to increased metastasis. Tumor metastasis assayed in vitro and in vivo showed that knockdown of PHKG1 or synergistic injection of phosphorylase inhibition based on the overexpression of vWF could inhibit metastasis. In summary, our research proved that lung adenocarcinoma–derived vWF may mediate tumor metastasis by regulating PHKG1 to promote glycogen metabolism and suggested potential targets for inhibition of lung adenocarcinoma metastasis.

KEYWORDS
glycogen, metabolism, PHKG1, tumor metastasis, vWF

1 | INTRODUCTION

Lung cancer is one of the most often occurring and deadliest cancers worldwide.[1,2] Hematogenous metastasis, which contributes to the poor prognosis of lung adenocarcinoma (LUAD) patients, is the main cause of LUAD-related death and a grand challenge in today's lung cancer management.[3]

Platelets can be activated and aggregated by tumor cells to cloak circulating tumor cells masking them from attack by the nature killer cells and, moreover, to facilitate tumor cell–endothelium adhesion

Abbreviations: EC, endothelial cells; KD, knockdown; LLC, Lewis lung carcinoma; LUAD, lung adenocarcinoma; NC, negative control; OD, optical density; OS, overall survival; PHKG1, phosphorylase kinase subunit G1; vWF, von Willebrand factor.

Jiayi Gu and Yingxue Qi have contributed equally to this work and share first authorship.

and extravasation.[4,5] Platelet surface adhesion receptor GPIbα can interact with von Willebrand factor (vWF) to form tumor thrombus, which is essential for hematogenous metastasis involving platelets.[6] However, the mechanism through which the vWF-platelet interaction contributes to the metastatic process remains unclear.

von Willebrand factor is a multimeric procoagulant plasma glycoprotein.[6] In addition to its critical role in hemostasis, the metastatic role of vWF has been widely reported.[7,8] Recent studies on the origin of vWF have found that not only endothelial cells and megakaryocytes could express vWF,[9] but some tumor cells of nonendothelial origin can also express vWF de novo, and this tumor-derived vWF expression was associated with increasing tumor, node, metastasis (TNM) stages, a global classification of malignant tumors.[10–13] As metastatic disease often develops in LUAD patients and remains the leading cause of their deaths,[14] we inferred that the same vWF expression pattern exists in patients with LUAD.

Phosphorylase kinase subunit G1 (PHKG1) is the catalytic gamma subunit of glycogen phosphorylase kinase (PhK). As the only enzyme known to catalyze the activation of glycogen phosphorylase, PhK is responsible for catalyzing the rate-limiting step of glycogen decomposition.[15] It can regulate glycogenolysis by activating glycogen phosphorylase. The glucose 1-phosphate released from glycogen enters the glycolysis to produce adenosine triphosphate (ATP). This extra energy burst allows tumor cells to perform high-energy tasks.[16] Previous studies have shown the upregulation of the γ subunit PHKG1 leads to increased PhK activity.[17] Camus et al. determined that PHKG1 is a kinase target of compounds that inhibit angiogenesis and demonstrated the promotion of PHKG1 in angiogenesis and endothelial cell migration.[16] The key role of PhK in the regulation of glycogen metabolism and the importance of glycogen metabolism in the development of cancer suggest that PHKG1 may be related to tumor metastasis.[18]

In this study, we proved that lung cancer cells could express vWF de novo, and LUAD-derived vWF could affect cell glycogen metabolism by upregulating PHKG1 through the PI3K/AKT pathway, thereby promoting the metastasis of LUAD cells. Our research may propose new potential targets for inhibiting LUAD metastasis.

2 | MATERIALS AND METHODS

2.1 | Cell culture, reagents, and animals

Lung cancer cells (95D and A549) were obtained from the Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in RPMI-1640 media with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin (Invitrogen Corporation) in a 37°C incubator with 5% CO_2.[19,20] Human umbilical vein endothelial cells (HUVECs) were maintained according to standard protocols.[21]

Plasmids used for transfection are listed in Table S1.

C57BL/6J mice were obtained from JSJ laboratories. Animal experiments were conducted in mice using protocols approved by the IACUC of East China University of Science and Technology. Animal husbandry protocols followed the Declaration of Helsinki.

2.2 | Human blood collection

Written, informed consent was obtained from all participants prior to their inclusion in studies. Venous blood was collected from healthy adult volunteers at East China University of Science and Technology. In addition, the use of donor-derived human platelets was approved by IRB in Shanghai Pulmonary Hospital.

2.3 | Quantitative real-time polymerase chain reaction (qRT-PCR)

For qRT-PCR, the total RNA levels were normalized with GAPDH. The results were calculated using the $2^{-\triangle\triangle CT}$ method and are expressed as the mean ± SD.[22] Forward (F) and reverse (R) primers are listed in the Table S2.

2.4 | Western blot and enzyme-linked immunosorbent assay (ELISA)

The antibodies used in Western blot are listed in Table S3. The antibody binding was detected using a Tanon Imaging System (5200S).

A commercial ELISA kit (CHE0155, 4A Biotech Co.) was used to quantify vWF in culture media.

2.5 | Immunofluorescent staining of cultured cells

The immunofluorescent staining was carried out as previously described.[13] The fluorescence images were caught using confocal microscope (Nikon).

2.6 | Flow cytometry

The flow cytometry used to detect the binding affinity of tumor cells with the GPIbα peptide was carried out as previously described.[23]

2.7 | Adhesion between tumor cells and platelets

The adhesion between tumor cells and platelets was conducted as previously described.[24] BCECF (sc-202492, Santa Cruz)-labeled

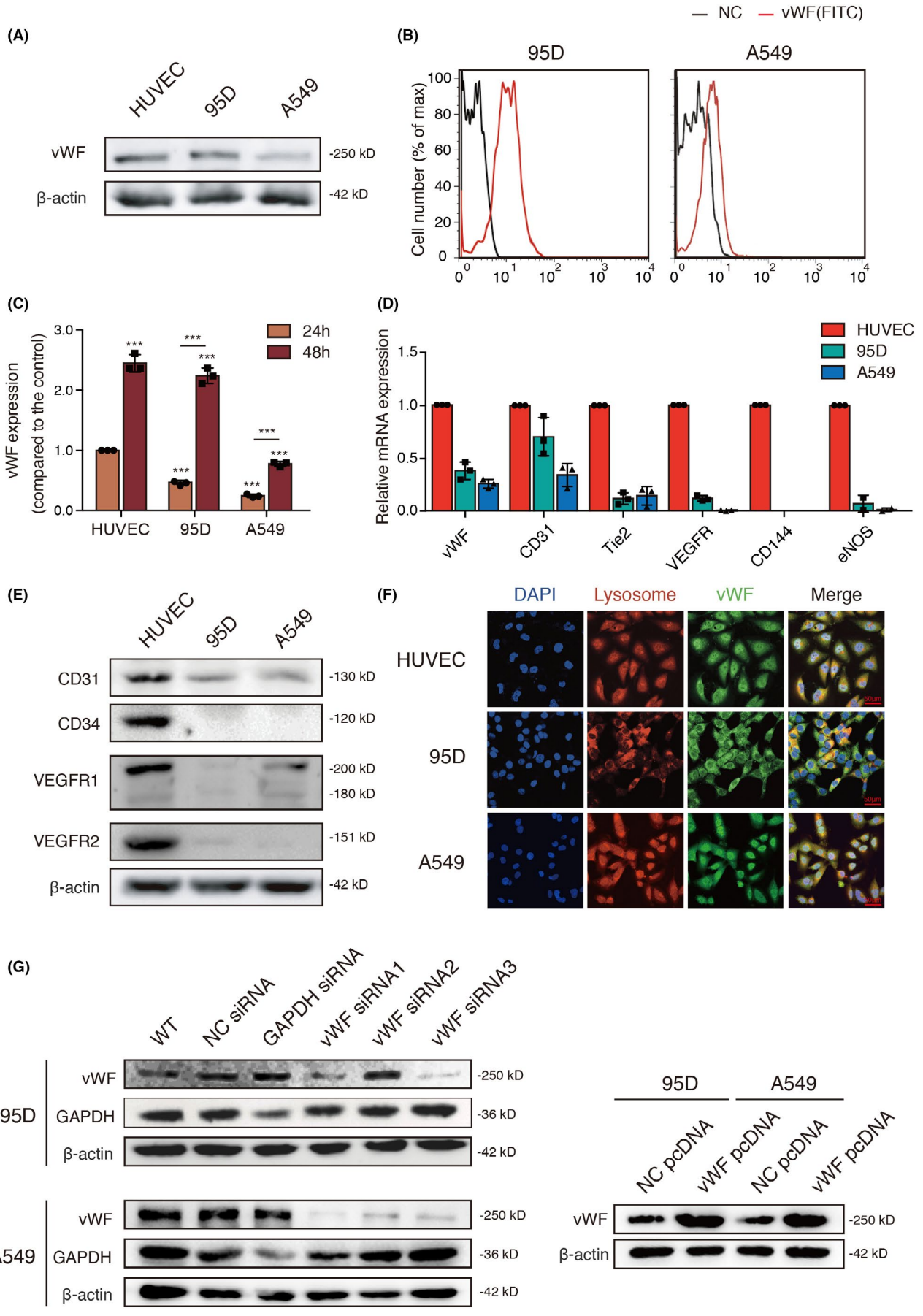

(A)
HUVEC
95D
A549
vWF
β-actin
-250 kD
-42 kD
(B)
— NC — vWF(FITC)
95D
A549
Cell number (% of max)
(C)
vWF expression (compared to the control)
24h
48h
HUVEC
95D
A549
(D)
Relative mRNA expression
HUVEC
95D
A549
vWF
CD31
Tie2
VEGFR
CD144
eNOS
(E)
HUVEC
95D
A549
CD31
CD34
VEGFR1
VEGFR2
β-actin
-130 kD
-120 kD
-200 kD
-180 kD
-151 kD
-42 kD
(F)
DAPI
Lysosome
vWF
Merge
HUVEC
95D
A549
(G)
WT
NC siRNA
GAPDH siRNA
vWF siRNA1
vWF siRNA2
vWF siRNA3
95D
A549
vWF
GAPDH
β-actin
-250 kD
-36 kD
-42 kD
95D
A549
NC pcDNA
vWF pcDNA
NC pcDNA
vWF pcDNA
vWF
β-actin
-250 kD
-42 kD

FIGURE 1 von Willebrand factor (vWF) was expressed de novo in lung adenocarcinoma. A, Western blot analysis of vWF and GPIbα expression in lung adenocarcinoma cell lines and HUVECs. B, The FACS analysis of vWF expression (red) in indicated cancer cell lines. FITC-labeled normal mouse IgG was used as control. C, ELISA analysis of vWF expression in supernatant of lung adenocarcinoma cells and HUVECs after 24 h and 48 h of cultivation. D, RT-PCR analysis of endothelial cell–specific genes in mRNA level (CD31, Tie2, VEGFR, CD144, and eNOS). E, Western blot analysis of endothelial cell–specific genes in protein level (CD31, CD34, and VEGFR1/2). F, Confocal microscopy and immunofluorescence staining to detect vWF expression (green) pathway positioning in 95D and A549 cell lines and HUVECs. DAPI (blue) marked the nucleus, and different organelles were marked with specific marker antibodies (lysosome; 60× magnification). G, Western blot analysis of vWF expression in lung adenocarcinoma cell lines and HUVECs. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

platelets were added into pretreated tumor cells for 4 hours of coincubation (platelet to tumor cells ratio was 3000:1). The nonadherent platelets were discarded. The fluorescent intensity of adherent tumor cells was observed with a fluorescence plate reader (PerkinElmer).

2.8 | Wound-healing assay

The wound-healing assay was carried out as previously described.[25]

2.9 | Transwell assay

The Transwell assay was carried out as previously described.[26]

2.10 | Glycogen content assay

The commercial glycogen PAS Staining Kits (G1281, G1360, Solarbio) were used to qualitatively analyze glycogen storage levels in cells and tissues. A commercial glycogen assay kit (BC0340-50, Solarbio) was used to quantify Glycogen content in cells and tissues.

2.11 | L-lactate assay

Tumor cells were seeded in a six-well plate and incubated for 24 hours. Media was collected, and lactate was measured using the L-Lactate Assay Kit (D799851-0050, Sangon Biotech) according to the manufacturer's instructions. The absorbance of the control group was subtracted from the absorbance of the test group, and the resulting value was substituted into the standard curve. The lactate content for each group was calculated according to the manufacturer's instructions.

2.12 | Animal experiences

2.12.1 | Experimental lung metastatic model

In the Lewis lung carcinoma (LLC) model, 6–8-week old normal C57BL/6J mice were randomly divided into four groups. There were five mice per group. For groups 1–4, mice were injected with 2.5×10^5 pretreated cells, respectively, through the lateral tail vein. The anti-mouse GPIbα mAb 2B4 was 50 μg per mouse in group 4.

2.12.2 | Xenograft model

A total of 2.5×10^5 LLC expressing a vWF cDNA ($n = 10$) or a PHKG1 shRNA ($n = 5$) was injected subcutaneously into the inner thigh of 6–8-week old $vWF^{-/-}$ C57BL/6J mice. LLC-expressing NC shRNA ($n = 5$) LLC cells were injected as a control. The mice in group 4 were treated with 40 μg/mouse CP-91149 (186392-40-5, Pfizer) when the volume of tumor reached 80 mm^3. The mice were killed after 3 weeks by cervical dislocation. The solid tumors were saved at −80°C for subsequent PAS staining and glycogen content assay.

2.13 | Statistical analysis

Statistical analysis was performed using Prism 6 software. All experiments were carried out at least three times. Statistical significance between two samples and among multiple samples was assessed by using the one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Each bar represents the mean ± SD of three independent experiments. Correlations were determined by Pearson's correlation. *P*-values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | vWF was expressed de novo in LUAD

To explore whether vWF was expressed in LUAD cells de novo, we determined the vWF expression in two different lung tumor cell lines (95D and A549) and found that 95D and A549 also expressed vWF (Figure 1A, B). Additionally, as shown in Figure S1A, vWF was expressed mainly in the cytoplasm. ELISA demonstrated that vWF synthesized in tumor cells was an exocrine protein, which was secreted into the supernatant at levels that increased with time (Figure 1C). Interestingly, vWF secretion in the tumor cells did not respond to thrombin (Figure S1B).

The expressions of some endothelial cell-specific genes were tested to evaluate whether vWF expression was indicative of the acquisition of an endothelial cell phenotype. There was no detectable expression

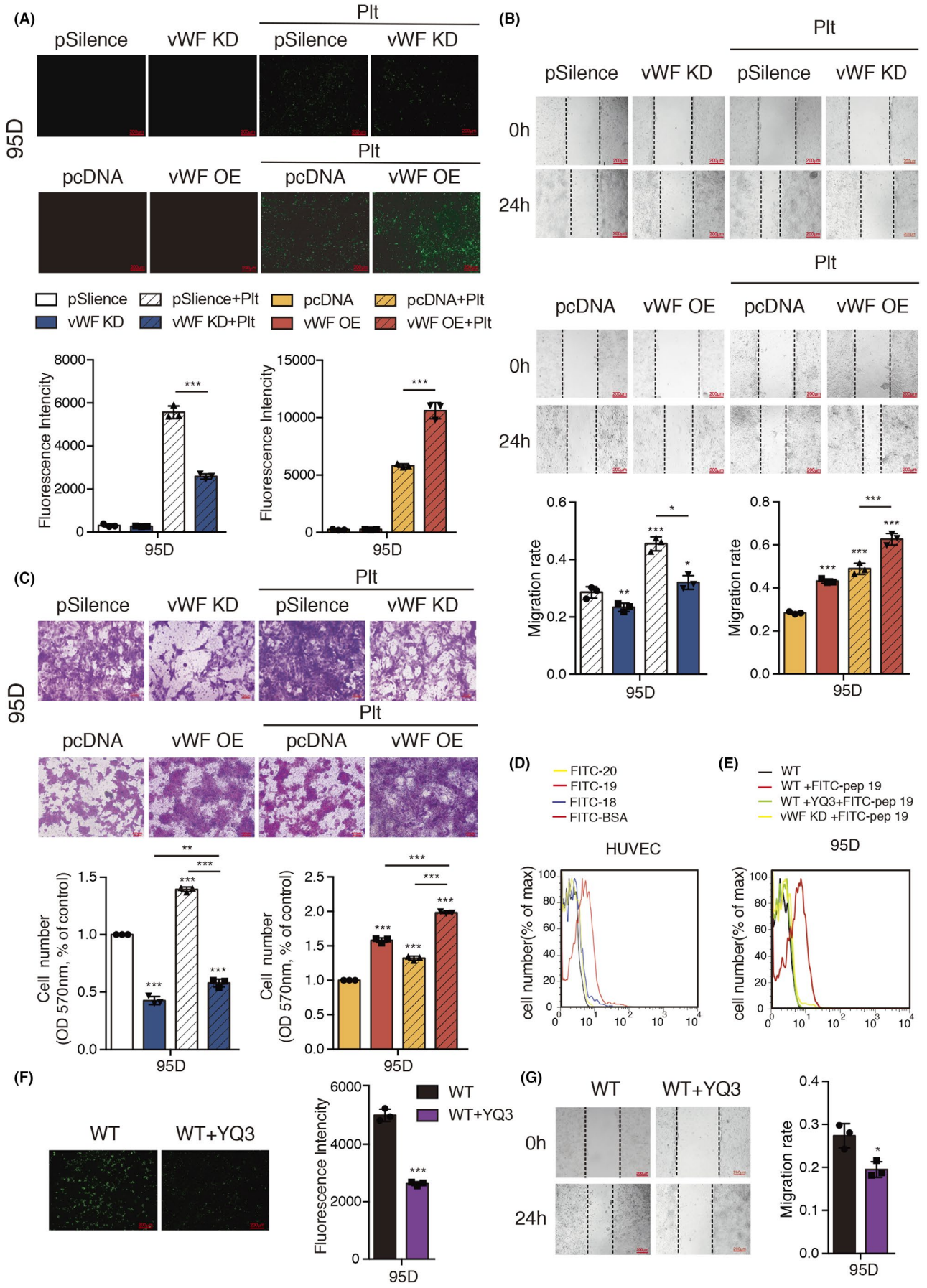

(A)
Plt
pSilence
vWF KD
pSilence
vWF KD
95D
Plt
pcDNA
vWF OE
pcDNA
vWF OE
pSlience
pSlience+Plt
pcDNA
pcDNA+Plt
vWF KD
vWF KD+Plt
vWF OE
vWF OE+Plt
Fluorescence Intencity
8000
6000
4000
2000
0
***
95D
15000
10000
5000
0
***
95D
(B)
Plt
pSilence
vWF KD
pSilence
vWF KD
0h
24h
Plt
pcDNA
vWF OE
pcDNA
vWF OE
0h
24h
Migration rate
0.6
0.4
0.2
0.0
*
***
**
*
95D
0.8
0.6
0.4
0.2
0.0
***
***
***
***
95D
(C)
Plt
pSilence
vWF KD
pSilence
vWF KD
95D
Plt
pcDNA
vWF OE
pcDNA
vWF OE
Cell number (OD 570nm, % of control)
1.5
1.0
0.5
0.0
**
***
***
***
95D
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
95D
(D)
FITC-20
FITC-19
FITC-18
FITC-BSA
HUVEC
cell number(% of max)
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
(E)
WT
WT +FITC-pep 19
WT +YQ3+FITC-pep 19
vWF KD +FITC-pep 19
95D
cell number(% of max)
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
(F)
WT
WT+YQ3
Fluorescence Intencity
6000
4000
2000
0
WT
WT+YQ3
***
95D
(G)
WT
WT+YQ3
0h
24h
Migration rate
0.4
0.3
0.2
0.1
0.0
*
95D

FIGURE 2 Functional consequences of von Willebrand factor (vWF) expression in lung adenocarcinoma cells on platelet-mediated tumor metastasis in vitro. A, Adhesion between 95D tumor cells to BCECF-labeled platelets was observed under a fluorescence microscope and was detected with a fluorescence plate reader. Average fluorescence intensity is shown in lower graphs. B, C, Wound-healing assay and Transwell showing the migration ability of 95D tumor cells transfected with vWF siRNA or vWF pcDNA in the absence or presence of platelets. These tumor cells which passed through the Transwell membranes were dissolved in methanol and quantified by a microplate reader (OD = 570 nm). Scale bar: 200 μm in wound-healing assay and 100 μm in Transwell. D, Binding of HUVECs to different FITC-labeled GPIbα peptide fragments detected by flow cytometry and quantitated by mean fluorescence intensity. E, Binding of indicated 95D cells to FITC-labeled GPIbα pep19 fragment detected by flow cytometry and quantitated by mean fluorescence intensity. F, Adhesion between 95D tumor cells to BCECF-labeled platelets, with or without YQ3, was observed under a fluorescence microscope. G, Wound-healing assay showing the migration ability of 95D tumor cells with or without YQ3 in the presence of platelets. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

of other endothelial markers except CD31 and VEGFR in LUAD cells (Figure 1D, E). However, the expressions of CD31 and VEGFR in LUAD cells were significantly lower than those in HUVECs. To confirm this finding, we compared the publicly available RNA-seq data of several LUAD cells with HUVEC (Figure S1C). Furthermore, immunofluorescence (IF) staining was conducted to further detect the vWF expression pathway localization. As shown in Figures 1F, S1D, and S1E, dyed vWF overlapped with lysosome, ribosome, and Golgi to some extent in tumor cells. These results suggested that vWF expression in LUAD was not a consequence of acquiring an endothelial cell phenotype.

3.2 | Functional consequences of vWF expression in LUAD cells on platelet-mediated tumor metastasis in vitro

95D and A549 cells with vWF knockdown (KD) or overexpression (OE) were used to investigate the effect of LUAD-derived vWF on tumor metastasis (Figure 1G). As shown in Figures 2A and S2A, compared with the control group, the adhesion of LUAD cells to freshly purified platelets was inhibited in vWF KD cells. The extent of tumor migration, as determined by wound-healing and Transwell assay, showed that knocking down vWF inhibited tumor cell migration, and this inhibition was alleviated with the presence of platelets, and vice versa (Figures 2B, C and S2B, C).

Because of the promotion on LUAD-derived vWF–mediated tumor metastasis by platelets, we next explored whether LUAD-derived vWF could bind to GPIbα, similar to vWF in HUVECs. The purified recombinant GPIbα fragments (pep19) with the highest binding capacity with HUVECs was screened out by fluorescence-activated cell sorting (FACS) (Figure 2D); as we expected, different lung tumor cells had strong binding affinity for pep19 (Figures 2E and S2D). YQ3 is a mouse anti-human GPIbα monoclonal antibody screened by our group, which could inhibit the vWF-platelet interaction.[23] When vWF was knocked down or YQ3 (a kind of vWF-platelet binding inhibitor) was added, the binding of the tumor cells to pep19 was inhibited (Figures 2E and S2D).

In platelet adhesion and wound-healing assay, not only the adhesion between platelets and tumor cells (Figures 2F and S2E) but also the migration of LUAD cells (Figures 2G and S2F) was markedly reduced when YQ3 was added. These results demonstrated that in the process of platelet-mediated metastasis, the expression of vWF in LUAD cells enhanced the vWF-GPIbα interaction, thereby promoting tumor cell migration.

3.3 | Functional consequences of vWF expression in LUAD cells on platelet-mediated tumor metastasis in vivo

The results of in vitro data suggested a potential role for tumor cell–derived vWF in enhancing LUAD cell metastasis. We established an experimental metastasis model of C57BL/6J mice to further explore the function of LUAD-derived vWF in tumor metastasis in vivo. First, the vWF expression in the LLC mouse LUAD cell line was knocked down by GFP-labeled lentivirus infection (Figure 3A), and the efficiency of the vWF KN was confirmed by Western blot (Figure 3B). Consistent with the in vitro observations, as shown in Figure 3C, the vWF KD LLC cells had an apparently decreased number of surface pulmonary nodules. The same metastasis inhibition was observed when a rat anti-mouse mAb, 2B4, was used to inhibit the platelet-vWF interaction. 2B4 is a rat anti-mouse GPIbα monoclonal antibody screened by our group, which could inhibit the platelet-vWF interaction.[23] These data highlighted the importance of cancer-derived vWF on platelet-mediated metastasis. This finding was also confirmed by the results of H&E-stained lung tissue observed under a microscope (Figure 3D). In addition, neither WT tumor cells nor vWF KD tumor cells caused significant liver metastasis in experimental metastasis models (Figure 3E). However, injection of WT tumor cells caused more pronounced liver inflammation and infiltration than did injection of vWF KD tumor cells or the addition of 2B4 (Figure 3E). Moreover, we also investigated overall survival related to vWF expression of late stage III-IV LUAD patients through The Cancer Genome Atlas (TCGA) database. The results from the Kaplan-Meier survival analysis revealed that lower vWF expression was associated with poor survival (Figure 3F). Taken together, these data suggested that tumor cell–derived vWF could potently promote tumor metastasis by interacting with platelet in vivo.

3.4 | PHKG1 was a key regulatory protein in the process of tumor metastasis mediated by vWF-platelet interaction

To identify the dominant signaling events in cancer cells critical for the platelet-vWF interaction, we first performed whole-transcriptome sequencing to identify genes that were significantly altered in tumor cells after vWF KD (135 genes changed) or YQ3 addition (47 genes changed) in the absence or presence of platelets (1034 genes changed) (Figures 4A-C and S3A). Among these genes,

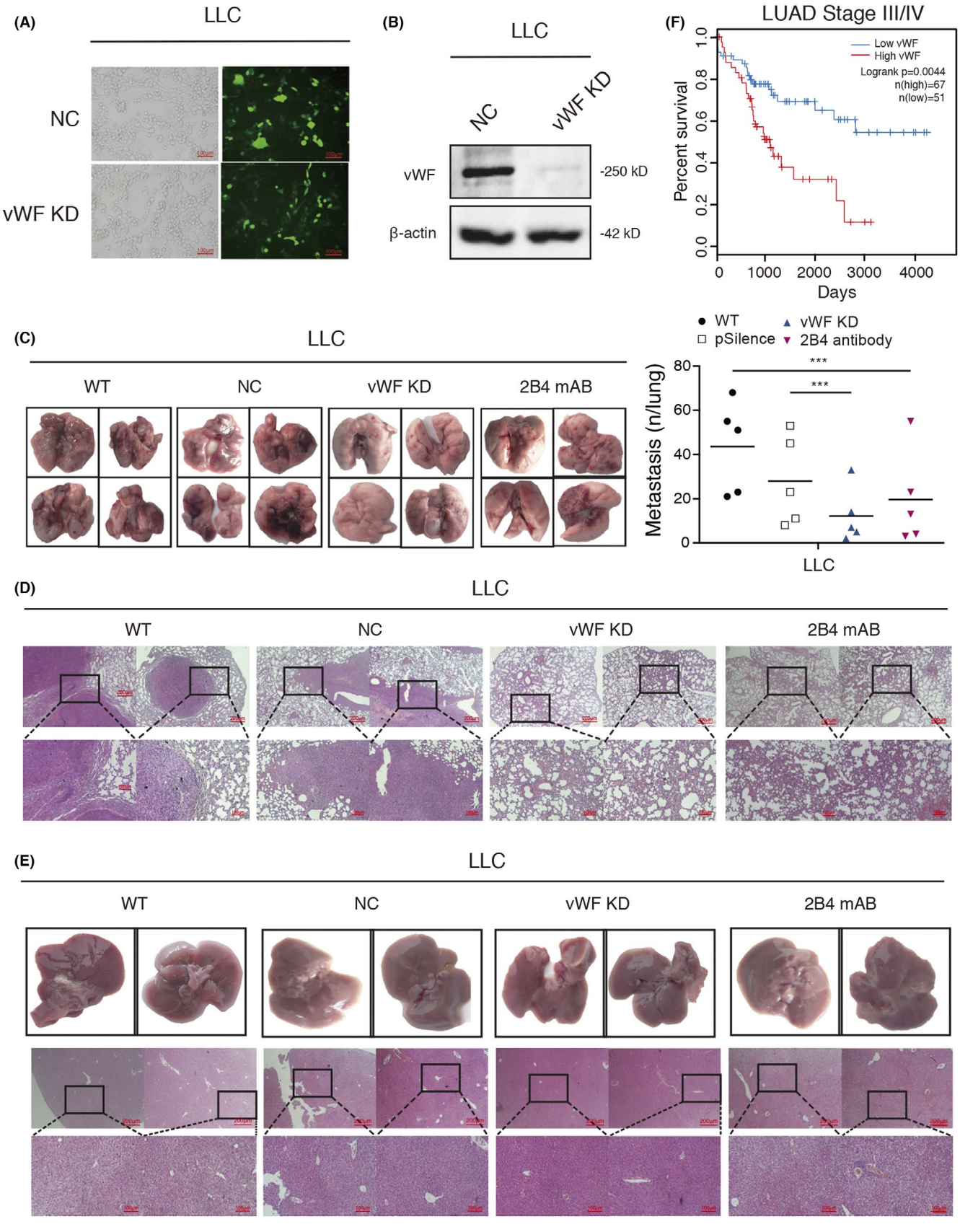

(A)
LLC
NC
vWF KD
(B)
LLC
NC
vWF KD
vWF
β-actin
-250 kD
-42 kD
(F)
LUAD Stage III/IV
Low vWF
High vWF
Logrank p=0.0044
n(high)=67
n(low)=51
Percent survival
Days
(C)
LLC
WT
NC
vWF KD
2B4 mAB
WT
pSilence
vWF KD
2B4 antibody
Metastasis (n/lung)
LLC
(D)
LLC
WT
NC
vWF KD
2B4 mAB
(E)
LLC
WT
NC
vWF KD
2B4 mAB

FIGURE 3 Functional consequences of von Willebrand factor (vWF) expression in lung adenocarcinoma cells on platelet-mediated tumor metastasis in vivo. A, Efficiency of vWF knockdown (KD) in Lewis lung carcinoma (LLC) cells by GFP-labeled lentivirus infection imaged by fluorescence microscope. B, Western blot analysis of vWF expression in LLC cells with vWF KD lentivirus infection. C-E, Pulmonary metastasis was assessed after (C) LLC cells injection through the lateral tail vein (n = 5/group). Metastasis was analyzed 14 days after injection of LLC tumor cells. Representative examples of the lungs (n=4/group) with metastatic foci were depicted. D, E, Representative histologic evidence from lung and liver sections of the different groups. Four percent of paraformaldehyde–embedded lungs of all mice were cut completely, stained with hematoxylin and eosin, examined histologically, and detected by microcopy. F, Kaplan-Meier analysis of overall survival (OS) related to the vWF expression in LUAD patients according to the data from The Cancer Genome Atlas (TCGA) database. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

the exostoses-like protein 1 (EXTL1) and PHKG1 genes were common differentially expressed genes (Figure 4D). PHKG1 is a catalytic subunit of glycogen metabolism–related PhK,[27] which mediates glycogen metabolism and glycolysis. The results of the Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis also revealed that pathways related to carbohydrate metabolic processes and glycolysis/gluconeogenesis were downregulated and were the most enriched in the vWF KD 95D groups (Figure 4E, F).

It has been reported that the upregulation of PHKG1 is related to cancer progression.[16,28,29] The LUAD-related gene expression data downloaded from TCGA database showed that compared with normal individuals, patients with non–small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) have higher PHKG1 expression (Figure 4G). In addition, higher PHKG1 expression was associated with poor survival of LUAD patients (Figure 4H). 95D and A549 with PHKG1 knocked down or overexpressed were used to evaluate the effect of PHKG1 on tumor cell migration (Figure S3B). Wound-healing and Transwell assay both revealed that PHKG1 overexpression accelerated cell migration, and vice versa (Figure 4I, J). These data suggested that PHKG1, which was regulated by the interaction of platelets and LUAD-derived vWF, could promote LUAD metastasis.

3.5 | Lung adenocarcinoma–derived vWF positively regulated PHKG1 through the PI3K/AKT pathway

To further explore the mechanism of platelet-vWF regulating LUAD metastasis, we first investigated the correlation between LUAD-derived vWF and PHKG1. Coexpression correlation analysis based on TCGA data showed a positive correlation between the levels of vWF and PHKG1 protein expression in LUAD (Figure 5A). qRT-PCR (Figure 5B) and Western blot (Figure 5C) were used to detect the mRNA and protein expression of vWF and PHKG1 of solid tumors in the previous spontaneous metastasis model of mice, respectively. Compared with the control group, knockdown of vWF suppressed the expression of PHKG1, and the addition of vWF-platelet inhibitor 2B4 also had the same inhibitory effect. Next, we further validated it in vitro. As shown in Figures 5D, E and S4A, B, the levels of PHKG1 were markedly downregulated in the vWF KD group, and vice versa. These data were consistent with the results of solid tumor detection and proved that PHKG1 is a downstream target of vWF.

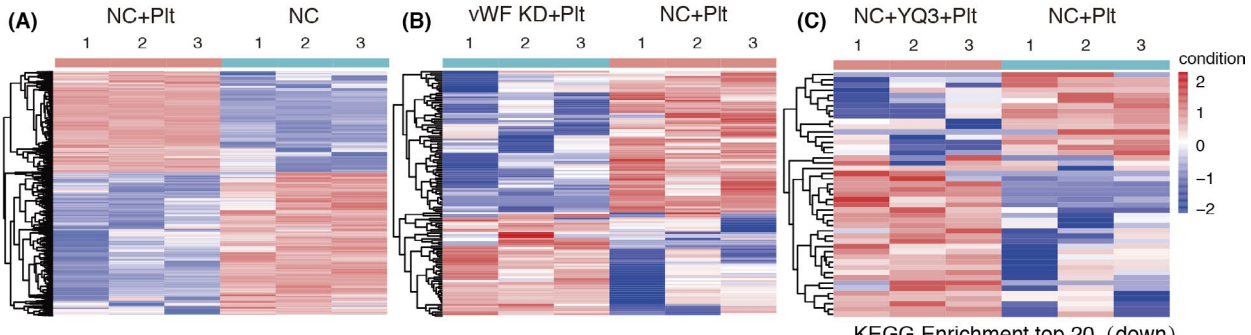
KEGG enrichment analysis revealed that downregulated genes in vWF KD 95D cells with platelets were significantly enriched in the PI3K-AKT signaling pathway (Figure 4F). The expression levels of p-PI3K and p-AKT were downregulated in 95D and A549 following vWF knockdown (Figures 5F and S4C). We next explored whether the PI3K/AKT signaling pathway is involved in the expression of PHKG1 regulated by vWF. As shown in Figures 5G and S4D, after the addition of PI3K inhibitor or AKT inhibitor, the promotion of PHKG1 expression by overexpression of vWF was effectively inhibited. These data proved that vWF from LUAD could positively regulate PHKG1 through the PI3K/AKT signaling pathway.

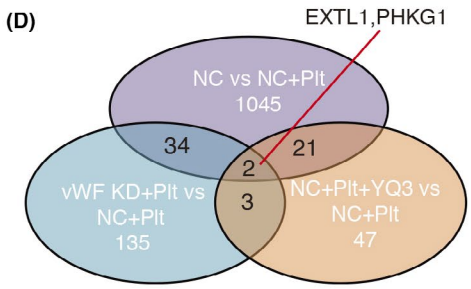
Given that PHKG1 participates in the glycogen metabolism of cells. We evaluated the effect of vWF on glycogen content in LUAD cells. Increased tumor-derived vWF expression could reduce glycogen stores in LUAD cells as shown by PAS staining and glycogen content assay, and vice versa (Figures 5H, I and S4E, F). We also examined the metabolic consequence of vWF-induced glycogen mobilization in LUAD cells. The glycolysis level of cells was judged by comparing the lactate production of each group. As shown in Figures 5J and S4G, vWF could promote the production of lactate, which matched with the results of glycogen reaction experiments. In summary, these data suggested that vWF can positively regulate PHKG1 through the PI3K/AKT pathway and further promotes glycogenolysis and glycolysis in LUAD cells.

3.6 | Knockdown PHKG1 could reverse the effect of vWF overexpression on LUAD metastasis in vitro

It has been verified that upregulation of vWF could increase the expression of PHKG1, thereby promoting glycogenolysis and glycolysis of tumor cells. Furthermore, vWF overexpression could promote tumor metastasis. In view of the relationship between vWF and PHKG1, we have proposed a hypothesis that knocking down PHKG1 or adding PHKG1-related inhibitors could reverse the effect of vWF overexpression on tumor metastasis enhancement.

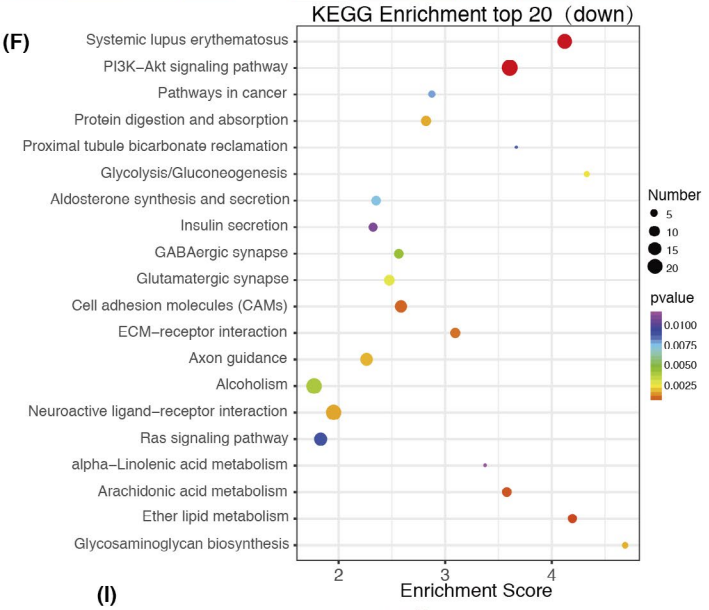
To confirm this hypothesis, we first detected PHKG1 expression in different groups. Western blot data showed that transfection of PHKG1 shRNA could effectively downregulate the level of PHKG1 in the presence of overexpressed vWF, while the addition of glycogen phosphorylase inhibitor (CP-91149)[30] had no effect on the expression of PHKG1 (Figures 6A and S5A). PAS staining (Figures 6B and S5B) and a glycogen analysis kit (Figures 6C and S5C) were used to qualitatively and quantitatively detect glycogen content. vWF-mediated glycogenolysis was obviously prevented by knockdown of PHKG1 or CP-91149. This result was also verified in the detection

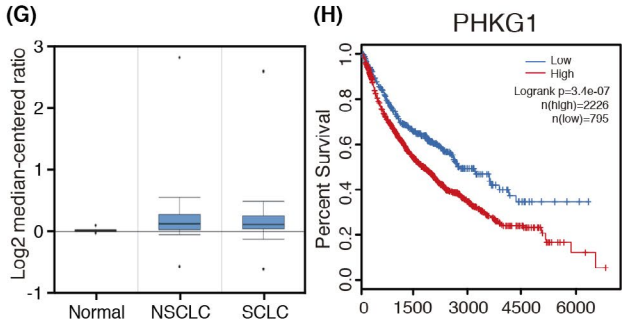


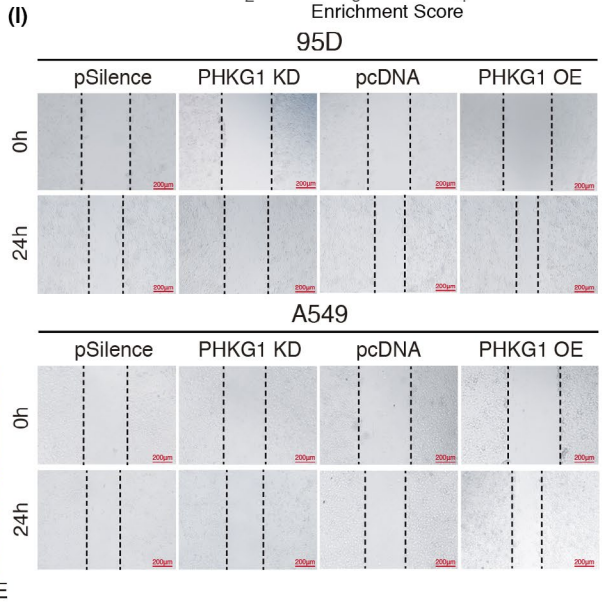


(E)

GO enrichment（down）	p-value
Carbohydrate metabolic process	< 0.0001
Immune response	0.0010
Intracellular signal transduction	0.0080
Cell differentiation	0.0249
Integral component of plasma membrane	0.0454







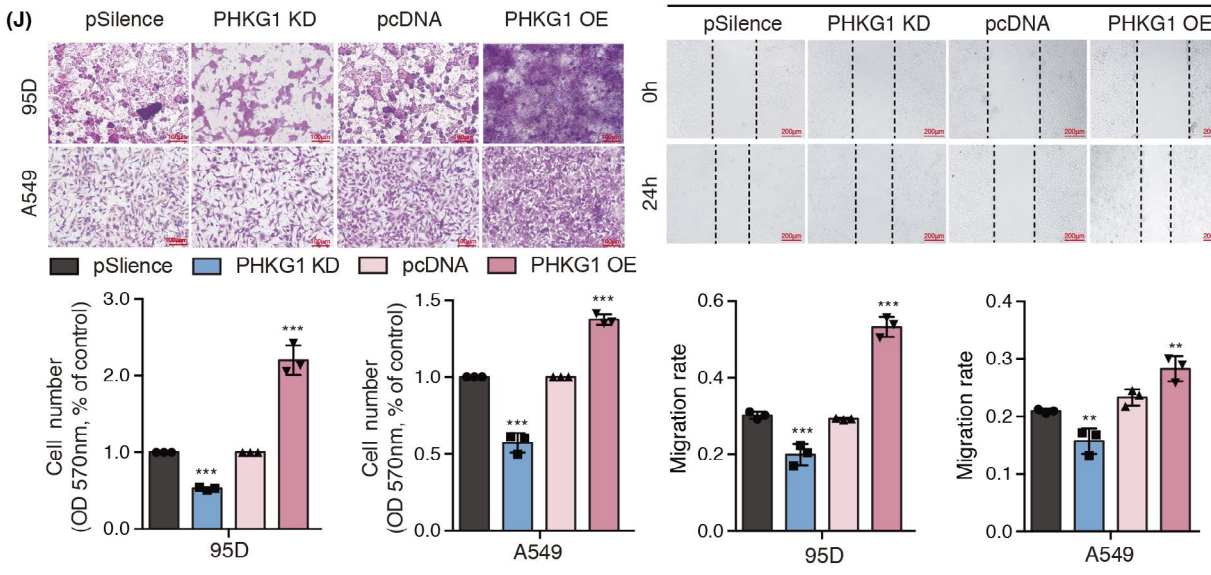


FIGURE 4 Phosphorylase kinase subunit G1 (PHKG1) was a key regulatory protein in the process of tumor metastasis mediated by von Willebrand factor (vWF)-platelet interaction. A-C, Heat maps showing differentially regulated gene expression as analyzed by whole-transcriptome sequencing in different cells ($n = 3$). NC siRNA–transfected 95D cells (NC) versus NC 95D cells cocultivated with platelets (tumor cells to platelet ratio was 1:3000) (A); vWF siRNA 1–transfected 95D cells (vWF KD)versus NC 95D cells in the presence of platelets (B); NC cells versus NC cells with YQ3 in the presence of platelets (C). D, Number of differential genes between indicated groups. E, F, Enriched pathways with indicated *P*-values of downregulated genes in vWF KD 95D cells compared with NC 95D cells in the presence of platelets, using Gene Ontology (GO) enrichment analysis (E) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (F). G, Differential PHKG1 mRNA levels (log2 intensity) in non–small cell lung cancer (NSCLC), small cell lung cancer (SCLC), and normal person according to the data from The Cancer Genome Atlas (TCGA) database. H, Kaplan-Meier analysis of overall survival (OS) related to PHKG1 expression in lung adenocarcinoma patients according to the data from the TCGA database. The log-rank test was used to calculate the OS rate for comparison between different groups. I, J, Wound-healing and Transwell assay showing the migration ability of 95D and A549 transfected with PHKG1 siRNA or PHKG1 pcDNA. Scale bar: 200 μm in wound-healing assay, 100 μm in Transwell assay. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

of lactate (Figures 6D and S5D). Interestingly, knockdown of PHKG1 or addition of CP-91149 could reverse the increase of glycogenolysis and lactate caused by overexpression of vWF. However, they could not completely eliminate this promotion of vWF compared with the control group or the PHKG1 KD group. Wound-healing and Transwell analysis were performed to test the reversal effect of PHKG1 knockdown in LUAD metastasis mediated by vWF overexpression in vitro. As shown in Figures 6E, F and S5E, F, knockdown of PHKG1 can reverse the effect of vWF overexpression on cell migration. Similarly, this inhibitory effect on cell migration could also be achieved after adding CP-91149.

3.7 | Inhibition of PHKG1 could reverse the effect of vWF overexpression on LUAD metastasis in vivo

It has been proved that knocking down the expression of PHKG1 or inhibiting the glycogen metabolism involved in PHKG1 could reverse the effect of overexpression of vWF on LUAD metastasis in vitro. To test this in vivo, we constructed an experimental metastasis model and a xenograft model with $vWF^{-/-}$ C57BL/6J mice, respectively. Consistent with the in vitro observations, as shown in Figure 7A, compared with the control group, the number of surface lung nodules injected with vWF-OE LLC cells was significantly increased. A marked reduction in the number of nodules was observed when CP-91149 was used to inhibit PHKG1-mediated glycogen metabolism, indicating that the promotion of lung metastasis by vWF overexpression was inhibited (Figure 7A). The H&E staining results of lung tissue sections also verified this finding (Figure 7C). Interestingly, in the experimental metastasis model, no obvious liver metastasis was found in the liver of any group of mice (Figure 7B).

In the mouse xenograft model, the subcutaneous solid tumor volume of mice injected with vWF OE LLC cells was larger than that of mice injected with control LLC cells. However, this promotion was apparently inhibited after using CP-91149 (Figure 7D). Moreover, the mass of solid tumors in each group also matched the tumor volume (Figure 7E). The PAS staining and glycogen content assay results of solid tumor sections showed that the vWF overexpression group contained lower levels of glycogen than the control group, while the CP-91149 group had a significant increase in glycogen content (Figure 7F, G). In summary, these data showed that increasing the expression of vWF can promote the metastasis of LUAD in vivo, and this is related to the glycogen metabolism mediated by PHKG1. Inhibiting PHKG1 could reverse this promotion.

4 | DISCUSSION

von Willebrand factor has been regarded as a typical EC marker in the clinic for a long time. As one of the major platelet adhesion ligands, its prometastatic effect has been widely reported.[31] Interestingly, several reports demonstrated that vWF induces tumor cell apoptosis in an integrin binding–dependent manner[32] or acts as an antimetastatic protein.[24] However, recently, vWF was found to be expressed de novo in several different cancer cells of nonendothelial origin.[33] The high expression of this endogenous vWF was closely related to the increase of tumor and lymph node metastasis. However, whether LUAD cells express vWF de novo and vWF's function in tumor metastasis remain unclear.

We first tested vWF expression in two cultured lung tumor lines and HUVECs in several independent experiments and confirmed that both lung cancer cells secreted vWF without stimulation (Figure 1A-C) and vWF de novo expression in the lung cancer cells was not a consequence of EC phenotype acquisition (Figure 1D-E).

Recent studies have reported that tumor cell–derived vWF may have direct functional consequences that are distinct from those of HUVECs.[34] The in vitro experiments showed that the lung cancer–derived vWF affected the cell-platelet adhesion and the tumor cell migration in vitro. In the presence of platelets, these effects were particularly obvious. Therefore, we speculated that vWF from lung cancer was involved in platelet-mediated tumor metastasis. Lung cancer–derived vWF, just as endothelial-derived vWF, could bind to platelet surface receptors. Interestingly, the platelet-vWF inhibitor could obviously prevent tumor-platelet adhesion and tumor cell migration in the presence of platelets (Figure 2D-G). Consistent with the in vitro observations, sham-transfected cells were significantly more conducive to grafting than the vWF KD tumor cells or the cells treated with the platelet-vWF inhibitor in mice pulmonary experimental and spontaneous metastasis models (Figure 3). On the basis of these results, we proposed that lung cancer–derived vWF confers

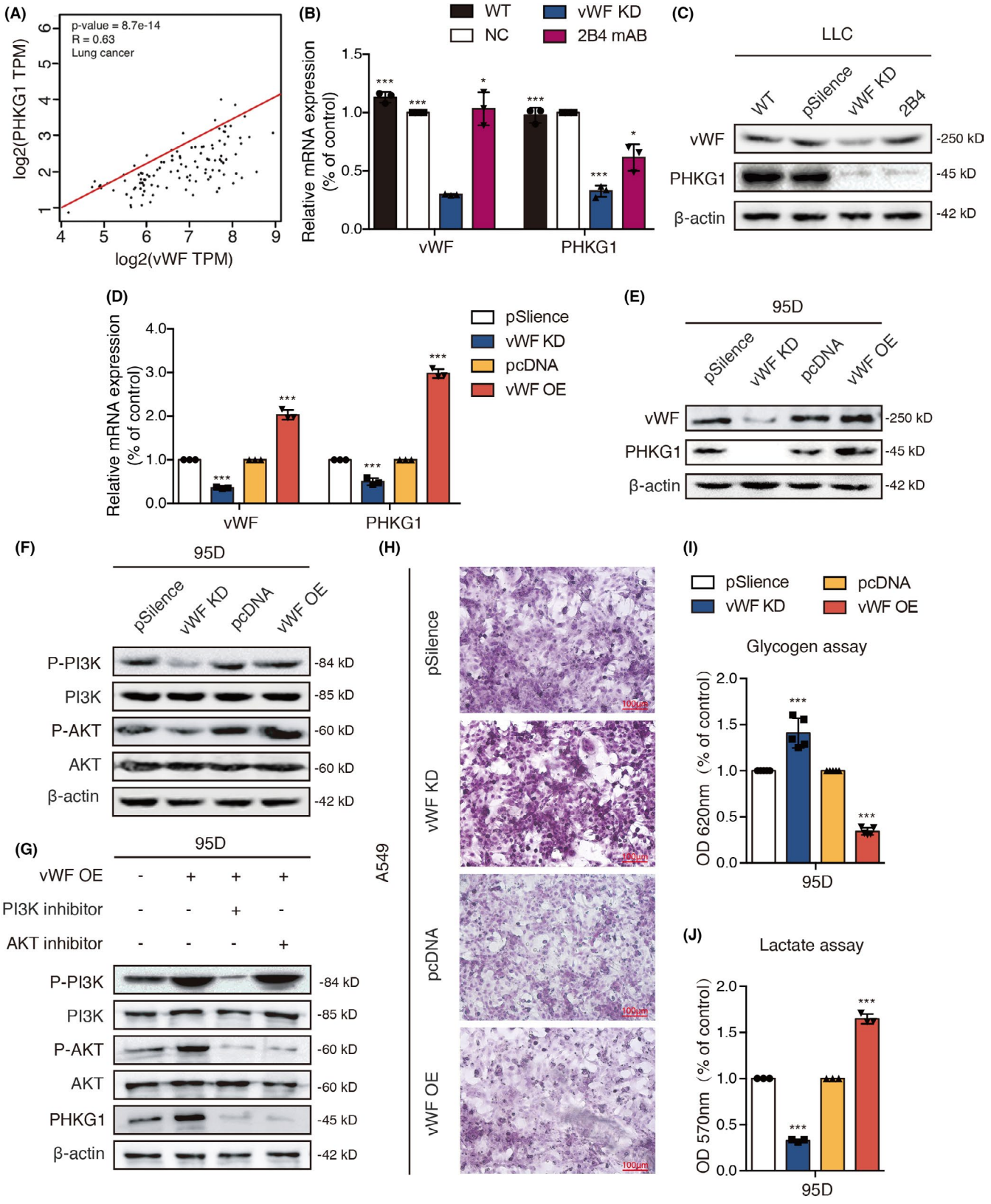


FIGURE 5 Lung adenocarcinoma–derived von Willebrand factor (vWF) positively regulated phosphorylase kinase subunit G1 (PHKG1) through the PI3K/AKT pathway. A, Coexpression correlation analysis of vWF and PHKG1 in lung adenocarcinoma based on The Cancer Genome Atlas (TCGA) database. B, C, qRT-PCR and Western blot analysis of vWF and PHKG1 protein or mRNA expression in solid tumors in a mouse spontaneous metastasis model. D, E, qRT-PCR and Western blot analysis of vWF and PHKG1 in protein or mRNA levels in 95D cells transfected with vWF siRNA or vWF pcDNA. F, G, Western blot analysis with antibodies directed against PI3K, P-PI3K, AKT, P-AKT, and PHKG1 was performed. H, Glycogen stores were visualized by PAS staining in 95D cells transfected with vWF siRNA or vWF pcDNA. Scale bar: 100 μm. I, J, Glycogen assay and lactate assay on 95D cells transfected with vWF siRNA or vWF pvDNA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

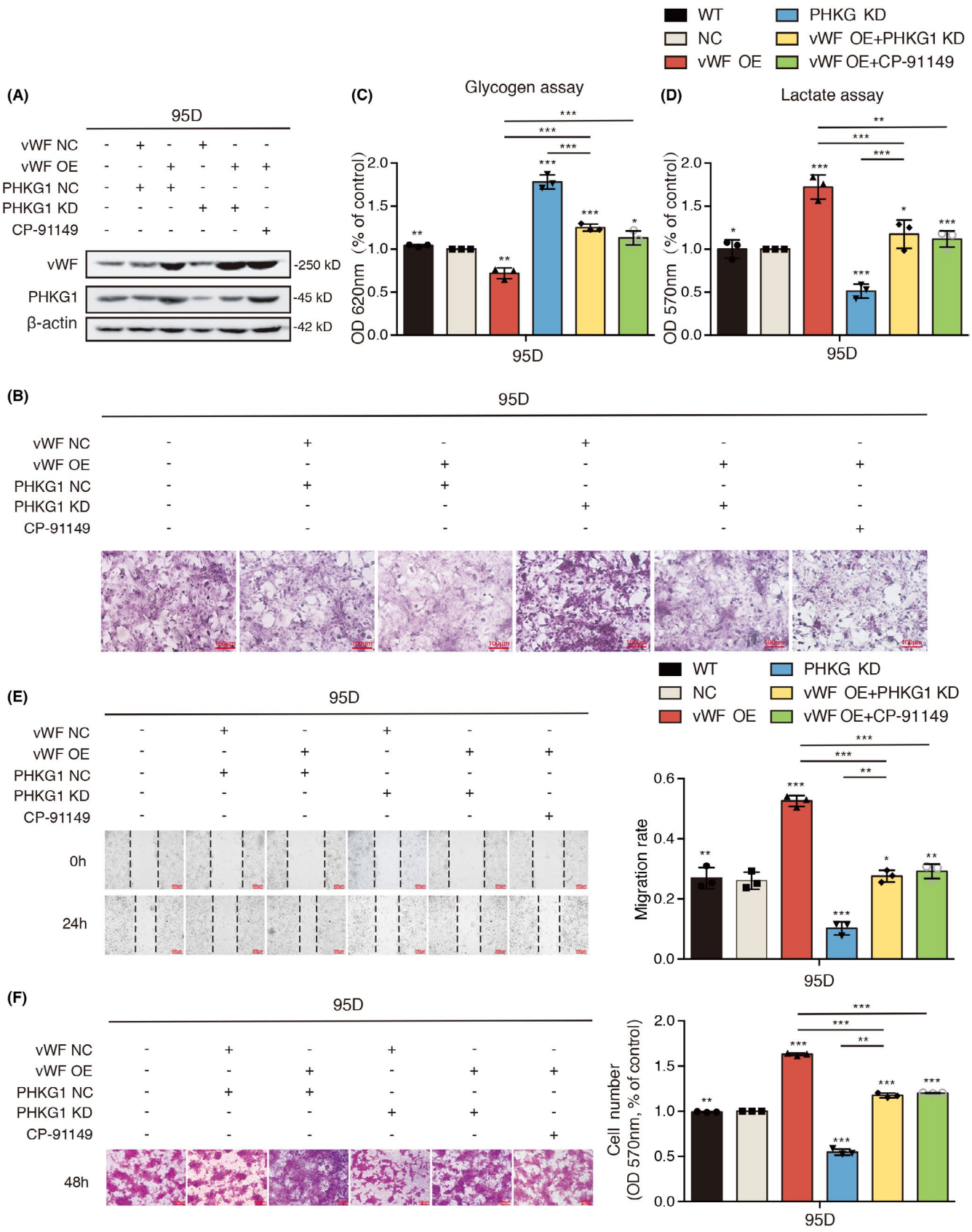
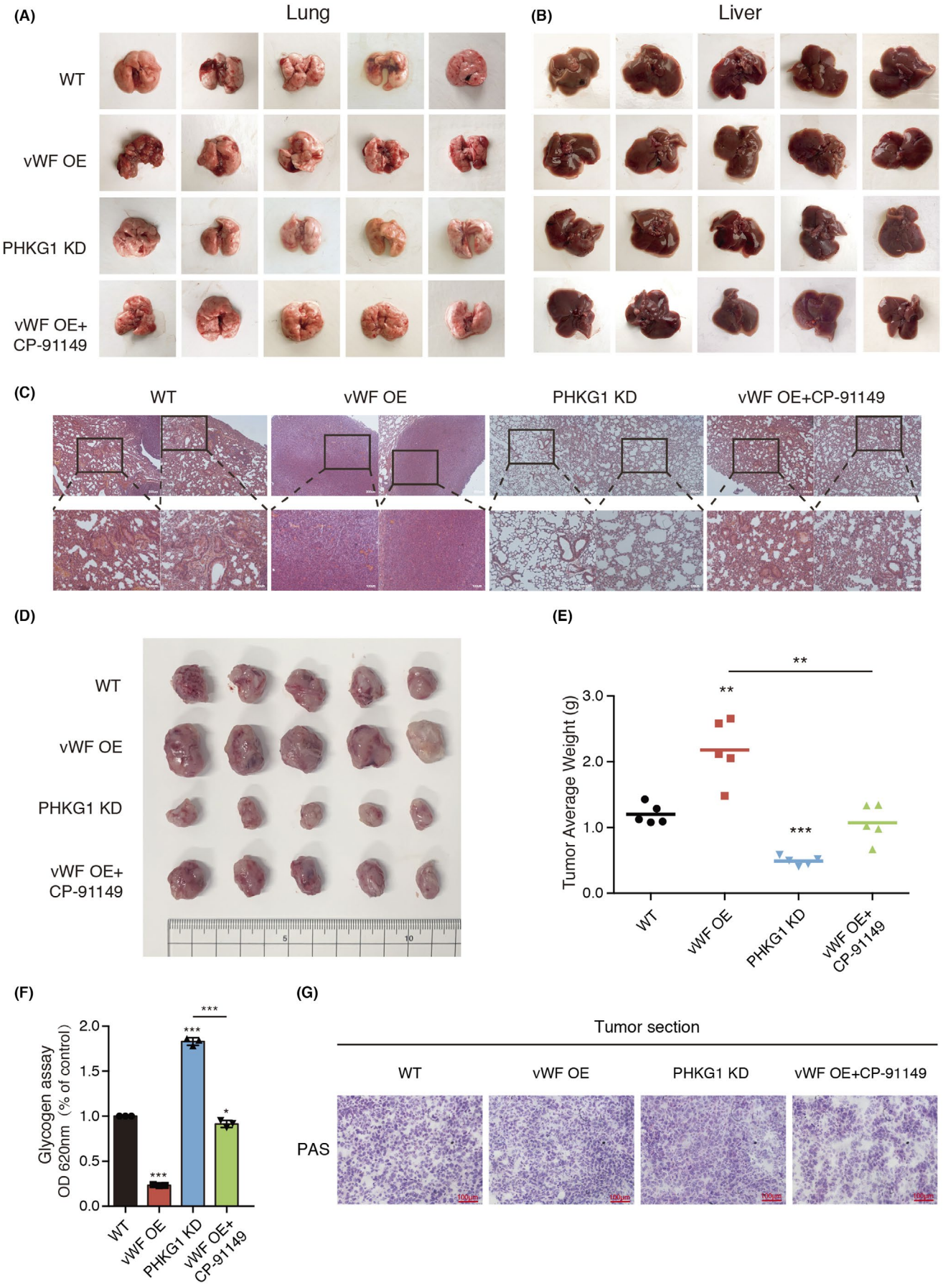


FIGURE 6 Knockdown phosphorylase kinase subunit G1 (PHKG1) could reverse the effect of von Willebrand factor (vWF) overexpression on lung adenocarcinoma metastasis in vitro. A, Western blot analysis of vWF and PHKG1 in protein level in 95D cells transfected with vWF pcDNA or PHKG1 shRNA or both vWF pcDNA and PHKG1 shRNA. 95D cells from inhibitor group were pretreated (1 h) with the glycogen phosphorylase inhibitor CP-91149 (0.5 μM). B, Glycogen stores were visualized by PAS staining in 95D cells. Scale bar: 100 μm. C, D, Glycogen assay and lactate concentration in 95D cells. E, F, Wound-healing assay and Transwell showing the migration ability of 95D tumor cells. Scale bar: 200 μm in wound-healing assay and 100 μm in Transwell. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$


(A)
Lung
WT
vWF OE
PHKG1 KD
vWF OE+
CP-91149
(B)
Liver
(C)
WT
vWF OE
PHKG1 KD
vWF OE+CP-91149
(D)
WT
vWF OE
PHKG1 KD
vWF OE+
CP-91149
(E)
Tumor Average Weight (g)
3.0
2.0
1.0
0.0
**
**
***
WT
vWF OE
PHKG1 KD
vWF OE+
CP-91149
(F)
Glycogen assay
OD 620nm (% of control)
2.0
1.5
1.0
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WT
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vWF OE+
CP-91149
(G)
Tumor section
WT
vWF OE
PHKG1 KD
vWF OE+CP-91149
PAS

FIGURE 7 Inhibition of phosphorylase kinase subunit G1 (PHKG1) could reverse the effect of von Willebrand factor (vWF) overexpression on lung adenocarcinoma metastasis in vivo. A, B, Pulmonary and liver metastasis was assessed after LLC cells injection through the lateral tail vein ($n = 5$/group). Concentration of CP-91149 was 40μg/mouse. Metastasis was analyzed 21 d after injection of Lewis lung carcinoma (LLC) tumor cells. C, Representative histologic evidence from tumor sections of the different groups. Four percent of paraformaldehyde–embedded lungs of all mice were cut completely, stained with hematoxylin and eosin, examined histologically, and detected by microcopy. D, E, Image (D) and weight (E) of xenograft tumors. F, G, Glycogen assay and PAS staining of xenograft tumors. Scale bar: 100 μm. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

a metastatic advantage to cancer cells by enhancing their platelet adhesion capacity and migration ability.

To identify the key regulatory proteins and pathway of the platelet-vWF interaction in tumor cells, we performed whole-transcriptome sequencing and found that PHKG1 was essential to platelet–tumor cell–derived vWF interaction–mediated lung tumor metastasis (Figure 4A-F). PHKG1 is a catalytic subunit (γ subunit), and its upregulation leads to increased PhK activity.[17] PhK, as the only enzyme known to catalyze glycogen phosphorylase activation, plays a key role in glycogenolysis regulation.[15] Recently, the importance of metabolism in maintaining the tumorigenic state has become clearer,[35,36] and glycogen metabolism plays a critical role in cancer development.[18] PHKG1 is amplified in a variety of tumor types, including lung cancer,[16,28,29,37] suggesting that upregulation of PHKG1 might be associated with cancer progression.[16] Furthermore, some studies have indicated that glycogen phosphorylase inhibition in cancer cells reduces metastasis.[38] We found that PHKG1 led to a poor prognosis and promoted tumor migration (Figure 4G-J).

We further explored the correlation between lung cancer–derived vWF and PHKG1 and found that PHKG1 was a downstream target of lung cancer–derived vWF and is positively regulated by vWF (Figure 5A–E). Moreover, the upregulation of PHKG1 expression caused by vWF further promoted glycogenolysis and glycolysis (Figure 5H–J). The glycogen stored in the cell entered into the glycolytic pathway to provide a large amount of energy for the subsequent life activities of lung cancer cells.[39,40] It provided a reasonable explanation for the previously discovered overexpression of vWF to promote lung cancer migration. KEGG analysis showed that these downregulated genes, including PHKG1, were closely related to the PI3K/AKT signaling pathway (Figure 4F). Our research verified that tumor-derived vWF could regulate PHKG1 expression through inducing the phosphorylation of the PI3K/AKT signaling pathway in lung cancer cells (Figure 5F, G).

Interestingly, vWF overexpression could effectively increase glycogenolysis, lactate production, and cell migration. However, this promotion was inhibited after knocking down PHKG1 or adding the glycogen phosphorylase inhibitor CP-91149[30,41] (Figure 6B–G). To further explore the effect of lung cancer–derived vWF on tumor metastasis, we used $vWF^{-/-}$ mice, which were often used in the research of vWF function in tumor development and metastasis.[42,43] Consistent with the in vitro observations, in the experimental metastasis model and xenograft model, adding CP-91149 could effectively inhibit tumor metastasis in mice caused by lung cancer–derived vWF (Figure 7).

Therefore, our results provide evidence for the mechanism of lung cancer–derived vWF regulating PHKG1-mediated tumor metastasis in the presence of platelets. As shown in the graphical abstract, we inferred that after cancer cells invade into blood vessels, cancer cell–derived vWF binds to platelet GPIbα to form tumor thrombi, which can facilitate tumor cells' evasion of the killing effect of tumor killer cells in the blood and tumor cells' adherence to HUVECs, which advances the invasion. At the same time, in the presence of platelets, lung cancer–derived vWF induces the secretion of PHKG1 in tumor cells by activating the PI3K/AKT signaling pathway. The upregulation of PHKG1 expression promotes glycogen metabolism in tumor cells, and glycogen enters the glycolytic pathway to provide a large amount of energy required for migration and invasion, thereby promoting tumor metastasis. Here, we proved that lung cancer cells could express vWF de novo, and LUAD-derived vWF could affect cell glycogen metabolism by upregulating PHKG1, thereby promoting the metastasis of LUAD cells. Our study is a further exploration into the molecular mechanism of the platelet-vWF interaction in tumor metastasis and suggests potential targets for the inhibition of tumor metastasis. Therefore, developing new specific inhibitors targeting tumor cell–derived vWF or blocking glycogen mobilization in lung tumor cells with PHKG1 inhibitors may be a new therapeutic strategy for LUAD metastasis treatment.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

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