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PIK3CB is involved in metastasis through the regulation of cell adhesion to collagen I in pancreatic cancer

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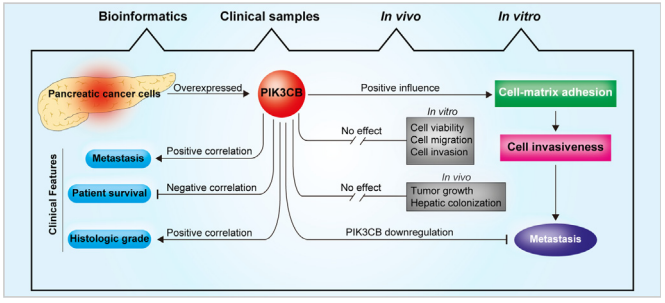
HIGHLIGHTS

- PIK3CB is highly expressed in PAAD.
- PIK3CB is involved in PAAD progression and metastasis.
- *PIK3CB*-depletion does not significantly affect cell viability, migration or invasion capabilities of human PAAD cells *in vitro*.
- *PIK3CB*-depletion does not significantly influence growth or hepatic colonization abilities of human PAAD cells *in vivo*.
- PIK3CB influences cell invasiveness via cell-to-collagen I interaction in human PAAD cells *in vitro*.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Pancreatic adenocarcinoma (PAAD) is an aggressive malignancy, with a major mortality resulting from the rapid progression of metastasis. Unfortunately, no effective treatment strategy has been developed for PAAD metastasis to date. Thus, unraveling the mechanisms involved in PAAD metastatic phenotype may facilitate the treatment for PAAD patients.
Objectives: *PIK3CB* is an oncogene implicated in cancer development and progression but less is known about whether PIK3CB participates in PAAD metastasis. Therefore, the objective of this study is to explore the mechanism(s) of PIK3CB in PAAD metastasis.
Methods: In our study, we examined the PIK3CB expression pattern using bioinformatic analysis and clinical material derived from patients with PAAD. Subsequently, a series of biochemical experiments were

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Metastasis
Pancreatic cancer

conducted to investigate the role of PIK3CB as potential mechanism(s) underlying PAAD metastasis *in vivo* using nude mice and in vitro using cell lines.
Results: We observed that PIK3CB was involved in PAAD progression. Notably, we identified that PIK3CB was involved in PAAD metastasis. Downregulation of *PIK3CB* significantly reduced PAAD metastatic potential *in vivo*. Furthermore, a series of bioinformatic analyses showed that *PIK3CB* was involved in cell adhesion in PAAD. Notably, *PIK3CB* depletion inhibited invasion potential specifically via suppressing cell adhesion to collagen I in PAAD cells.
Conclusion: Collectively, our findings indicate that PIK3CB is involved in PAAD metastasis through cell-matrix adhesion. We proposed that PIK3CB is a potential therapeutic target for PAAD therapy.
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Introduction

Pancreatic adenocarcinoma (PAAD) is a highly lethal malignancy with limited treatment options [1–3], due to its late diagnosis, and frequent early metastasis [4–7]. Therefore, an improved understanding of the underlying mechanisms of PAAD metastatic potential is required to optimize clinical therapy.

Dysregulation of PI3K signaling pathway is frequently correlated with tumorigenesis and cancer therapy resistance [8,9]. Although the framework of PI3K signaling pathway has been described in several cancers, the role of PI3K isoforms is not systematically studied in PAAD. p110α (PIK3CA), p110β (PIK3CB), p110γ (PIK3CG) and p110δ (PIK3CD) are the subunits of class I PI3Ks [10]. In particular, *PIK3CA* is the most frequently mutated oncogene across human cancers [11–13]. In contract, literature on PIK3CB has been limited. In most of these studies, PIK3CB has been shown to play critical roles in cell growth and invasion, and the PIK3CB specific inhibitors could prevent formation of integrin adhesion contacts, resulting in defective platelet thrombus generation [14–16]. In particular, the role of PIK3CB in development of PTEN-deficient PAAD cells was recently described. PIK3CB promotes PTEN-deficient PAAD progression through activating the AKT signaling pathway [17]. Most studies focus on the role of PIK3CB under PTEN-deficient condition, but its role in wild type PAAD cells remains unclear.

The tumor microenvironment (TME) is a heterogeneous and dynamic cellular milieu interacting with cancer cells [18]. Extracellular matrix (ECM) is a non-cellular network comprising of structural and functional proteins, such as fibrous proteins, glycoproteins, and proteoglycans [19]. These ECM proteins play important roles in tumor metastasis by forming a niche that influences tumor cell adhesion and migration. Importantly, interaction between PAAD cells and ECM is associated with progression and metastasis [20–22]. Tumor cells migrate through ECM and enters into the lymphatic or vascular system to initiate tumor metastasis [23]. Collagen is abundant in the tumor ECM. It facilitates tumor delivery and promotes tumor metastasis [24–27], potentially via the adhesion between collagen and cancer cells [28]. Multiple studies proposed that collagen I enhances tumor progression. Excessive deposition of collagen I in ECM could also promote the metastasis behavior in PAAD [29]. Collagen fibers can act as highways to facilitate cancer cell migration and metastasis [30–32]. Nevertheless, the mechanisms by which PAAD cells manipulate adhesion to collagen I are still not well understood.

In the present study, we explored the function of PIK3CB with clinical material derived from patients, in *vitro* with cell lines, as well as *in vivo* using murine model. We found that PIK3CB was involved in metastasis in PAAD, potentially by influencing cell invasiveness via cell-to-collagen I interaction in PAAD cells.

Material and methods

Compliance with ethics requirements

This study has received approval from the Ethics Committee of Kyushu University (#24-222, 25-117). This study conforms to the Ethical Guidelines for Human Genome/Gene Research legislated by the Japanese Government and the Declaration of Helsinki.

Clinical material derived from PAAD patients

Clinical samples were collected from 110 patients following surgical resection of PAAD at the Department of Surgery and Oncology, Graduate School of Medical Science, Kyushu University. The corresponding clinical information and clinicopathologcal features of patients were arranged and summarized in Supplementary Table 1.

Immunohistochemical (IHC) staining and analysis

IHC staining was carried out as previously described [33]. We incubated the tissue sections (4 μm) with p110β (PIK3CB protein) antibody (ab151549, Abcam) for 12 h at 4 °C. Then, we stained the tissue sections with EnVision System-HRP Labeled Polymer Anti-Rabbit (#K4003; Dako). We identified tumor cells according to morphological signatures. The tumor cells were observed in twenty fields at 200× magnification per section. We evaluated the p110β protein staining [34] and assigned the patients into p110β-high group and p110β-low group according to the staining.

Cell lines

PAAD SUIT-2 cells were purchased from Japanese Cancer Resource Bank (Tokyo, Japan). PAAD AsPC-1 and CFPAC-1 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Human umbilical vein endothelial cells (HUVECs, C2517A) were purchased from Lonza (Walkersville, MD, USA). SUIT-2, AsPC-1 and CFPAC-1 cells stablely expressing GFP were established as previously described [35].

Plasmid transfection

Two *PIK3CB* shRNA vectors (SHCLNV-NM_006219) were purchased from Sigma. The luciferase expression vectors (#LVP326) were purchased from GenTarget. We prepared and transfected the vectors into SUIT-2, CFPAC-1 and AsPC-1 cells. Puromycin from Takara (#631305) and blasticidin S hydrochloride from Sigma (#15205) were used to select stable clones, for 3 weeks cell culture. We used a non-target shRNA (SHC016V-1EA, Sigma) as a silencing negative control.

Western blotting

Western blot was carried to evaluate protein expression levels as described previously [33]. The transferred proteins were detected with primary antibodies as follows: p110β antibody (#3011), p110α antibody (#4249), p110γ antibody (#5405), akt antibody (#4691), and phospho akt antibody (#4060) were purchased from Cell Signaling Technology. The β-actin antibody

(ab8227) was from Abcam. We detected the immunoblot signals using ChemiDoc XRS (Bio-Rad Laboratories).

Cell viability assay

We seeded PAAD cells in 96-well plates (1×10^3 cells/well) or in ultra-low attachment surface and round bottom (Product Number 7007; Corning Inc., Corning). The Cell Viability Assay Kit (CellTiter-Glo Luminescent, G7570, Promega) was used to evaluate the cell viability as described previously [36]. Background values were obtained from a medium-contining well and subtracted.

Migration and invasiveness assay

The migration capacity and invasiveness of cancer cells were assessed using uncoated or Matrigel-coated transwell chambers (8 μm pore size; Becton Dickinson, Franklin Lakes, NJ, USA) as previously described [37]. Migration capacity was examined 24 h after cell seeding, and invasiveness was examined 48 h after cell seeding. In migration and invasiveness assays, hematoxylin and eosin were used for staining of migrated or invaded PAAD cells. The Three-dimensional (3D) collagen I matrix invasion assay was performed as previously described [38].

Cell adhesion assay

HUVEC cells were cultured in 96-well plates until cells reached 70% confluency. Cells were then co-cultured with 1×10^5 GFP-labeled PAAD cells in minimal essential medium, supplemented with 2.5 mg/mL bovine serum albumin for 1 h. PAAD cells adhering to the HUVEC monolayer were examined after washing. Adhering cells were quantified according to fluorescence intensities measured by a microplate reader (Tecan, Männedorf, Switzerland). In addition, 2×10^5 PAAD cells were seeded into 24-well plates coated with Fibronectin (354408, Corning, NY, USA) or collagen I (354411, Corning, NY, USA) in minimal essential medium. After 1 h, cultured cells were gently washed three times with PBS to remove non-adherent cells and then stained with hematoxylin and eosin.

In vivo analysis of murine model

PAAD cells (1×10^6) were transplanted into the pancreas of nude mice (BALB/c AJcl nu/nu female) via injection. Pancreas or liver were resected and examined 6 weeks after orthotopic transplantation. For liver colonization model, we injected wild type or *PIK3CB*-depleted luciferase-expressing PAAD cells into the spleen of mice. 5 min after the injection, the spleens were extirpated. Luciferin activity was measured using the IVIS Spectrum following injection of 150 mg d-luciferin into the intraperitoneal cavity of mice.

Database-based bioinformatics data mining

The mRNA expression datasets and clinical data in our study were obtained from GTEx database (The Genotype-Tissue Expression project), Arrayexpress database (E-GEOD-71729; www.ebi.ac.uk/arrayexpress) [39,40], TCGA database (The Cancer Genome Atlas Program), ICGC database (ICGC-CA), KM-plotter database [41] and UCSC Xena [42]. We conducted bioinformatic analyses using R language.

Statistical analysis

Values were expressed as the mean ± SEM in results of in vitro experiments. Kaplan–Meier survival curves were compared using the log-rank test. In bioinformatic analyses, comparisons of RNA expression were carried out using Kruskal-Wallis test and Wilcox test. The chi-squared test was performed to assess relationships between p110α protein expression and clinicopathological features. A p value of 0.05 was used as the criteria for significance.

Results

PIK3CB is highly expressed in PAAD tumors and correlates with poor survival in PAAD patients

To determine the expression pattern of *PIK3CB* mRNA, we performed database analysis using datasets from TCGA, GTEx and Arrayexpress databases. We observed that *PIK3CB* mRNA level was significantly increased in PAAD tumor tissues compared with normal tissues in both TCGA-GTEx conjoint database analysis and Arrayexpress database analysis (Fig. 1A, TCGA database, wilcox test, p = 2.0299e-38); Arrayexpress database, wilcox test, p = 0.0005). Meanwhile, dataset from TCGA database revealed that expression level of *PIK3CB* mRNA was positively correlated with advanced PAAD tumor grade. (Fig. 1B, wilcox test). To validate the reliability of *in silico* results, We examined PIK3CB protein (p110β) expression in clinical tissues derived from patients using IHC staining. We observed that p110β expression was upregulated in PAAD tumor tissues than those in normal tissues (Fig. 1C). The results revealed that PIK3CB expression was upregulated in PAAD tumor tissues compared with normal tissues at both RNA and protein levels in patients. We next assessed the association between *PIK3CB* mRNA and survival in ICGC database and KM-plotter database, showing that patients with higher *PIK3CB* mRNA expression had poorer survival than the patients with lower *PIK3CB* mRNA expression (Fig. 1D, ICGC, Overall Survival (OS), long-rank test, p = 0.0031; KM-plotter, OS, p = 0.0005 and Disease-Free Survival (DFS), p = 0.0017) . Similar survival outcomes were recently reported by Tian J *et al* [17]. To validate the *in silico* survival results, p110β expression of 110 patients was determined by IHC staining and the corresponding clinical survival information was arranged for survival analysis. We then examined the relationship between p110β expression level and survival status. A similar trend of survival curves was observed. The survival curves indicated that high p110β expression phenotype exhibited a poorer survival outcome of PAAD patients (Fig. 1E, OS, long-rank test, p = 0.0032 and DFS, p = 0.0022). Altogether, our findings indicate that PIK3CB is highly expressed in PAAD tumor tissues and negatively correlated with survival of PAAD patients. Furthermore, we examined *PIK3CB* DNA methylation status using UALCAN database, The Beta value were used to indicated the level of DNA methylation [43]. We observed that *PIK3CB* DNA promoter region was hypomethylated in tumor tissues (Fig. S1A, wilcox test, p = 0.0219). We also carried out Kaplan-Meier analysis for *PIK3CB* DNA methylation status, showing that *PIK3CB* DNA methylation level was positively correlated with survival of patients with PAAD (Fig. S1B, long-rank test, p = 0.0070). Our findings suggested that DNA methylation status of *PIK3CB* might be related to its abnormal expression in PAAD.

PIK3CB is significantly associated with metastasis of PAAD

To investigate the clinical significance of PIK3CB expression, we analyzed the association between p110β and clinicopathological characteristics. The overall clinicopathological parameters of 110 patients with PAAD were summarized in Table S1. We quantified p110β protein expression in tumor tissues from 110 PAAD patients using IHC staining. We then assigned the patients into p110β-high expression group and p110β-low expression group according to quantification of IHC staining. High p110β expression was associ-

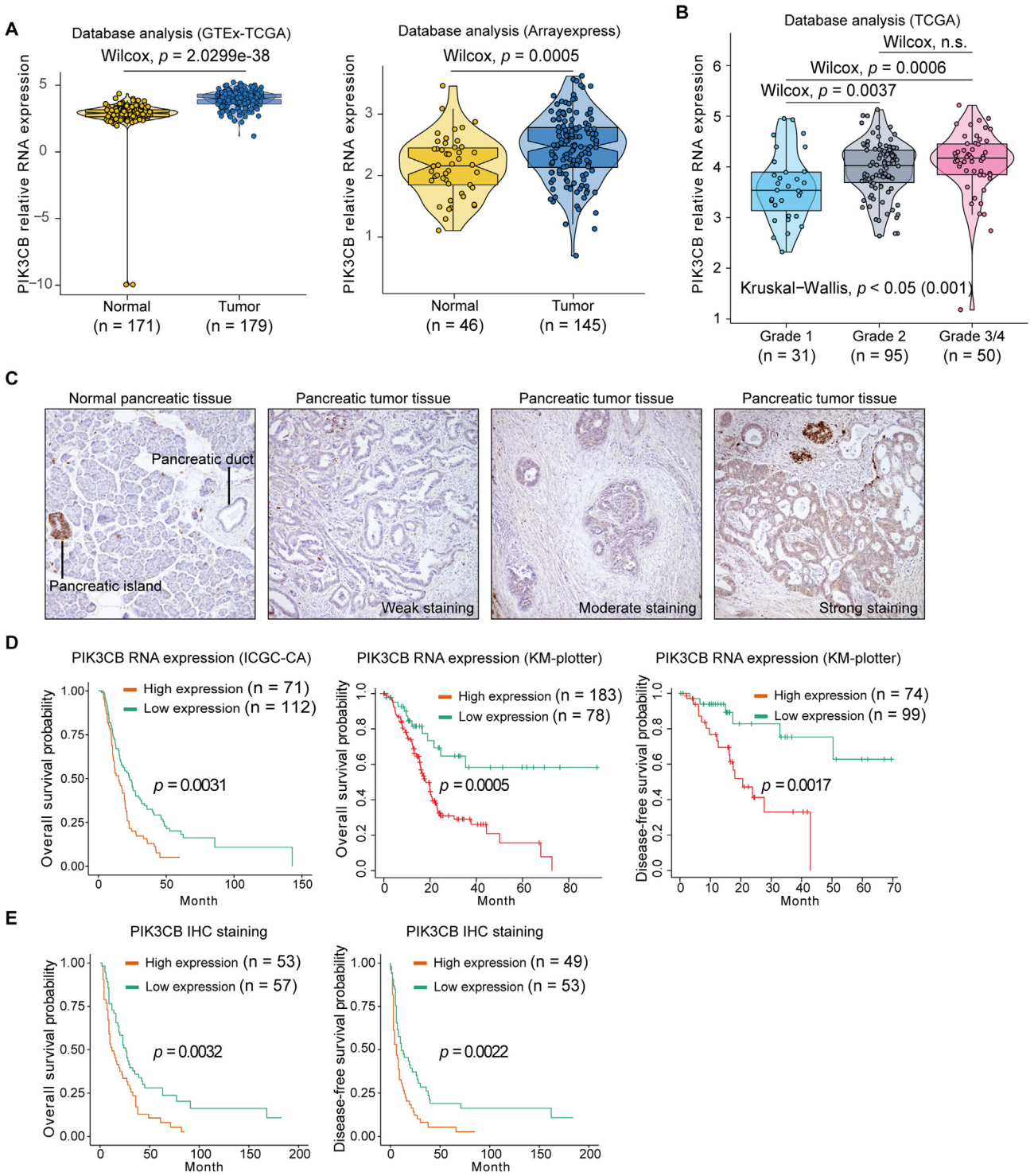


Fig. 1. The expression pattern of PIK3CB in human PAAD. (A) *PIK3CB* RNA level in human PAAD was determined by GTEx-TCGA conjoint analysis and Arrayexpress analysis. (B) Assessment of the relationship between *PIK3CB* RNA expression and PAAD tumor histologic grade. (C) p110β IHC staining in pancreatic normal and tumor tissues. (D) Kaplan-Meier estimation for individuals with PAAD according to *PIK3CB* RNA expression. Datasets were download from ICGC database and KM-plotter database. (E) Kaplan-Meier estimation for individuals with PAAD according to p110β expression determined by IHC staining.

ated with advanced tumor grade (Table 1, chi-squared test, $p = 0.012$). Notably, patients in p110β-high expression group had more frequent distant metastasis (Table 1, $p = 0.005$) and liver metastasis (Table 1, $p = 0.035$). Meanwhile, the Cox proportional hazard model analysis revealed that survival of patients with PAAD was significantly dependent on p110β expression (Table 2, relative risk = 1.661, $p = 0.018$; Table S2, $p = 0.003$). To verify the correlation between p110β expression and PAAD metastasis, we performed IHC staining to examine p110β expression in tumor tissues from metastatic sites or from primary tumor. IHC staining of p110β in metastatic tissues from liver and lung showed a higher p110β protein level, whereas primary tumor tissue showed a relative lower p110β expression level. (Fig. 2A). To further confirm the correlation between PIK3CB and metastasis, we also examined the expression level of *PIK3CB* mRNA in our previously established human PAAD cells with high metastatic potential (GEO accession: GSE144909)

Table 1
The associations of p110β expression level with clinicopathological features (n = 110).

Clinical features	p110β low expression group n = 57(51.8%)	p110β high expression group n = 53(48.2%)	*P* value
Age			0.298
<65	26(45.6)	19(35.8)	
≥65	31(54.4)	34(64.2)	
Sex			0.773
Female	20(35.1)	20(37.7)	
Male	37(64.9)	33(62.3)	
pT			0.815
pT1/pT2	5(8.8)	4(7.5)	
pT3/pT4	52(91.2)	49(92.5)	
pN			0.170
pN0	14(24.6)	13(24.5)	
pN1	41(71.9)	33(62.3)	
PN2	2(3.5)	7(13.2)	
UICC staging			0.123
I/II	52(91.2)	43(81.1)	
III/IV	5(8.8)	10(18.9)	
Residual tumor			0.280
R0	40(70.2)	32(60.4)	
R1	17(29.8)	21(39.6)	
Tumor histologic grade			0.012
Histologic grade 1	18(31.6)	6(11.3)	
Histologic grade 2	22(38.6)	19(35.8)	
Histologic grade 3	17(29.8)	28(52.8)	
Lymphatic invasion (ly)			0.191
–	17(29.8)	9(17.0)	
+	23(40.4)	21(39.6)	
++	17(29.8)	23(43.4)	
Vascular invasion (v)			0.905
–	22(38.6)	20(37.7)	
+	23(40.4)	20(37.7)	
++	12(21.1)	13(24.5)	
Perineural invasion (ne)			0.392
–	7(12.3)	11(20.8)	
+	20(35.1)	14(26.4)	
++	30(52.6)	28(52.8)	
Local recurrence			0.886
Negative	48(84.2)	9(15.8)	
Positive	44(83.0)	9(17.0)	
Distant metastasis			0.005
Negative	28(49.1)	12(23.1)	
Positive	29(50.9)	40(76.9)	
Liver metastasis			0.035
Negative	44(77.2)	31(58.5)	
Positive	13(22.8)	22(41.5)	

[34,44]. We observed that *PIK3CB* mRNA was increased in SUIT-2 cells and PANC-1 cells with highly metastatic potential compared with parental cell lines in microarray results (Fig. 2B) [44]. Collectively, our data suggested the involvement of PIK3CB in PAAD metastasis. We proposed that PAAD patients with higher expression of PIK3CB were potentially more likely to have metastasis. To investigate closely on the effects of PIK3CB on metastatic potential, we stably knocked down *PIK3CB* RNA in SUIT-2, AsPC-1 and CFPAC-1 cells. The reduction of PIK3CB was validated at protein level using western blot (Fig. 2C). We then investigated the influence of PIK3CB depletion on metastatic ability *in vivo*. The parental

Table 2
Multivariate analysis.

Clinical features	Risk	95% CI	*P* value
p110β expression	1.661	1.090–2.531	0.018
pT category	0.697	0.290–1.675	0.420
pN status	1.209	0.811–1.802	0.352
Residual tumor	2.346	1.493–3.685	<0.001
Lymphatic invasion	1.377	0.925–2.051	0.115
Vascular invasion	1.207	0.861–1.693	0.276

or *PIK3CB*-depleted SUIT-2 cells were transplanted into the pancreas of nude mice. The metastatic tumors were examined 6 weeks after injection. No significant differences were seen in peritoneal metastasis (Fig. 2D, top). Notably, depletion of PIK3CB in SUIT-2 cells reduced the liver metastatic capacity compared to parental SUIT-2 cells (Fig. 2D, chi-squared test, bottom, $p = 0.0389$). Depletion of *PIK3CB* in primary tumor tissues was also confirmed using IHC staining (Fig. 2E). The results indicate that *PIK3CB* depletion remarkably inhibits hepatic metastasis *in vivo*.

PIK3CB depletion does not significantly influence human PAAD cell viability, invasiveness and migration in vitro

Alterations of cell viability, migration and invasion abilities are often associated with the pre-metastatic stage of cancers [45,46]. To explore the precise contribution of PIK3CB in metastatic ability of PAAD, we proceeded to examine the influence of PIK3CB down-regulation on cell viability, migration and invasion. For cell viability assay, *PIK3CB* depletion did not significantly impact cell viability of PAAD cells (Fig. 3A). We then assessed the effect of *PIK3CB* depletion on invasiveness and migration of PAAD cells. As shown in Fig. 3B and 3C, *PIK3CB* depletion did not significantly affect PAAD cell migration and invasiveness. Overall, no significant difference was seen between *PIK3CB*-depleted and parental PAAD cells in cell viability, migration and invasion abilities.

PIK3CB depletion does not significantly influence growth or hepatic colonization abilities of human PAAD cells in vivo

We then examined the biological effect of PIK3CB *in vivo*. Parental or *PIK3CB*-depleted SUIT-2 cells were injected into the pancreas of nude mice. We weighed the primary tumor tissues 6 weeks after implantation but no significant differences in the weights of primary tumor tissues were observed between the parental SUIT-2-induced tumors *vs* *PIK3CB*-depleted SUIT-2-induced tumors (Fig. 3D). Additionally, we investigated the influence of PIK3CB depletion on hepatic colonization. We injected the GFP-labeled SUIT-2 cells (parental or *PIK3CB*-depleted) into the spleen of nude mice. The spleens were then removed 5 min after injection and the abdominal cavity was closed. In vivo imaging system (IVIS imaging system) was used for evaluation of GFP-labeled PAAD cells (Fig. 3E). Pancreatic tumors tissues on livers on day 21 after implantation of IVIS analysis were also showed (Fig. 3F). As shown in Fig. 3E and F, there was no obvious difference between *PIK3CB*-depleted SUIT-2 and parental SUIT-2 in regards to cancer cell hepatic colonization. These findings suggest that PIK3CB reduction does not significantly influence primary tumor growth or hepatic colonization abilities of PAAD cells *in vivo*.

PIK3CB is involved in cell adhesion potential

Next, we explored the biological significance of PIK3CB in PAAD using bioinformatic analysis. Gene ontology (GO) enrichment analysis of the *PIK3CB* high-correlated genes showed overrepresenta-

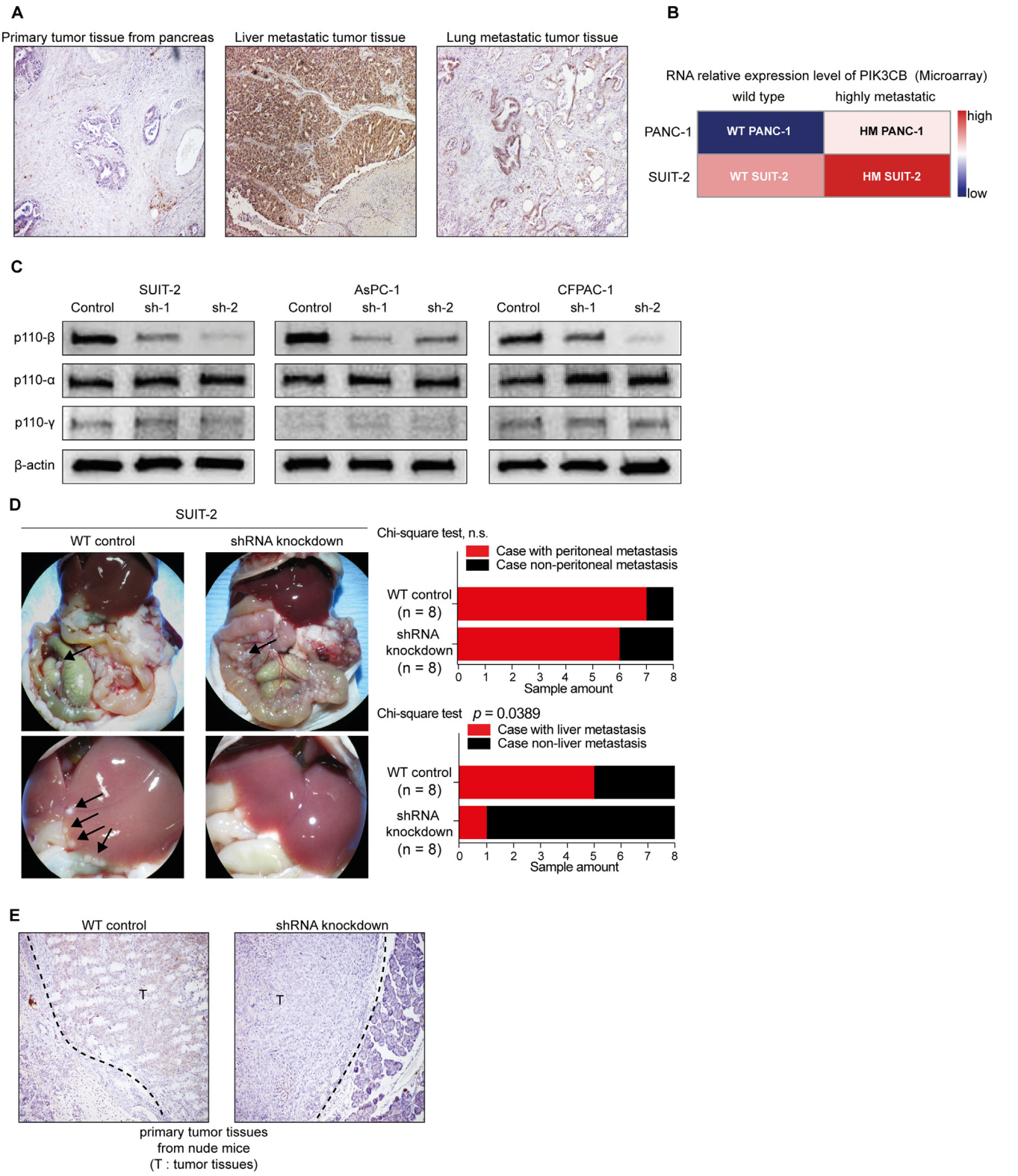


Fig. 2. Assessment of relationship between PIK3CB expression and PAAD metastasis. (A) p110β IHC staining in tumor tissues from primary tumor and paired metastatic sites. (B) Identification of *PIK3CB* RNA expression in parental and highly metastatic PANC-1 and SUIT-2 cells determined by microarray. (C) Western blot was performed to examine p110β expression in parental and *PIK3CB*-depleted PAAD cells. (D) Murine model of PAAD peritoneal and hepatic metastasis was established by transplanting parental SUIT-2 or *PIK3CB*-depleted SUIT-2 cells into tail of pancreas in nude mice. Metastatic tumor tissues are indicated with black arrows. (E) IHC staining of p110β in parental SUIT-2 or *PIK3CB*-depleted SUIT-2 cells induced primary tumor tissues.

tion of the adhesion related GO terms, suggesting that PIK3CB might be involved in cell adhesion ability (Fig. 4A and Fig. S2A) [47]. To validate the role of PIK3CB in cell adhesion of PAAD, the cell adhesion related gene sets were specifically selected for Gene Set Enrichment Analysis (GSEA) of PIK3CB. We then conducted GSEA analysis between low and high *PIK3CB* expression tumor tis-

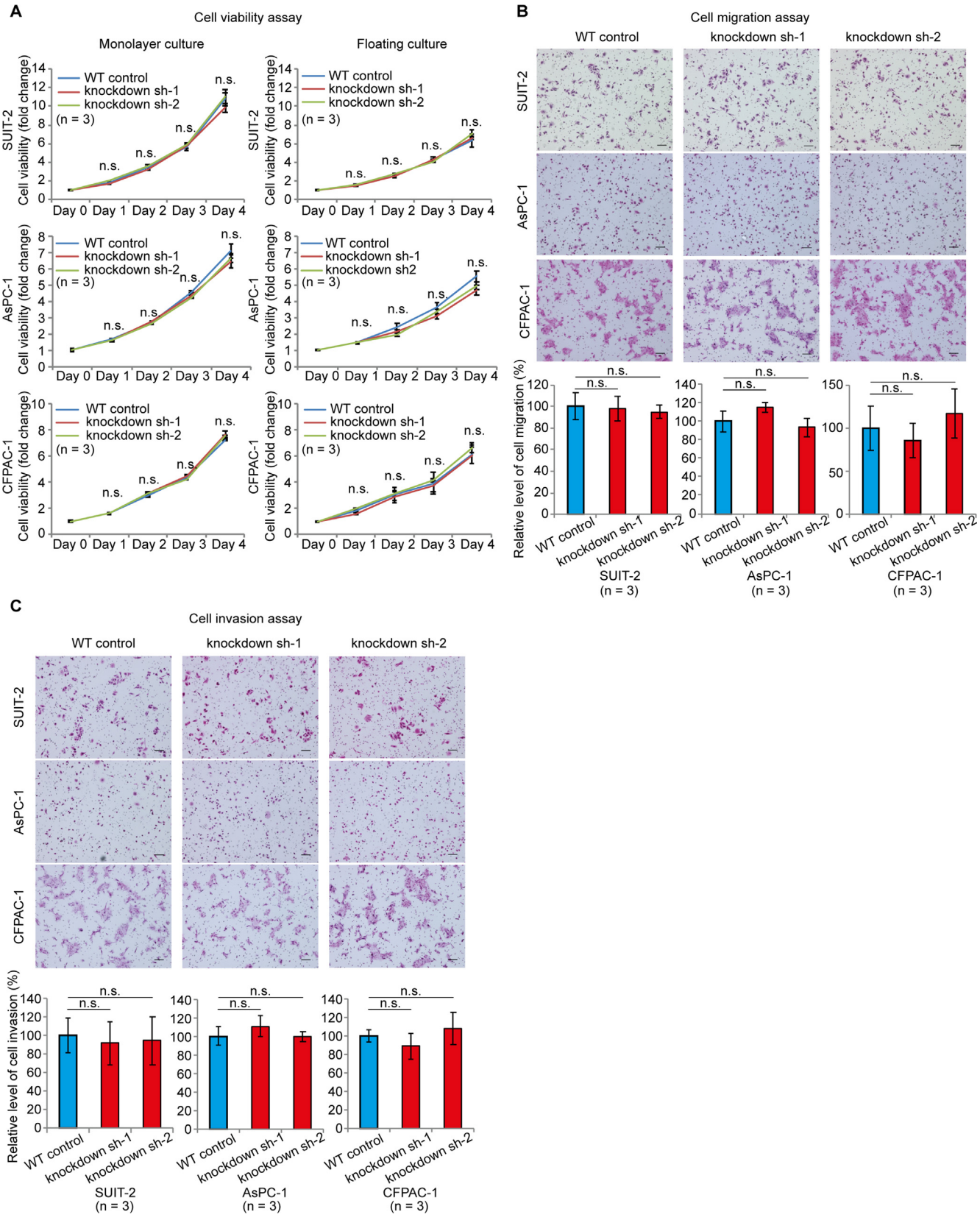


Fig. 3. Comparison of cell viability, migration and invasion abilities between parental and *PIK3CB*-depleted PAAD cells. (A) Quantification of cell viability by continuous monitoring of parental and *PIK3CB*-deplated PAAD cells. (B) Assessment of cell migration ability in parental and *PIK3CB*-deplated PAAD cells. (C) Assessment of cell invasiveness in parental and *PIK3CB*-deplated PAAD cells. (D) Comparison of primary tumor growth (weight) between parental SUIT-2 induced tumors and *PIK3CB*-deplated SUIT-2 induced tumors. (E) Tumor hepatic colonization evaluation using the IVIS imaging system. (F) Tumor hepatic colonization on day 21 of IVIS examination.

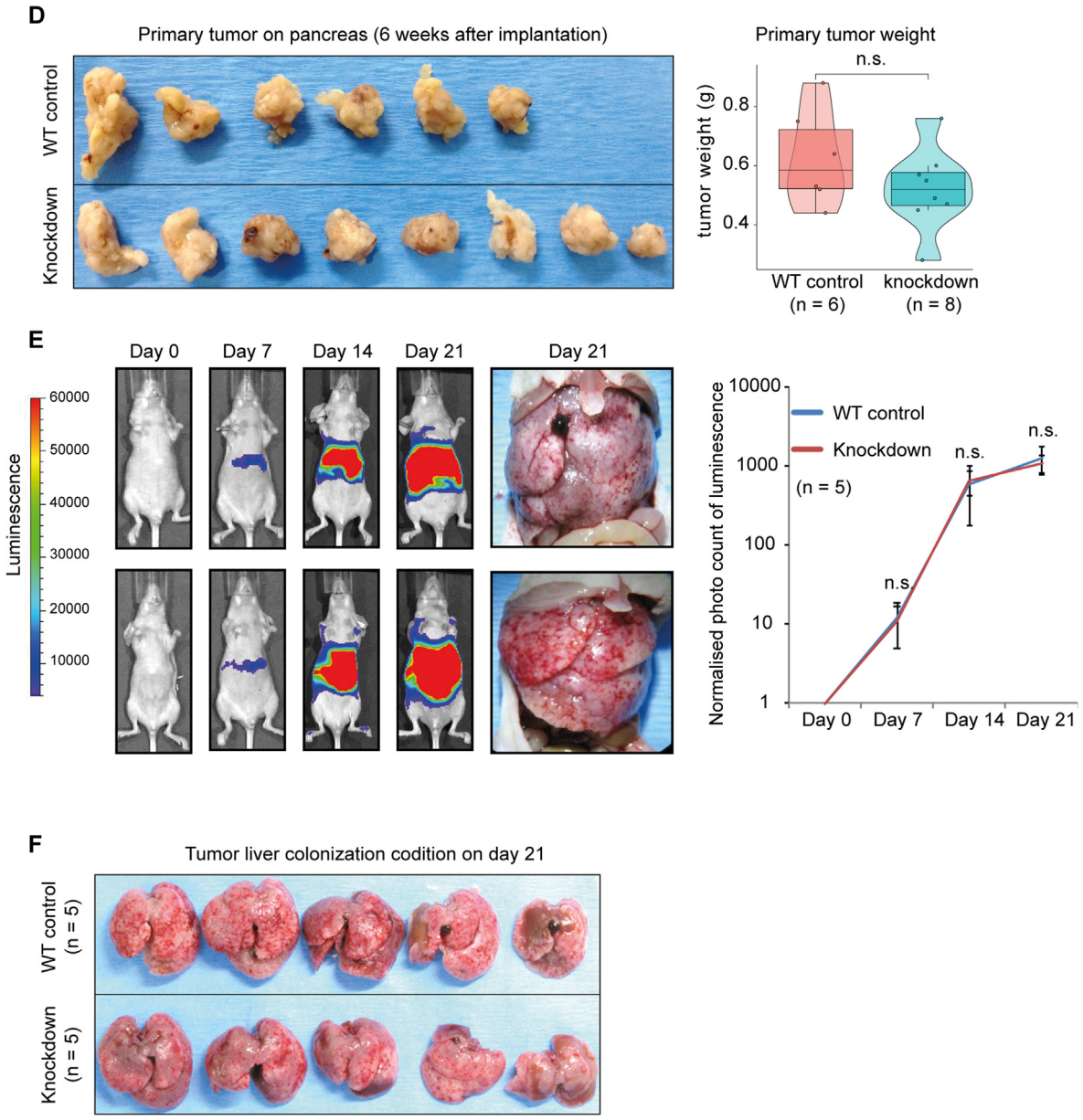


Fig. 3 (*continued*)

sues. The result showed that PAAD tumor tissues with high *PIK3CB* expression enriched in cell adhesion related GO terms (Fig. 4B), which suggested a significant association between *PIK3CB* and cell adhesion potential. To further validate the biological activity of PIK3CB in cell adhesion, we further performed Gene Set Variation Analysis (GSVA). We observed that high *PIK3CB* expression was associated with increased GSVA scores of cell adhesion related pathways (Fig. 4C and Fig. S2B). To elucidate the importance of cell adhesion in PAAD metastasis, we carried out GO analysis of differential expression RNAs between parental and highly metastatic PAAD cells, which were established in our previous studies [34,44]. The signatures of differential expression genes were evaluated using Cytoscape [48]. The GO terms exhibited an enrichment of differentially expressed genes in cell adhesion (Fig. 4D), suggesting that abnormal cell adhesion might contribute to PAAD metastasis. Collectively, based on the strong correlation between *PIK3CB* and adhesion potential in our *in silico* analysis, we proposed the possibility that PIK3CB might be involved in metastasis of PAAD via PIK3CB-mediated cell adhesion.

Downregulation of PIK3CB leads to reduced cell adhesion to Collagen I

In order to elucidate the role of PIK3CB in extracellular microenvironment adhesion, we next performed cell-matrix adhesion analyses. PAAD cellular adhesion was examined using three types of extracellular matrix, fibronectin, endothelial cell and collagen I protein. There were no significant differences in cell adhesion to fibronectin or endothelial cells between *PIK3CB*-depleted and parental PAAD cells (Fig. 5A and B). Of note, we observed dramatically decreased cell adhesion to collagen I in *PIK3CB*-depleted PAAD cells (Fig. 5C) as compared to parental cells. Furthermore, *PIK3CB*-depleted PAAD cells exhibited decreased invasion ability in 3D culture under condition with a collagen I matrix as compared to parental PAAD cells (Fig. 5D). These results suggest that *PIK3CB* downregulation leads to decreased PAAD cell invasion ability specifically by decreasing cell adhesion to collagen I.

AKT pathway has been proposed to be regulated by PIK3CB in PTEN-deficient PAAD cell lines [17]. To assess whether alteration in cell-collagen I adhesion ability in wild type PAAD cell lines

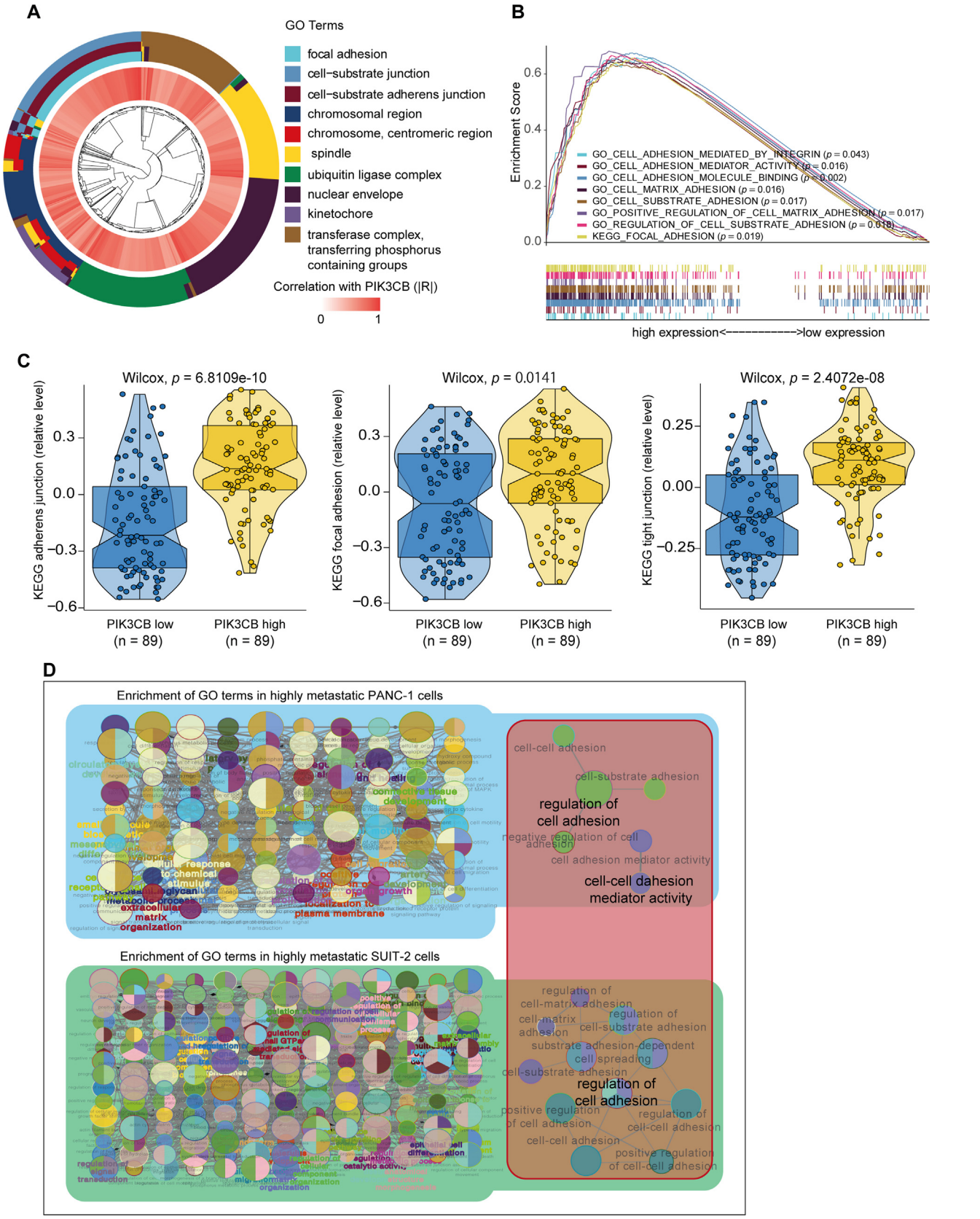


Fig. 4. Functional annotation of PIK3CB in PAAD. (A) *PIK3CB*-related GO terms. Spearman's correlation analysis was used to screen *PIK3CB* correlated genes according to the criteria that |R| > 0.4. Expression dataset from TCGA was used for the analysis. We carried out GO analysis to explore the biological function of *PIK3CB* using its correlated genes. (B) Enrichment blot from GSEA. We conducted GSEA between low and high *PIK3CB* expression tissues. The significantly changed cell adhesion related GO terms were selected and showed. The dataset from TCGA database was used for GSEA analysis. (C) Comparison of cell adhesion related pathways using GSVA. We conducted GSVA between low and high *PIK3CB* expression tissues. The significantly changed cell adhesion pathways (KEGG) were selected and showed. The dataset from TCGA database was used for GSVA analysis. (D) GO analysis of differential expression RNAs between parental and highly metastatic PAAD cells. Differentially expressed genes in highly metastatic PANC-1 and SUIT-2 cells were identified as fold change cutoff > 1, $p < 0.05$. Venn diagram representation showed that the differentially expressed genes are enriched in the cell adhesion related GO terms.

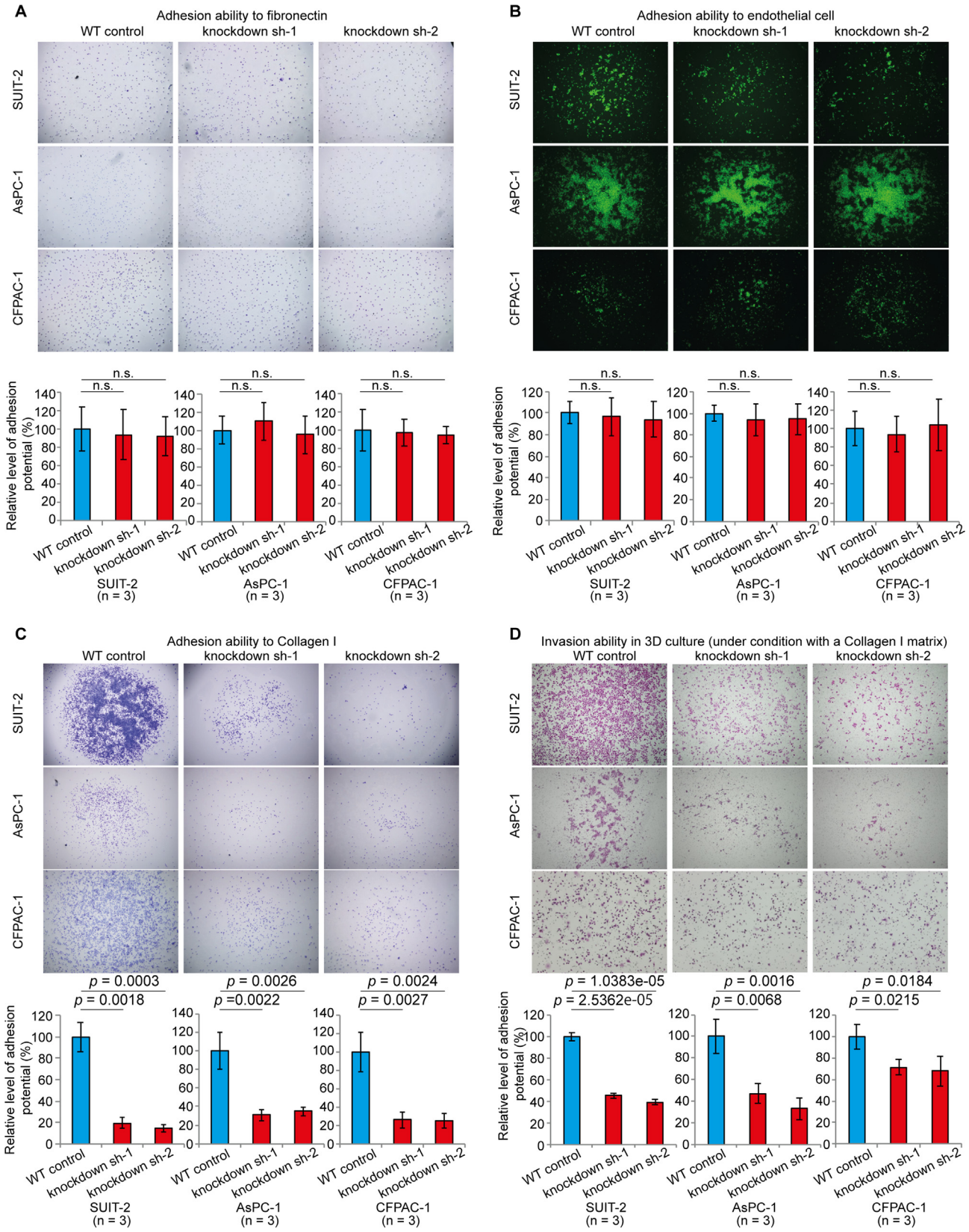
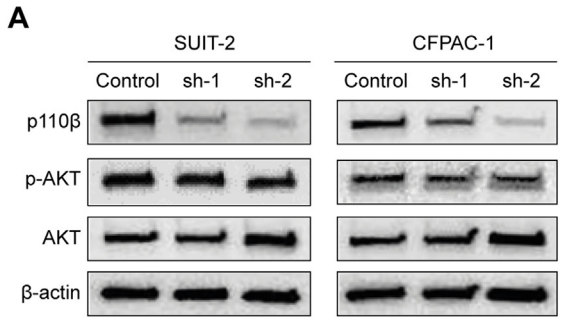
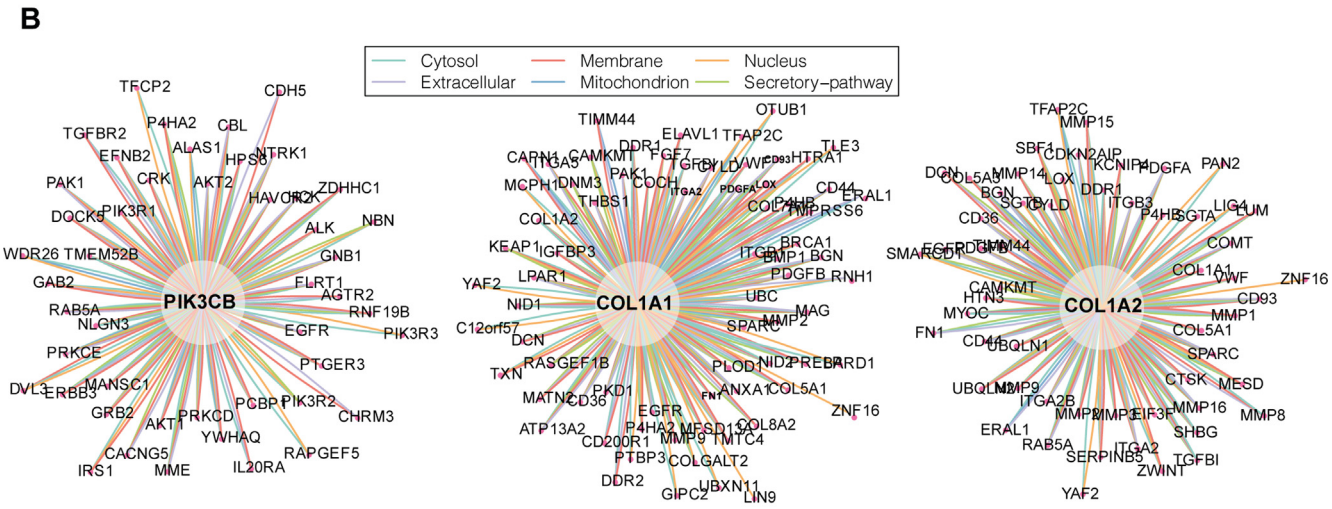


Fig. 5. Cell adhesion evaluation in parental and *PIK3CB*-deplated PAAD cells. (A) Analysis of cell adhesion capacity to fibronectin. (B) Analysis of cell adhesion capacity to endothelial cell. Green fluorescent protein was expressed as a marker in PAAD cells. (C) Analysis of cell adhesion capacity to collagen I protein. (D) 3D invasion assay. The parental or *PIK3CB*-depleted PAAD cells were 3D cultured under condition with collagen I.

was also working through an AKT-dependent pathway, we next investigated the effect of PIK3CB in AKT activation in wild type PAAD cell lines. We examined phosphorylation status of AKT under condition of *PIK3CB* knockdown. Our results revealed that *PIK3CB* depletion did not significantly change the total AKT protein expression or phosphorylation status of AKT protein (Fig. 6A), suggesting





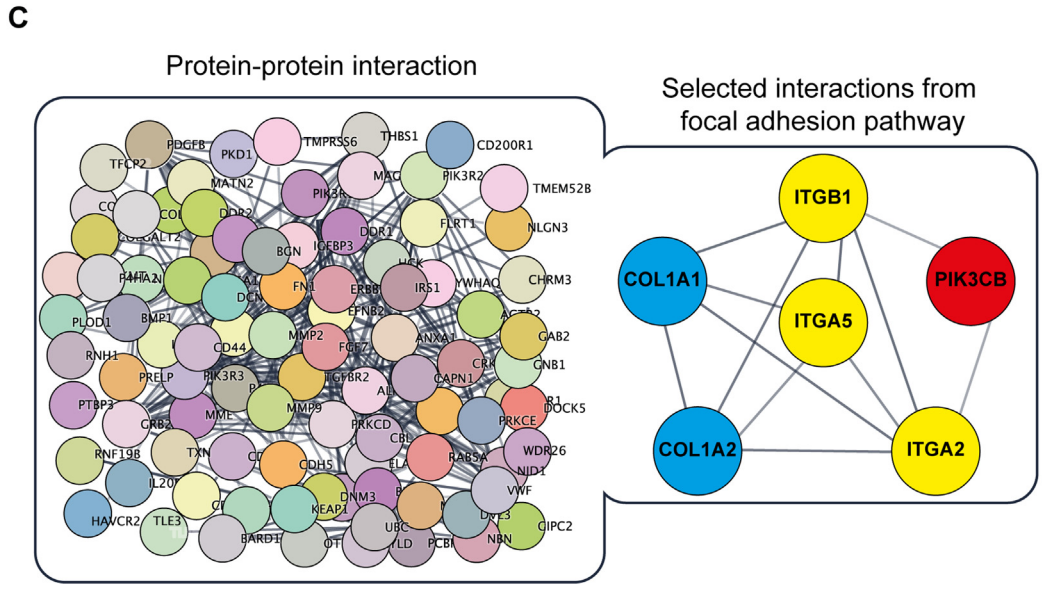


Fig. 6. Predicted functional association of PIK3CB in cell adhesion. (A) Western blot analyses of total AKT protein and phosphorylation AKT protein in parental and *PIK3CB*-deplated PAAD cells. (B) Identification of possible interaction proteins of PIK3CB, The interaction datasets were downloaded from ComPPI database (http://comppi.linkgroup.hu/protein_search). R language was used for visualization of interaction proteins. The colours of lines indicated the localisation of interaction proteins. COL1A1 and COL1A2. Protein-protein interaction datasets were download from Compartmentalized Protein-Protein Interaction Database. (C) Protein-protein interaction analysis. The datasets were generated by string database (https://string-db.org/). Cytoscape software was used for data visualization.

that the PIK3CB-mediated cell-collagen I adhesion may not be working through an AKT-dependent pathway in wild type PAAD cells. To further evaluated the possible role of PIK3CB in regulating cell-collagen I adhesion, we performed in silicon analysis using ComPPI database to predict the possible interacting proteins of PIK3CB and collagen I (COL1A1 and COL1A2) (Fig. 6B) [49,50]. These possible interacting proteins were subsequently used for protein-protein interaction network assay using Cytoscape string-APP [51,52]. As shown in Fig. 6C, we identified an interaction between PIK3CB and collagen I linked by ITGA2, ITGA5 and ITGB1, which are the subunits of integrin. The results suggest that PIK3CB might influence cell-collagen I adhesion through its interaction with integrin.

Discussion

In this work, we set out to uncouple the specific roles of PIK3CB in PAAD metastasis. Based on our findings, we revealed that PIK3CB was involved in PAAD progression. Like in previous study that reported by Tian J *et al* [17], we also observed that PIK3CB overexpression was associated with worse survival which may lead to poor prognosis of PAAD. Notably, we identified the role of PIK3CB in PAAD metastasis, *PIK3CB* knockdown dramatically reduced hepatic metastasis *in vivo*. Importantly, we elucidated that PIK3CB expression is involved in metastasis specifically through cell-collagen I adhesion in wild type PAAD cells via an AKT-independent manner. Finally, we proposed a the possibility that PIK3CB might regulate cell-collagen I adhesion via its interaction with integrin. Together, our results proposed that PIK3CB might be a potential target for PAAD metastasis.

PTEN-deficient tumors are dependent on PIK3CB for signaling and cell growth. PTEN-deficient cells require PIK3CB activity to sustain PI3K signaling pathway. Moreover, the PIK3CB-selective inhibitor inhibited phospho-AKT only in PTEN-deficient cells [53]. Recently, the function of PIK3CB in PTEN-deficient PAAD cells was described by Tian J *et al.* PIK3CB contributes the severity of PTEN-deficient PAAD through regulation of AKT signaling pathway [17]. Whereas the PIK3CB subunit is unnecessary for pancreatic tumorigenesis in a mouse model [54]. So far, the function of PIK3CB in AKT pathway was nicely described under PTEN-deficient condition, but its role in wild type PAAD cells is still unknown. Our study specifically elucidated the role of PIK3CB in wild type PAAD cells. Interestingly, *PIK3CB*-depletion does not affect either the AKT signaling or tumor cell malignant phenotypes such as cell viability, migration ability and invasiveness in wild type PAAD cells. We identified that PIK3CB was involved in cell-matrix adhesion through an AKT-independent manner in wild type PAAD cells. Our study fills in a gap of current studies which offers a new research direction for PIK3CB in PTEN-proficient cancers.

The ECM could function as cytoskeletal structure and serve to support cell adhesion, migration and invasion [55,56]. The dynamic interactions between tumor cells and ECM could therefore dictate tumor progression. The ECM have been shown to promote tumor metastasis via formation of a premetastatic niche, and are being increasingly studied as therapeutic targets [57–60]. In PAAD there is a dramatically increased deposition of extracellular matrix. Especially, collagens are the main structural proteins constituting PAAD extracellular matrix [61]. Studies proposed that deposition of collagen I in ECM promotes the metastasis [29], but its regulatory networks in PAAD have not been elusive so far. Our results revealed that *PIK3CB*-depletion specifically suppressed cell adhesion to collagen I in PAAD cells. Notably, the PIK3CB-mediated alteration of cell-collagen I adhesion could significantly influence invasion ability of PAAD cells. Our results identify a novel role of PIK3CB in regulating PAAD metastasis and suggest that PIK3CB acts in an AKT-independent manner on cell-matrix adhesion. We delineate a new regulatory network underlying tumor cell-collagen I adhesion which may provide a view to identifying potential therapeutic strategy or druggable targets for PAAD metastasis.

Integrins are a family of heterodimeric transmembrane receptors functioning in cell-matrix interactions that is crucial to cancer initiation, progression and metastasis [62,63]. In particular, the integrin family play a crucial role in mediating cancer cell interactions with collagen I. Integrin depletion in PAAD cells leads to abrogation of metastasis in mice [64]. Previous studies also showed that PIK3CB could modulate integrin-collagen affinity, influencing platelet adhesion [65]. No study to date, however, has investigated whether that PIK3CB affect cell-collagen I adhesion through integrin in PAAD. Combining with the previous studies, our protein-protein interaction results provide a possible explanation that the role of PIK3CB that we have identified in cell-collagen I adhesion might work through its interaction with integrin.

Conclusion(s)

In conclusion, we proposed that PIK3CB not only influences PAAD progression via AKT signaling under PTEN-deficient condition as described in previous studies, but also regulates metastasis in a collagen I-dependent and an AKT-independent manner in wild type PAAD cells. Our study provides a new viewpoint to explore PIK3CB function in PTEN-proficient cancers.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.02.002.

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