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Original Articles

PBK overexpression promotes metastasis of hepatocellular carcinoma *via* activating ETV4-uPAR signaling pathway

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ABSTRACT

Invasion and metastasis are the predominant causes of lethal outcomes in patients with hepatocellular carcinoma (HCC). However, the molecular mechanism underlying the invasive or metastatic process are still insufficiently understood. Here, we first integrated several public databases and identified a novel protein kinase, PDZ-binding kinase (PBK) that was frequently upregulated and correlated with poor prognosis in patients with HCC. Gain- or loss-of-function analysis revealed that PBK promoted migration and invasion of HCC cells both *in vitro* and *in vivo*. Mechanistically, PBK enhanced uPAR expression by activating its promoter activity. Chromatin immunoprecipitation (ChIP) assay showed that ETV4 directly bound to the core region of uPAR promoter while PBK could enhance the binding of ETV4 to uPAR promoter. In orthotopic mouse model, PBK knockdown markedly inhibited the lung metastasis of HCC cells, while this effect was significantly restored by uPAR overexpression. Finally, there was a positive correlation between PBK and uPAR, ETV4 and uPAR in HCC clinical samples. Collectively, these findings revealed that PBK acted as a crucial kinase by promoting invasion and migration via the ETV4-uPAR signaling pathway, and it therefore could be a promising diagnostic biomarker and therapeutic target for HCC metastasis.

1. Introduction

Despite new advances in treatment and diagnostic technology over recent decades, liver cancer remains the third leading cause of cancer-related mortality worldwide, with approximately 2.1 million new cases in 2018 [1]. Hepatocellular carcinoma (HCC), accounting for 85%–90% of primary liver cancer, is characterized by well-perfused vascularity, frequent intrahepatic recurrence, and extrahepatic metastasis, thus making the poor prognosis of these patients [2]. Indeed, metastasis, contributing to the major cause of HCC-related mortality, is normally formed by a series of complex processes of invasion-metastasis cascades [3]. Therefore, better understanding the predictive markers and molecular mechanisms of the progression and metastasis in HCC is of great importance to improve the prognosis and treatment.

Protein kinases are critical for regulating the majority of signal-transduction pathways, especially those participated in fundamental cellular processes [4,5]. For kinases involved in cancer, deregulated activity or expression of several of these kinases is often correlated with uncontrolled proliferation and metastatic development [6,7]. PBK (PDZ-binding kinase), a novel serine-threonine kinase, belongs to a member of mitogen-activated protein kinase (MAPK) family and functions as an upstream kinase for transforming oncogenes in some key signaling pathways [8,9]. Physiologically, PBK plays a critical role in the regulation of cytokinesis as well as DNA damage and repair through phosphorylation of different histone targets [10,11]. Cumulative evidences indicate that elevated expression of PBK contributes to the tumorigenesis or metastasis in various cancers [12,13], probably due to the undetected PBK expression in most normal tissues [14]. Additionally, PBK knockdown or treatment with a PBK inhibitor exhibits decreased tumorigenicity and growth inhibitory effects in some human malignancies [15,16]. Although PBK has been identified as an attractive molecular target for several types of cancers, the biological functions of PBK and the molecular mechanisms regarding these functions in HCC metastasis have not been thoroughly characterized to date.

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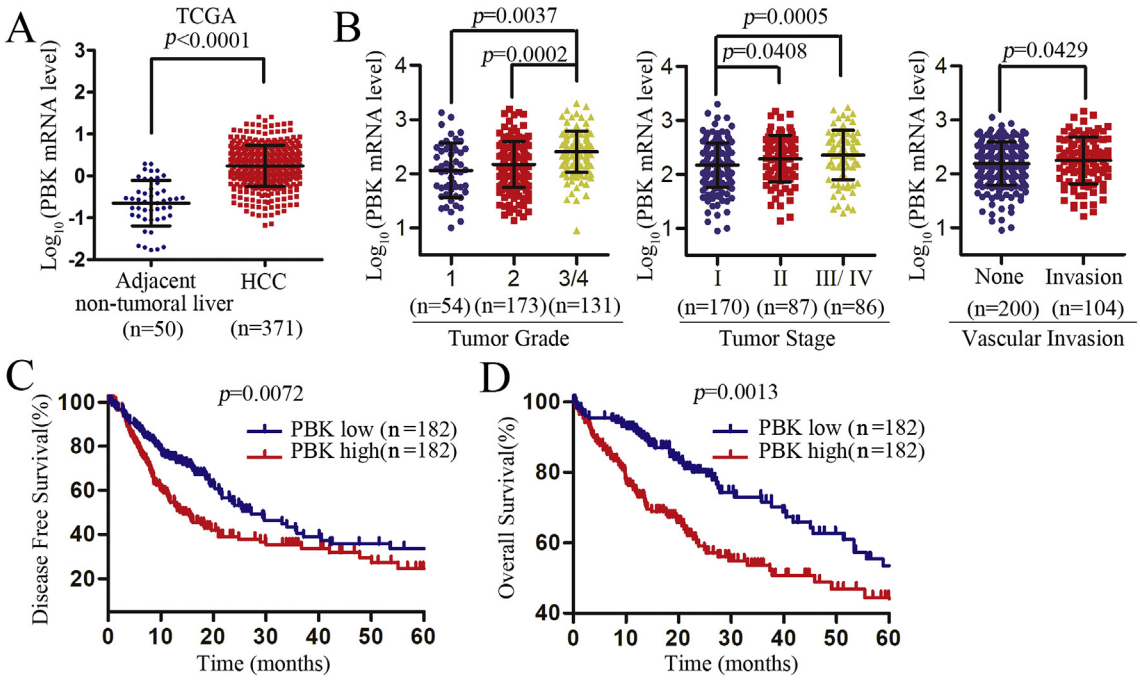

Fig. 1. PBK mRNA expression and clinicopathological analysis in HCC tissues from different public databases.
(A) The mRNA level of PBK from RNA-sequencing data on 50 primary human HCC tissues and 371 adjacent nontumor liver tissues of TCGA. (B) The mRNA level of PBK in HCC samples and their adjacent nontumor in TCGA cohort with tumor grade, tumor stage and vascular invasion, (C) Correlation between PBK expression and disease-free survival in TCGA HCC cohort. (D) Correlation between PBK expression and overall survival in TCGA HCC cohort.

Urokinase-type plasminogen activator (uPA) and its receptor, uPAR, are involved in the fibrinolytic system, and play a pivotal role in physiological functions and pathological processes [17]. By binding to the receptor uPAR, uPA activates some proteases related to the pericellular plasmin formation and extracellular matrix degradation, providing a key basis for tumor invasion and migration [18]. Numerous studies have confirmed that increased expression of uPAR is most likely to be associated with the aggressive phenotype in different advanced cancers [19–21]. Conversely, interference with the uPA/uPAR system appears to show the decreased propensity for invasion and metastasis in various tumors [22,23], suggesting uPA/uPAR as a potential biomarker for cancer diagnosis. In addition, ETV4, a key member of ETS transcription factor family, is identified as being downregulated following the uPA knockdown, which is known to be frequently overexpressed in advanced tumors with adverse prognosis [24,25]. Despite these reports highlight a critical role of ETV4 and uPAR in tumor development and progression, no studies have focused on the associations between PBK and ETV4/uPAR expression in HCC, and if so, the molecular mechanisms through which PBK regulates the ETV4/uPAR signaling pathway activation need further investigation.

In the present study, we describe the functional roles of PBK in HCC *in vitro* and *in vivo*. Moreover, we report the relationship between PBK and ETV4 or uPAR expression in HCC for the first time. Of note, we explore a novel molecular mechanism whereby the ETV4/uPAR signaling pathway is activated in the presence of PBK during the HCC metastasis, thereby providing a new potential therapeutic target for liver cancer.

2. Materials and methods

2.1. Public database and bioinformatics analysis

RNA sequencing data and patients' clinical pathological characteristics and survival outcomes were downloaded from TCGA (https://cancergenome.nih.gov/) Liver Hepatocellular Carcinoma (LIHC) dataset. The data from TCGA were log10 transformed and analyzed using GraphPad Prism7 software. In addition, Oncomine database (https://www.oncomine.org) was also used to analyze the differential expression levels of PBK between HCC and normal groups.

2.2. Plasmids and antibodies

PBK short hairpin RNA (shPBK-1 and shPBK-2) or nontargeting shRNA (shCont) were purchased from Shanghai Genechem Company Limited. Sequences of shPBK-1 and shPBK-2 targeting shRNA are 5′-CTTCTCTGTATGCACTAAT-3′ and 5′-CTGTGTCTTGCTATGGAAT-3′, respectively. Sequences of shCont is 5′-GCAACAAGATGAAGAGCAC CAA-3'. The plasmids used in our study including ETV4 expression vector, PBK expression vector and uPAR expression vector were obtained from OriGene. Anti-PBK (#4942), Anti-uPAR (#12863) were purchased from Cell Signaling Technology. Anti-ETV4 (sc-113) and Anti-GAPDH (sc-365062) were obtained from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were purchased from GE Healthcare UK Limited.

2.3. HCC specimens

Tumorous liver tissues and the corresponding adjacent nontumoral liver tissues were collected from 48 patients who underwent curative surgery for HCC at the First Affiliated Hospital of Chongqing Medical University in Southwest China. The patients were not subjected to any form of chemotherapy prior to the surgery. Informed consent was obtained from each patient recruited, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of Chongqing Medical University. Total RNA and proteins were acquired from these samples.

2.4. Cell culture

HepG2, Hep3B, SK-Hep-1 and PLC/PRF/5 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Huh-7 cell

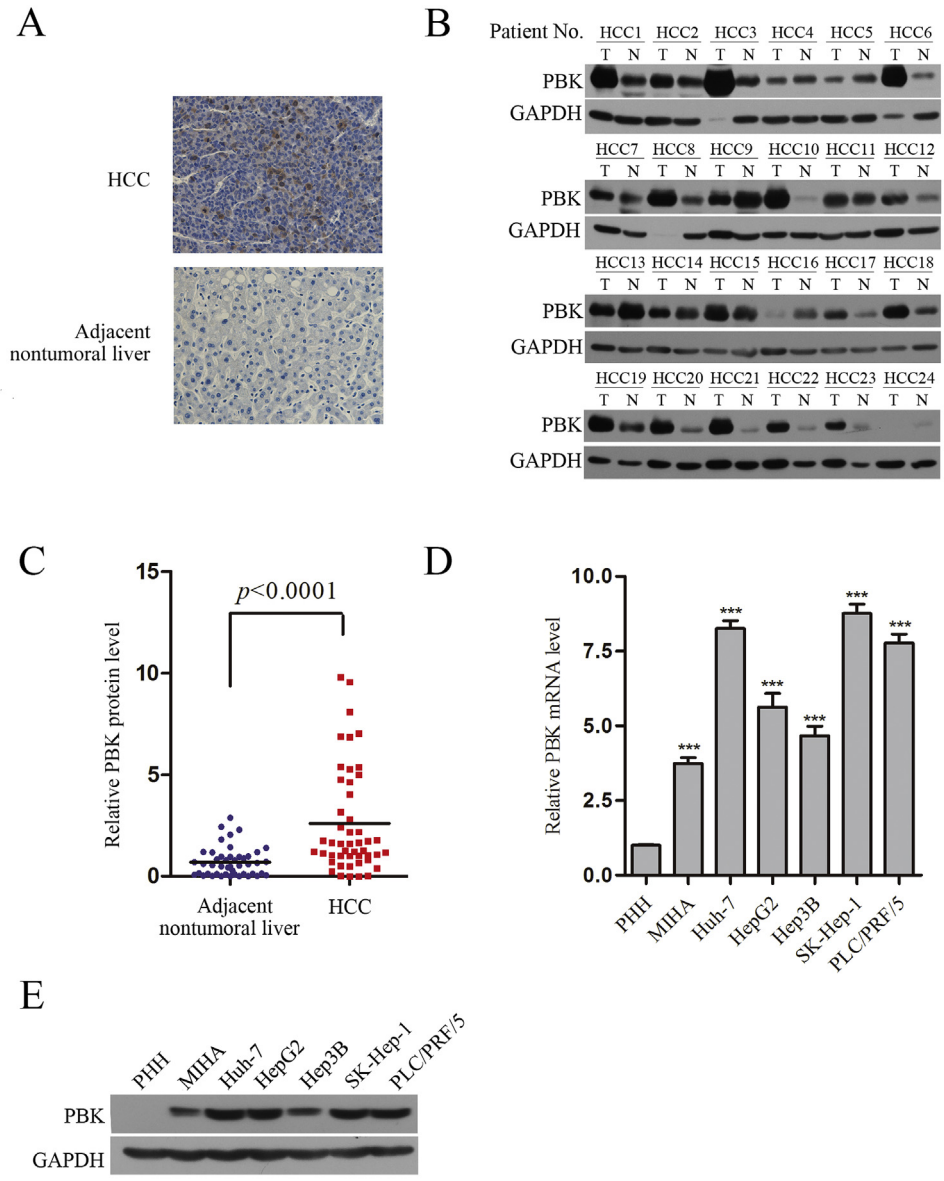

Fig. 2. PBK expression in HCC tissues and different HCC cell lines.
(A) IHC analysis of PBK protein expression in HCC samples and their corresponding non-tumoral liver tissues. Magnification, × 400. (B) Western blotting analysis of PBK in 24 out of 48 paired HCC tissues (T) and their adjacent non-tumor liver tissues (N). GAPDH was used as a loading control. (C) Quantitative analysis of PBK protein levels in 48 paired HCC tissues. (D, E) The mRNA and protein levels of PBK in different HCC cell lines. β-Actin was used as an internal control for qRT-PCR. ***$P < 0.001$, versus the PHH group. GAPDH was used as a loading control for western blotting analysis.

line was obtained from the Health Science Research Resource Bank (Osaka, Japan). MIHA cell line was obtained from Professor Ben C.B.Ko (The Hong Kong Polytechnic University). MIHA, Huh-7, Hep3B, SK-Hep-1 and PLC/PRF/5 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Gibco BRL). HepG2 was maintained in Eagle's minimum essential medium containing 10% FBS. Primary human hepatocytes (PHH) were purchased from Sciencell Research laboratories and cultivated in hepatocyte medium (Sciencell). All cells were maintained at 37 °C in 5% CO2. Cells were authenticated by short-tandem repeat (STR) fingerprinting by Beijing Microread Genetics Company Limited recently.

2.5. Western blotting analysis

Total proteins were extracted from cells and tissues using RIPA lysis buffer with a protease inhibitor cocktail (Roche, Germany). Protein lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then blocked with 5% nonfat milk for 1.5 h and incubated with primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies. The bolts were developed using ECL Western blotting reagents (Millipore, USA). GAPDH was used as a loading control.

2.6. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was prepared using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Relative quantification of gene expression level was conducted using FastStart Universal SYBR Green Master Mix (Roche, Germany) with β-actin mRNA as an endogenous control. The expression values of target genes were calculated using the $2^{--\triangle\triangle Ct}$ method. The sequences of gene-specific primers were listed in Supplementary Table SI.

2.7. Wound-healing assay

Cells with different treatments were seeded into six-well plates. After cells reached 90–95% confluence, wounds were made with a pipette tip by scratching of the cell monolayer. PBS was used to wash the wounded monolayers and remove cell debris. At 0 and 24 h after wounding, the distance between the wound edges was measured to assess the ability of cell motility.

Table 1
Correlation of clinicopathological features and PBK expression.

Clinicopatholigic Parameters	No.of Specimens	PBK Expression (Tumor/Nontumoral)		P Value
		Low	High	
Sex				
Female	6	4	2	0.09
Male	42	12	30	
Age (mean ± SD)		52.4 ± 16.80	52.8 ± 14.05	0.93
ALT				
≤40 IU/L	28	11	17	0.36
> 40 IU/L	20	5	15	
Carcino-embryonic antigen (CEA)				
≤5 ng/mL	41	13	28	0.67
> 5 ng/mL	7	3	4	
α-fetoprotein (AFP)				
≤20 ng/mL	28	11	17	0.36
> 20 ng/mL	20	5	15	
Tumor size				
≤3 cm	13	5	8	0.74
> 3 cm	35	11	24	
Multiple tumor				
No	43	15	28	0.65
Yes	5	1	4	
Grade				
1	7	5	2	0.05
2	29	9	20	
3	12	2	10	
Vascular invasion				
No	25	12	13	0.03
Yes	23	4	19	
Cirrhosis				
No	14	2	12	
Yes	34	14	20	0.10
HBV				
No	24	11	13	0.12
Yes	24	5	19	

NOTE: High tumoral PBK expression was considered > 1.5-fold upregulation relative to the adjacent nontumoral liver.

2.8. In vitro migration and invasion assay

Cell metastasis ability was assessed by the transwell migration and invasion assays. The invasion assay used inserts coated with Matrigel (Corning Life Sciences, #353097), while the migration assay used non-coated inserts (BD Biosciences, #354480), Briefly, 5×10^4 cells suspended in 350 μL of serum-free DMEM were added to the upper chamber, whereas 900 μL culture medium containing 10% FBS was placed in the lower chamber. After 12 h incubated, cells migrated through the membrane to the bottom chamber were fixed and stained with 0.1% crystal violet. Twenty minutes after staining, cells were calculated and imaged under the microscope.

2.9. Cytoskeletal staining

To label the cell cytoskeleton through the binding of phalloidin to F-actin, the cells following different treatments were seeded on coverslips and incubated for 24 h at 37 °C. Subsequently, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS and permeabilized using 0.5% Triton X-100 for 20 min. After PBS washing, F-actin was stained with DyLight™ 488 Phalloidin (CST, #12935; dilution 1:40) for 15 min at room temperature according to the manufacturer's instructions. The cells were then incubated with 4′,6-diamidino-2- phenylindole (DAPI) for nuclear counterstaining and images were captured by a Laser Scanning Confocal Microscopy (Leica TCS SP2).

2.10. Cell proliferation

Cell proliferation in response to PBK silencing and overexpression was determined by trypan blue exclusive assay and cell counting kit-8 (CCK-8) assay. For trypan blue exclusive assay, transfected cells were reseeded in 6 well plates and counted every 24 h for seven consecutive days. For CCK-8 assay, 1×10^3 treated cells were seeded in 96 well plates. CCK-8 working solution (MedChemExpress, #HY-K0301) was added to each well and mixture was incubated for another 3 h. The optical density (OD) values were measured every day with a microplate reader (Bio-Tek) at 450 nm. Cell viability was expressed as a multiple of the absorbance at the first day from three replicates in three independent experiments.

2.11. Colony formation assay

Transfected SK-Hep-1, Huh-7 or MIHA cells were seeded in 6 well plates and newly formed colonies were stained with 1% crystal violet solution after 10 days incubation. Colonies were scored and results from duplicate assays were expressed as the mean from three independent experiments.

2.12. Apoptosis assay

PBK-depleted cells were treated with 6 μg/ml sorafenib for 48 h and stained using Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology) according to the manufacturer's instruction. The percentage of apoptosis cells was tested by Flow Cytometry (BD Accuri C6).

2.13. Luciferase reporter assay

Distinct lengths of the uPAR promoter fragment were subcloned into the pGL3-basic vector to produce pGL3-1249, pGL3-1080, pGL3-884, pGL3-650, pGL3-475 and pGL3-338. These different vectors were co-transfected with shCont or shPBK. The pRL-TK vector was used to normalize the transfection efficiency. After transfection for 36 h, the cells were lysed for luciferase activity measurement by using the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The specific primers were designed by our group and the sequences are shown in Supplementary Table SI.

2.14. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed with genomic DNA samples from cross-linked cells using a specific antibody according to the manufacturer's protocol (Millipore). Briefly, cells were treated with 1% formaldehyde to crosslink protein and DNA to the uPAR promoter, and sonication was used to make small DNA fragments (200–1000 bp). The supernatants were incubated with the immunoprecipitating antibody and protein A/G magnetic beads at 4 °C overnight. Subsequently, the free DNA was retrieved from Protein/DNA complexes and purified using spin columns. The region 2 of the uPAR promoter was amplified from the immunoprecipitated DNA samples by qRT-PCR using the sense primer F 5′-CCAAAATGGAGGGCTCAACA-3′ and the antisense primer R 5′-AATGTGGCTGATTATTTGGC-3’.

2.15. In vivo xenograft model

All animal studies were conducted according to the guidelines for the use of laboratory animals of Chongqing Medical University [SCXK (JING) 2014–0004]. Male BALB/c nude mice (4-week age) were orthotopically injected in the liver with 1×10^6 treated Huh-7 cells in 50 μL DMEM/Matrigel (1:1 mixture). The mice were sacrificed after 4 weeks of treatment, and the liver was harvested, weighed, and photographed. The tumor volume was measured with calipers and calculated

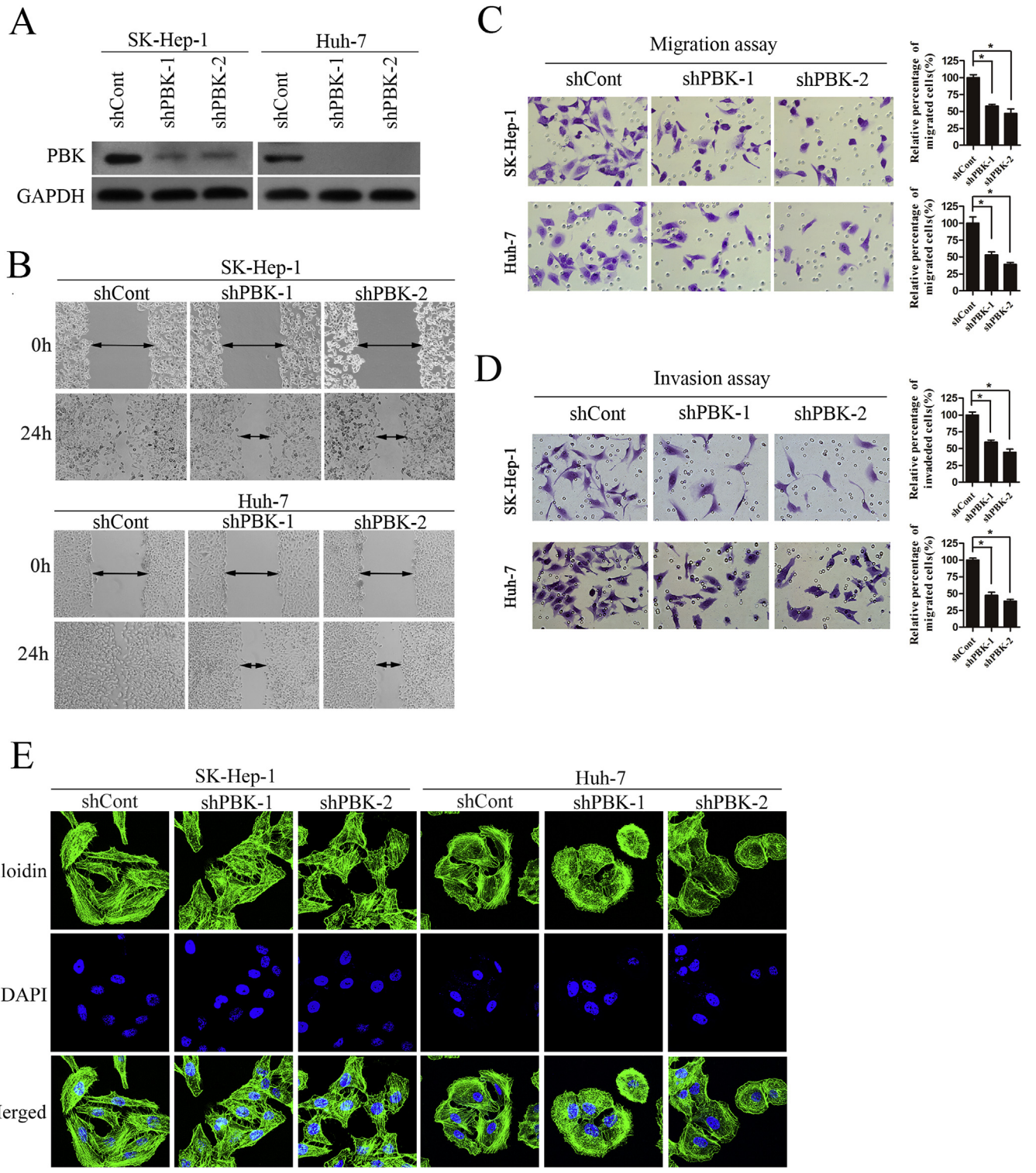

Fig. 3. PBK knockdown inhibited HCC cells migration and invasion *in vitro*.
(A) Western blotting analysis revealed that PBK in SK-Hep-1 and Huh-7 cells was significantly inhibited by PBK short hairpin RNA (shPBK-1 and shPBK-2) versus nontargeting shRNA (shCont). GAPDH was used as a reference gene. (B) Wound-healing assay revealed that downregulation of PBK dramatically decreased the cell mobility of SK-Hep-1 and Huh-7 cells. (C) Migrated ability was assessed by transwell migration assay in PBK-depleted SK-Hep-1 and Huh-7 cells. $^{*}P < 0.05$, versus the control. (D) Invasive ability was assessed by transwell invasion assay in PBK-depleted SK-Hep-1 and Huh-7 cells. $^{*}P < 0.05$, versus the control. (E) F-actin staining of PBK-depleted SK-Hep-1 and Huh-7 cells with DyLight™ 488 Phalloidin (green). Nucleus was stained with DAPI (blue). Magnification, × 630. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

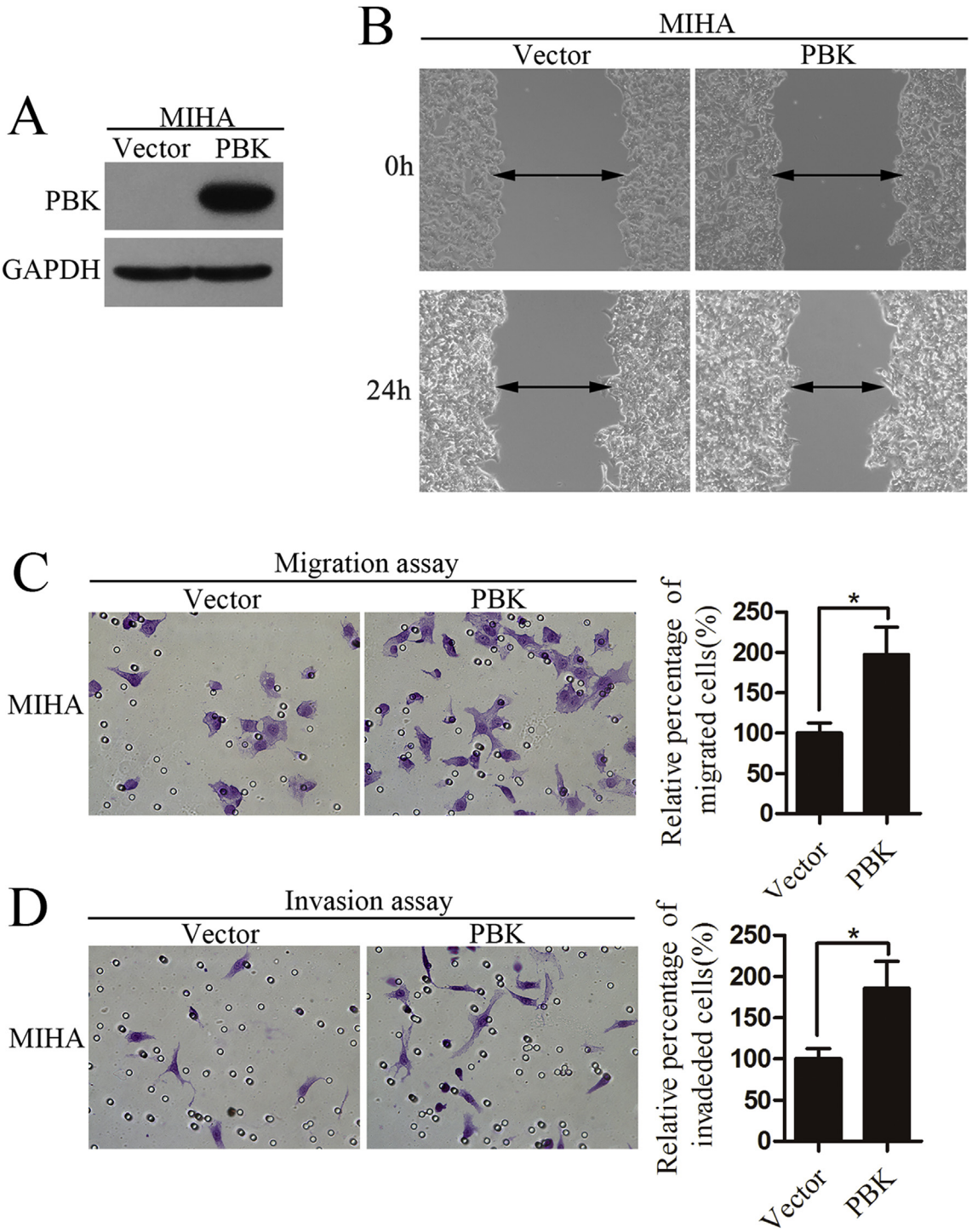

Fig. 4. PBK overexpression promoted MIHA cells migration and invasion *in vitro*.
(A) The efficacy of PBK overexpression was examined by western blotting analysis in MIHA cells. GAPDH was used as a reference gene. (B) Impairment of wound-healing ability was examined in MIHA cells with PBK overexpression. (C) Migrated ability was assessed by transwell migration assay in MIHA cells with PBK overexpression. (D) Invasive ability was assessed by transwell invasion assay in MIHA cells with PBK overexpression. *$P < 0.05$.

according to the following formula: Tumor volume (mm^3) = (length × $width^2$) × ½, as previously reported [26]. The liver tumor tissues were homogenized to detect the expression of PBK, ETV4 and uPAR. The presence or absence of metastatic nodules in the lung was histologically evaluated using Hematoxylin-Eosin staining after 8 weeks of injection.

2.16. Hematoxylin-Eosin staining and immunohistochemistry

Liver tissues taken from mice were fixed in 4% paraformaldehyde for 48 h, dehydrated and embedded in paraffin. Tissue sections, each measuring 3–5 μm in thickness, were cut from the paraffin blocks and performed with Hematoxylin-Eosin for nuclear counterstaining and immunohistochemistry for PBK staining. For immunohistochemistry, the tissues were microwaved-heated in sodium citrate buffer (10 mmol/

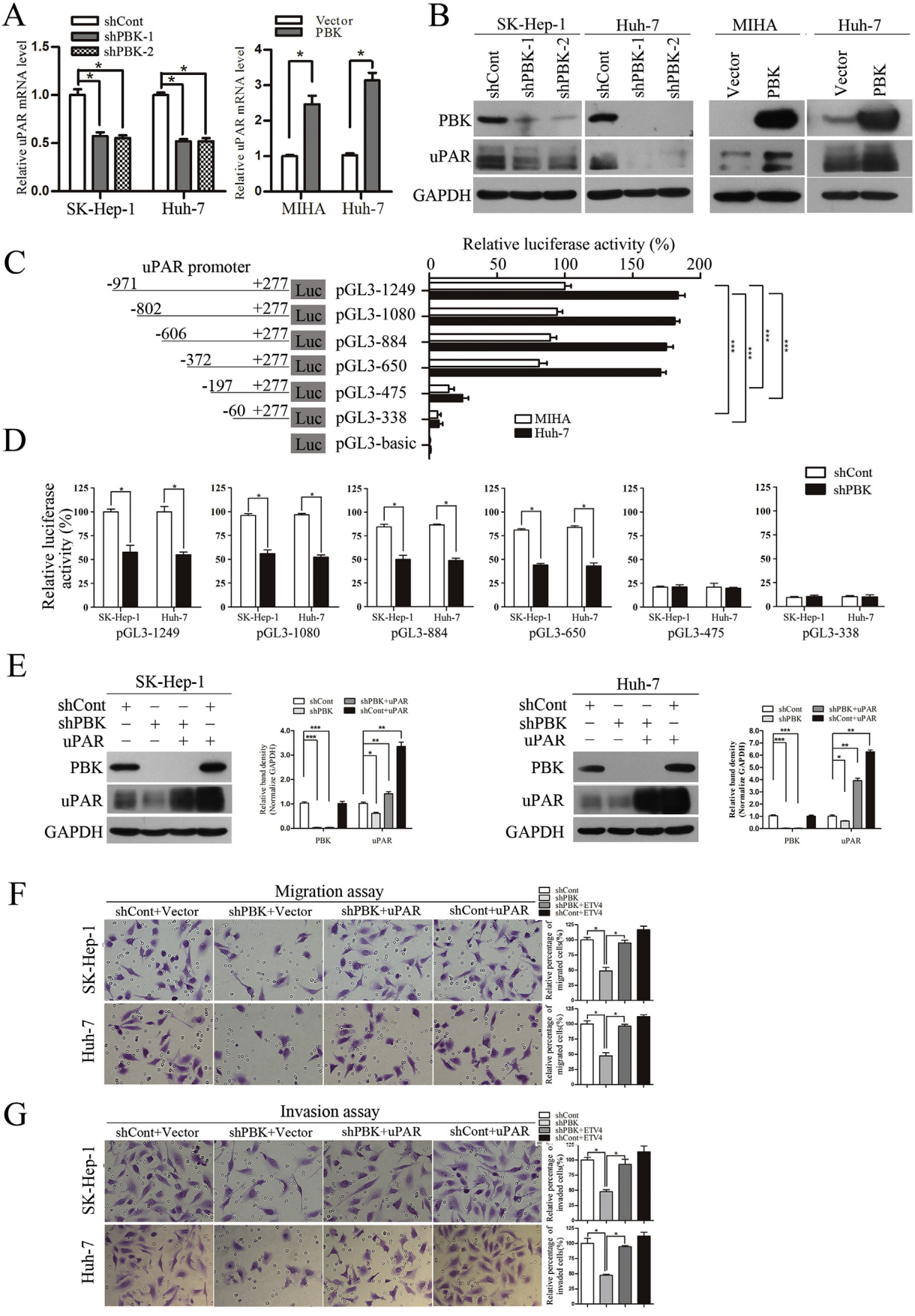

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Fig. 5. PBK enhanced uPAR expression via activating its promoter activity.
(A–B) PBK regulated uPAR mRNA (A) and protein (B) expression in SK-Hep-1, Huh-7 and MIHA cells. β-Actin was used as an internal control for qRT-PCR and GAPDH was used as a loading control for western blotting analysis. $^{*}P < 0.05$. (C) uPAR promoter activity was examined by using dual-luciferase reporter assay. Huh-7 and MIHA cells were transfected with pGL3-basic or reporter constructs containing various lengths of uPAR promoter region. Vs. −971/+227 (pGL3-1249) group. $^{***}P < 0.001$. (D) PBK depletion reduced uPAR promoter activity. $^{*}P < 0.05$. (E–G) Enrichment of uPAR significantly rescued the effects of PBK knockdown in SK-Hep-1 and Huh-7 cells for both migration and invasion. Intensities of bands were normalized to the amount of GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

L, pH 6.0) for antigen retrieval, permeabilized using 0.5% Triton X-100 for 20 min, and quenched with 3% H_2O_2 to block endogenous peroxidase activity for 10 min. Next, the slides were incubated with primary PBK antibody (diluted at 1:100) overnight at 4 °C and subsequently with secondary antibodies for 1 h. The sections were incubated with diaminobenzidine (DAB) for immunoreactivity detection. The analysis was performed by two independent pathologists, as previously described [27]. HScore was calculated based on the intensity of the staining and the percentage of positive cells per the formula: HScore = 1 × (% light staining) + 2 × (% moderate staining) + 3 × (% strong staining). HScores ranged from 0 to 300 The proportion of tumor cells with positive nuclear staining was scored as follows: 0 (< 10%); 1 (10%–30%); 2 (30%–50%); and 3 (> 50%) [28].

2.17. Statistical analysis

The nonparametric χ^2 test and Spearman's σ rank test were used to evaluate the correlation between PBK expression and the clinicopathologic parameters. Survival rates for PBK expression were estimated using the Kaplan–Meier's method. Equivalences of the survival curves were tested by log-rank statistics. Correlation analysis between PBK, ETV4 and uPAR mRNA in HCC specimens from TCGA dataset was analyzed by Pearson rank test. For cell model data, results are expressed as mean ± SD from three independent experiments. Statistics were performed with the non-parametric Mann-Whitney *U* test. A value of $p < 0.05$ was considered significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) All analyses were carried out using SPSS statistical software (SPSS version 19.0, Chicago, IL, USA).

3. Results

3.1. PBK overexpression was positively correlated with poor prognosis in HCC

To rapidly prioritize kinases for HCC progression and metastasis, we analyzed gene expression from RNA-sequencing data on 50 primary human HCC tissues and 371 adjacent non-tumoral livers of The Cancer Genome Atlas (TCGA), and found that PBK in HCC tissues was significantly upregulated compared with the adjacent non-tumoral tissues (Fig. 1A). Moreover, PBK expression was significantly correlated with poor differentiation, aggressive tumor stage and vascular invasion in TCGA data set (Fig. 1B). Analysis of disease-free and overall survival using the Kaplan–Meier method revealed HCC patients with high PBK expression had a shorter disease-free survival and overall survival rate than those with low PBK expression (Fig. 1C). The level of PBK mRNA in HCC samples was further analyzed from Oncomine database. Consistently, high-expression of PBK was found in different HCC cohorts from Wurmber, Roessler, Chen and Mas liver (Supplementary Fig. 1A). Furthermore, we found that high PBK mRNA level was closely associated with tumor grade and vascular invasion in Wurmbach liver cohort (Supplementary Fig. 1B).

To further validate this observation, we detected PBK protein expression in 48 clinical primary HCC samples and their adjacent nontumoral tissues collected in our group. Immunohistochemical analysis showed increased expression of PBK in 54.1% (26/48) of HCC samples, with being generally localized to the cytoplasm and nucleus of the tumor cells (Fig. 2A). Western blotting analysis further showed that a marked upregulation of PBK was detected in most of the HCC cases (32/48, 66.67%) (Fig. 2B and Supplementary Fig. 2). Moreover, the average level of PBK protein in HCC was found to be significantly higher than that in nontumoral liver (Fig. 2C). In addition, clinicopathological analysis confirmed that increased PBK protein level was correlated with poor differentiation and vascular invasion (Table 1). Subsequently, the mRNA and protein levels of PBK were examined in a panel of liver cancer cell lines (Huh-7, HepG2, Hep3B, SK-Hep-1 and PLC/PRF/5), immortalized liver cell lines (MIHA) and primary human hepatocyte (PHH). Five liver cancer cell lines showed higher level of PBK compared with MIHA cells while PHHs exhibited almost hardly detected level of PBK (Fig. 2D and E). Taken together, these findings suggested that PBK was frequently upregulated in clinical HCC samples and liver cancer cells lines.

3.2. PBK knockdown inhibited HCC cells migration and invasion in vitro

The above data revealed that PBK might play a critical role in HCC metastasis, and we therefore first silenced endogenous PBK expression in two HCC cell lines (SK-Hep-1 and Huh-7, showing relatively higher level of PBK in the above-mentioned cell lines) by using lentivirus expressing shRNA targeting PBK (shPBK-1 and shPBK-2) (Fig. 3A). To assess the cell mobility, we performed wound-healing assay in SK-Hep-1 and Huh-7 cells, and uncovered that the mobility of PBK-silenced cells was significantly decreased relative to the control cells (Fig. 3B). To evaluate the effect of PBK in cell migration and invasion, transwell assay were utilized and found that PBK knockdown could significantly attenuate the migrated ability of HCC cells (Fig. 3C). Moreover, knockdown of PBK also impaired cell invasion through Matrigel (Fig. 3D). Actin filaments are reported to play an important role in promoting cell invasion and therefore we examined the formation of actin by using phalloidin staining. The result showed that the majority of PBK-depleted cells lost their actin filaments as compared with that in control cells (Fig. 3E). Additionally, we further examined the effect of PBK knockdown on cell proliferation using trypan blue exclusive assay, cell counting kit-8 assay (CCK-8) and colony formation assay in SK-Hep-1 and Huh-7 cells. Our results revealed that PBK silencing had no significant effect on cell growth in above-mentioned cell lines (Supplementary Figs. 3A–C). Moreover, we also examined whether PBK knockdown would alter sorafenib sensitivity of HCC cells. PBK knockdown could not alter the cellular susceptibility of SK-Hep-1 and Huh-7 cells to sorafenib treatment which was evidenced by using CCK-8 assay (Supplementary Figs. 4A and B). Flow cytometry also indicated that PBK depletion could not significantly increase the apoptotic rate of sorafenib-treated cells (Supplementary Fig. 4C). Collectively, these data indicated that PBK could enhance the ability of HCC migration and invasion without promoting proliferation.

3.3. PBK overexpression promoted MIHA cells migration and invasion in vitro

To further elucidate the role of PBK in HCC progression and metastasis, the immortalized liver cell line (MIHA) was transiently transfected vector expressing PBK (Fig. 4A). PBK overexpression enhanced wound healing capacity and migrated ability of MIHA cells, as evidenced by wound-healing and transwell migration assays (Fig. 4B and C). Consistently, PBK overexpression in MIHA cells could promote the

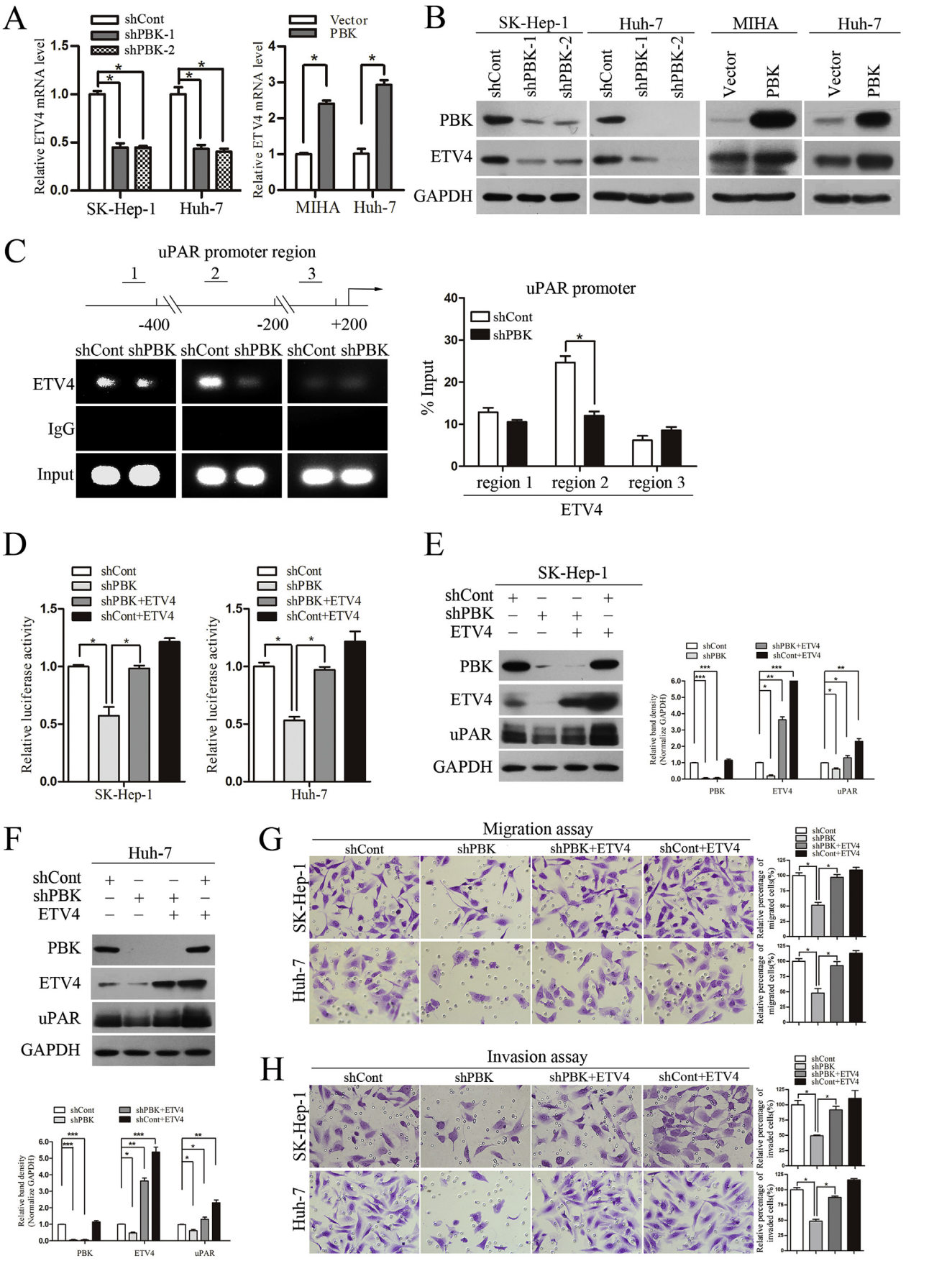

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Fig. 6. PBK activated uPAR transcription by promoting the binding of ETV4 to uPAR promoter region.
(A, B) PBK regulated ETV4 mRNA (A) and protein (B) expression in SK-Hep-1, Huh-7 and MIHA cells. β-Actin was used as an internal control for qRT-PCR and GAPDH was used as a loading control for western blotting analysis. $^{*}P < 0.05$. (C) The interaction between ETV4 and the regulatory region of uPAR promoter was confirmed by ChIP assay. ChIP assay with anti-ETV4 was performed in SK-Hep-1 cells tranfected with plasmids expressing shPBK or shCont. IgG served as an internal control. $^{*}P < 0.05$. (D) Upregulation of ETV4 could significantly enhance the luciferase activity of uPAR promoter in PBK-silencing SK-Hep-1 and Huh-7 cells. $^{*}P < 0.05$. (E–G) Enrichment of ETV4 significantly rescued the effects of PBK knockdown in SK-Hep-1 and Huh-7 cells for both migration and invasion. Intensities of bands were normalized to the amount of GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

invasive ability through Matrigel as determined by transwell invasion assay (Fig. 4D). However, elevated expression of PBK could not significantly promote the cell proliferation in MIHA cell line (Supplementary Figs. 5A–C). Collectively, PBK overexpression accelerated MIHA cells migration and invasion *in vitro*.

3.4. PBK enhanced uPAR expression via activating its promoter activity

To explore the underlying mechanism by which PBK regulate migration and invasion in HCC cells, we first screened 84 representative metastasis-related genes in PBK-depleted HCC cells by using Human Tumor Metastasis PCR Array. Among the altered genes that are involved in regulation of metastasis, ETV4, uPAR and CXCR4 were the leading genes that was significantly downregulated in PBK-depleted Huh-7 cells (Supplementary Fig. 6A). The protein levels of ETV4, uPAR and CXCR4 were further confirmed by using western blotting analysis and showed that PBK knockdown markedly inhibited ETV4 and uPAR protein levels without affecting CXCR4 protein (Supplementary Fig. 6B). Therefore, we focus on uPAR and ETV4 for further investigation.

Consistently, gene silencing of PBK inhibited both mRNA and protein levels of uPAR in Huh-7 and SK-Hep-1 cells, while PBK overexpression promoted uPAR mRNA and protein expression in MIHA and Huh-7 cells (Fig. 5A and B). Together, these data suggest PBK might regulate uPAR expression in transcription-dependent manner. To test this hypothesis, various lengths of the uPAR 5′-flanking region, including −971/+227 (pGL3-1249), −802/+277 (pGL3-1080), −606/+277 (pGL3-884), −372/+277 (pGL3-650), −197/+277 (pGL3-475) and −60/+277 (pGL3-338), were cloned and transiently transfected into Huh-7 and MIHA cells. A dual-luciferase reporter assay revealed that all six uPAR promoter-driven luciferase constructs displayed higher luciferase activities in Huh-7 cells than those in MIHA cells. Notably, construct pGL3-1249 exhibited maximum luciferase activity, while the luciferase activity was sharply reduced in pGL3-475 (Fig. 5C), suggesting that the −372/-197 region might be the core region of uPAR promoter. Furthermore, the inhibitory effect of PBK knockdown on uPAR promoter was abolished in construct pGL3-475, suggesting that the region regulated by PBK is located at −372/-197 of uPAR promoter (Fig. 5D). Next, we determined whether uPAR plays a major role in the PBK-mediated regulation of migration and invasion of HCC cells. Impaired migration and invasion ability induced by PBK was substantially restored by uPAR overexpression in Huh-7 and SK-Hep-1 cells (Fig. 5E–G).

3.5. PBK activated uPAR transcription by promoting the binding of ETV4 to uPAR promoter region

ETV4, an actively transcribed factor of ETS family, was reported to be associated with tumor invasive progression through directly binding to the uPAR promoter in prostate cancer [25]. Here, in HCC cells, we found that PBK knockdown significantly inhibited both the mRNA and protein levels of ETV4, while PBK overexpression promoted ETV4 expression in MIHA and Huh-7 cells (Fig. 6A and B). Based on above findings, we hypothesized that PBK depletion inhibited the uPAR promoter activity possibly by inhibiting ETV4 expression in HCC cells. We first predicted the putative binding site of ETV4 on uPAR promoter by using the software PROMO, revealing that the binding site of ETV located in the region of −236/-229 (Supplementary Fig. 7). ChIP assay further revealed that the recruitment of ETV4 to the core region of uPAR promoter was significantly decreased in PBK-depleted cells (Fig. 6C). Moreover, ETV4 overexpression restored uPAR promoter activity and protein level in PBK-silencing cells (Fig. 6D–F). Impaired migration and invasion ability of PBK-depleted cells was markedly restored in the presence of ETV4 overexpression (Fig. 6G and H). Collectively, these results suggested that PBK promoted the migration and invasion of HCC cells via enhancing the binding of ETV4 to uPAR promoter.

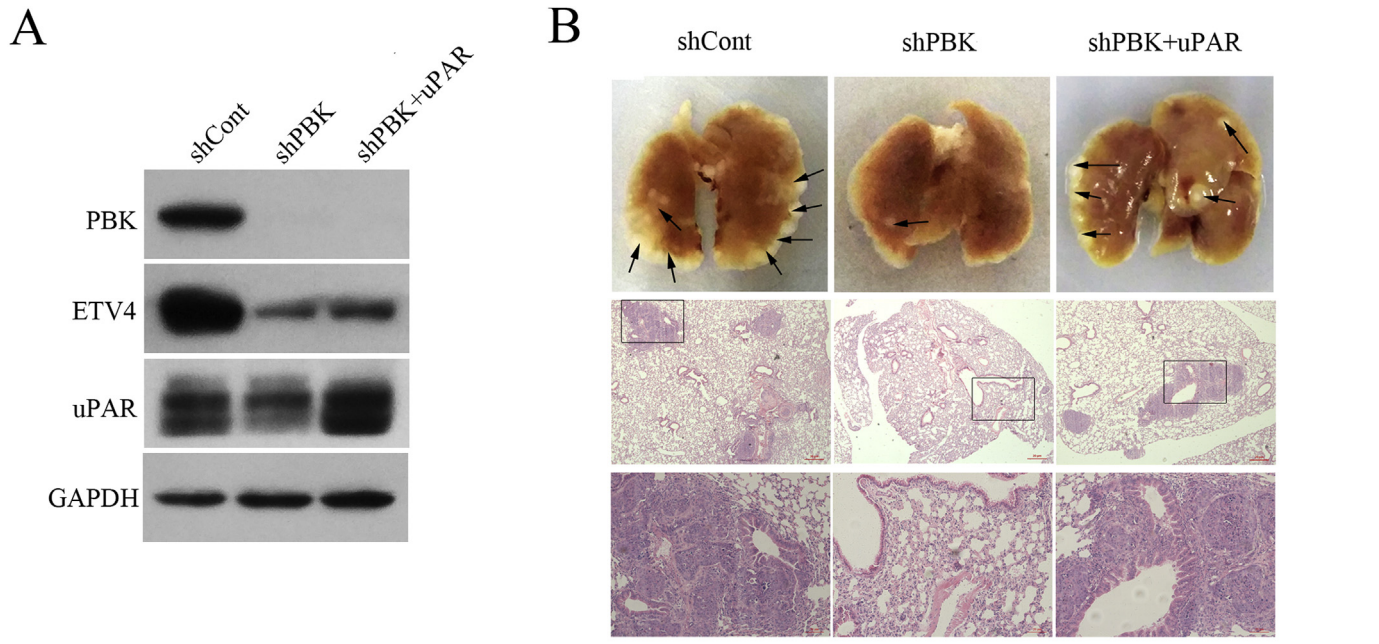
3.6. PBK knockdown inhibited lung metastasis of HCC cells in vivo

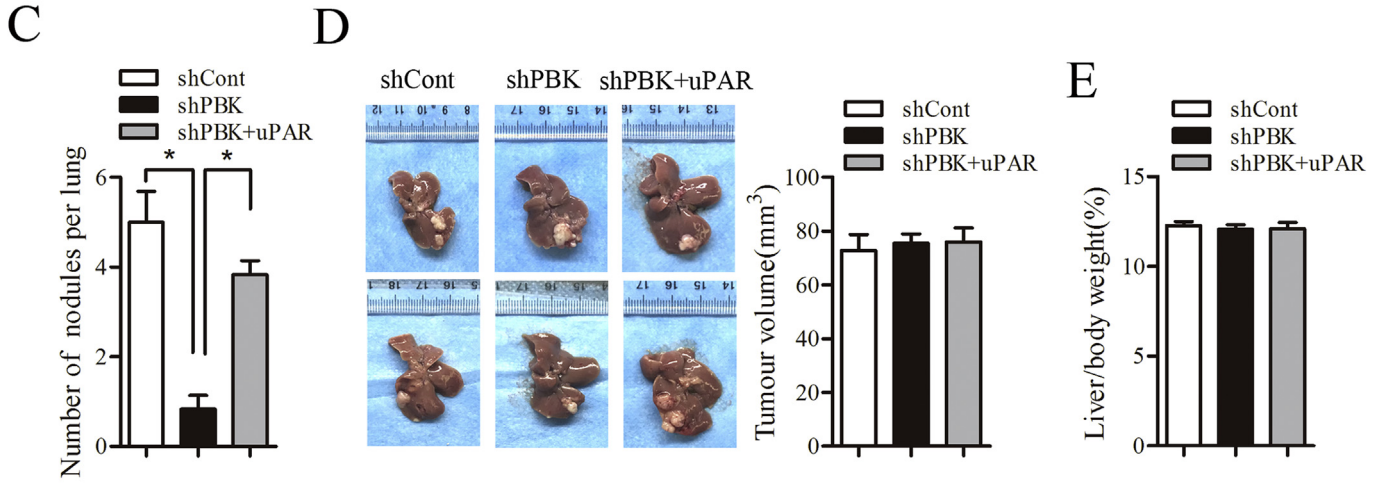
To confirm the role of PBK in HCC metastasis *in vivo*, Huh-7 cells infected with lentivirus expressing shCont, shPBK, and shPBK + uPAR were injected into the liver of nude mice orthotopically. Loss of PBK expression in liver tumors was validated by western blotting analysis. It was associated with a reduce expression of uPAR and ETV4. (Fig. 7A). Interestingly, the size and number of the metastatic nodules in the lung were dramatically decreased in shPBK groups compared with controls, whereas the effect was significantly restored by uPAR overexpression (Fig. 7B and C). However, the size of liver tumors as well as weight ratio between liver and body in xenograft mice had no significant difference in each group, indicating that PBK knockdown did not affect malignant tumor progression (Fig. 7D and E). In addition, the correlation between PBK, ETV4 and uPAR expression was analyzed in 370 paired human HCC tissues from TCGA. Correlation analysis revealed a positive correlation between PBK and uPAR (Pearson $r = 0.2231$, $P < 0.0001$). Similarly, a significant correlation was observed between ETV4 and uPAR in HCC tissues (Pearson $r = 0.2944$, $P < 0.0001$) (Fig. 7F). These data suggested a PBK-ETV4-uPAR regulatory axis might exist *in vivo*.

4. Discussion

In the past few years, accumulating researches have shed light on the functional roles of protein kinases in various types of tumors, which provided new insights into the underlying mechanisms of tumorigenesis and metastasis [29,30]. After integrating the metastasis-related kinases in several public databases, we identified PBK, a novel serine-threonine kinase, as a potential target in HCC progression. Although PBK plays a tumorigenic or aggressive role in multiple cancers by regulating some important oncogenes [16,31], the functional roles of PBK in HCC have not been thoroughly characterized, with even less attention to the underlying mechanisms. Our findings not only provide novel evidences that PBK can effectively promote the invasion and migration of HCC *in vitro* and *in vivo*, but also further explore a new molecular mechanism regarding the function of PBK.

Recently, aberrant PBK expression was reported in various tumors, with increased regulation correlated to poor prognosis [8,13]. Consistent with previous observations in other cancers [12,32], our study confirmed that PBK was frequently upregulated expression in HCC samples and showed significant correlations with poor clinicopathological characteristics of HCC patients. Importantly, higher PBK levels predicted shorter overall survival in these patients, suggesting PBK might act as a promising biomarker for HCC prognosis. Regarding the function of PBK in HCC, a recent study by Yang and





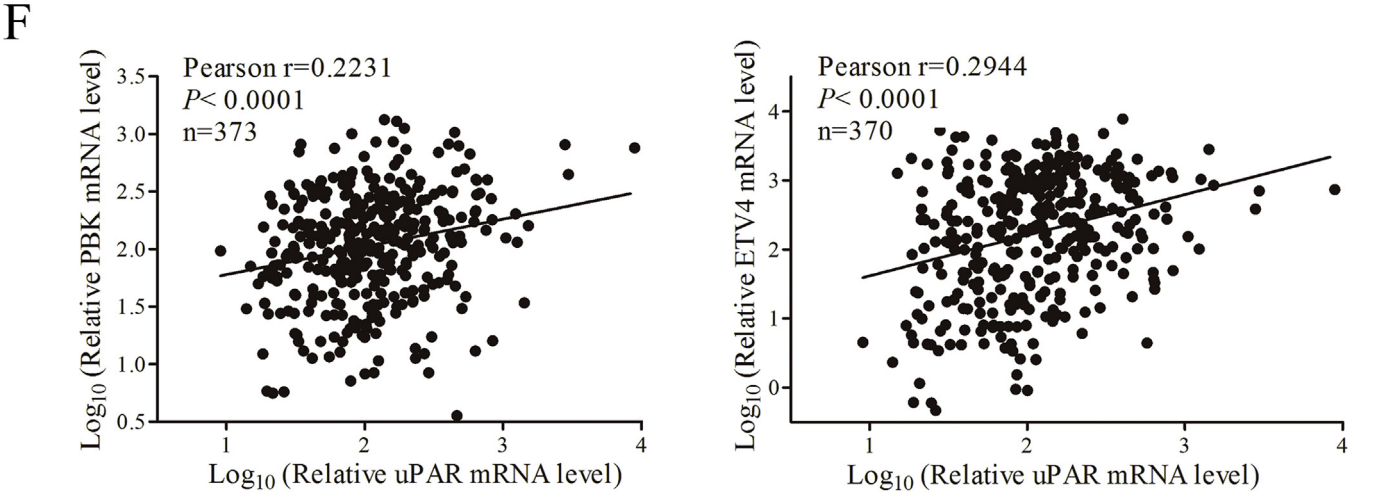


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Fig. 7. PBK depletion inhibited lung metastasis of HCC cells *in vivo*.
Huh-7 cells stably transfected with plasmids expressing shCont, shPBK and shPBK + uPAR were injected orthotopically into the liver of nude mice. (A) PBK, ETV4 and uPAR protein expression in liver tumors were measured by western blotting analysis. (B) Representative images of lung metastasis nodules formed by the injection of Huh-7 cells expressing shCont, shPBK and shPBK + uPAR into the liver of nude mice. Representative images of lung tumor sections were stained by Hematoxylin-Eosin (HE). Scale bar, 20 μm. (C) The presence or absence of metastatic nodules in the lung was histologically evaluated according to Hematoxylin-Eosin staining. $^{*}P < 0.05$ (D) Images (left) and volume (right) of xenograft liver tumors in nude mice (n = 6). (E) Liver/body weight ratios of nude mice. N = 6 for each group. (F) Correlation between PBK, ETV4 and uPAR mRNA in HCC specimens from TCGA data set. Correlation analysis was conducted using Pearson rank test.

colleagues revealed that PBK exerted a promoting effect on cell proliferation and migration by activating the FoxM1/β-Catenin pathway [33]. However, our *in vitro* and *in vivo* study showed that PBK knockdown had no significant effect on cell growth of HCC cells. Additionally, compared with their study on cell migration [33], we established the orthotopic hepatocellular carcinoma model to directly observe cancer metastasis with the exception of *in vitro* confirmation. Consequently, our results provided more solid evidences that PBK overexpression could significantly enhance the migrated ability of HCC cells. Although Guo and colleagues revealed the tumor-promoting effect of PBK in different HCC cell lines [34], our findings further confirmed that elevated expression of PBK could also significantly promote the migrated and invasive ability in the immortalized liver cell line (MIHA). In addition, our study firstly revealed that PBK-depleted HCC cells appeared to have some cytoskeletal changes, showing the disorganized and rearranged actin filaments. Taken together, our study complemented the functional roles of PBK in HCC metastasis *in vitro* and *in vivo*, as well as identified PBK as a predictive biomarker for HCC patients.

To further explore the molecular mechanisms through which PBK could regulate HCC progression, we analyzed several metastasis-related genes in PBK-depleted HCC cells, and identified uPAR as a potential downstream target of PBK. In fact, a critical role of uPAR was reported to degrade the surrounding extracellular matrix (ECM), contributing to a major part of the metastatic process to distant organs [18]. Based on these, we hypothesized that PBK could promote HCC metastasis by upregulating the uPAR expression. A dual-luciferase reporter assay found that PBK could enhance HCC migration and invasion via activating uPAR promoter activity. More importantly, our orthotopic injection model further confirmed that enrichment of uPAR could significantly rescue the metastatic behaviors of PBK knockdown in HCC cells *in vivo*. Therefore, these data confirmed that PBK could promote HCC metastasis via regulating the uPAR signaling. Considering that uPAR was well-demonstrated to collaborate with some factors to accelerate cancer progression [35,36], we focused on a novel transcription factor, ETV4. Emerging evidences have showed that ETV4 served some important functions in cellular responses, including cancer development, progression and metastasis [25,37]. A recent study from Qi and colleagues revealed that ETV4 could regulate uPA through direct binding to its promoter uPAR in prostate cancer [25]. Interestingly, our study showed that PBK silencing in HCC cells could markedly decrease the expression of ETV4 and uPAR. Subsequent analysis revealed that the promoter uPAR contained a potential binding site for ETV4 and ETV4 could directly bind to uPAR promoter region as evidenced by ChIP assay. Additionally, PBK knockdown inhibited the migration and invasion of HCC cells via reducing the interaction of ETV4 with uPAR promoter. Therefore, our study provided a new regulatory mechanism for PBK via activating ETV4-uPAR signaling pathway in HCC metastasis. Nevertheless, it could not be ruled out that many events might be involved in HCC progression by upregulation of PBK via activating this pathway, so our future work will be conducted to discover these links.

In summary, our study presented convincing evidence for the critical role of PBK in HCC progression and metastasis. In addition, as PBK down-regulation could reduce the aggressive behavior of HCC cells by regulating ETV4/uPAR signaling pathway, it is a novel potential therapeutic target for HCC treatment.

Declarations of interest

None.

Competing interest

The authors have no competing financial interests to disclose.

Conflicts of interest

The authors of this manuscript have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.028.

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