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Protein Kinase C Iota is Required for Pancreatic Cancer Cell Transformed Growth and Tumorigenesis

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Abstract

Pancreatic cancer is the fourth leading cause of cancer deaths in the United States with an overall 5-year survival rate of <5%. Pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer, is highly resistant to conventional chemotherapies underscoring the critical need for new molecular targets for pancreatic cancer chemotherapy. The *KRAS* proto-oncogene is mutated in >90% of PDAC. Protein kinase C iota (PKC ι) is required for oncogenic Ras-mediated transformed growth in lung cancer and intestinal epithelial cells. However, little is known about the role of PKC ι in pancreatic cancer. In this study, we evaluated the expression of PKC ι in human pancreatic cancer and the requirement for PKC ι for the transformed growth and tumorigenicity of PDAC cells. We find that PKC ι is significantly over-expressed in human pancreatic cancer and high PKC ι expression correlates with poor patient survival. Inhibition of PKC ι expression blocks PDAC cell transformed growth *in vitro* and tumorigenicity *in vivo*. Inhibition of PKC ι expression in pancreatic tumors also significantly reduces tumor angiogenesis and metastasis. Analysis of downstream PKC ι effectors implicates the Rac1-MEK/ERK1/2 signaling axis in PKC ι -mediated transformed growth and cellular invasion. Taken together, our data demonstrate a required role for PKC ι in the transformed growth of pancreatic cancer cells and reveal a novel role for PKC ι in pancreatic cancer cell metastasis and angiogenesis *in vivo*. Our results strongly indicate that PKC ι will be an effective target for pancreatic cancer therapy.

Keywords

pancreatic cancer; transformed growth; invasion; metastasis; protein kinase C iota; Rac1; ERK1/2; VEGF

Introduction

Pancreatic cancer is highly lethal, with patients having a median survival time of <6 months and an overall 5-year survival rate of <5%. The deadly nature of pancreatic cancer is attributed to late detection, rapid growth, a propensity to invade and metastasize, and resistance to conventional chemotherapy. Even patients that undergo “curative” surgery have only a 20% 5-year survival rate (1). Due to the frequent failure of conventional therapies, there is an urgent

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need for new molecularly-targeted therapies that can improve the outcome for those diagnosed with pancreatic cancer.

Oncogenic *KRAS* mutations are found in >90% of all advanced pancreatic cancers (2). Anti-sense inhibition of oncogenic K-ras expression in PDAC cell lines blocks cellular transformation, demonstrating a continued requirement for oncogenic K-ras-mediated signaling to maintain the transformed phenotype (3). Currently, there are no clinically effective therapeutic agents that inhibit oncogenic K-ras activity. Farnesyl transferase inhibitors (FTIs) were introduced into the clinic to target mutant Ras, but have not proven to be therapeutically effective in pancreatic cancer (reviewed in (4)). Thus, oncogenic K-ras signaling is critical to pancreatic cancer, but downstream K-ras effector pathways may be better targets for molecularly-targeted therapy in pancreatic cancer.

Our lab and others have identified PKC ζ as an important effector of oncogenic K-ras *in vitro* and *in vivo* (5-11). Here, we tested the hypothesis that PKC ζ plays a requisite role in pancreatic cancer cell transformed growth and tumorigenesis. We find that PKC ζ is highly expressed in human pancreatic cancers and that high PKC ζ expression predicts poor survival. We show that PKC ζ is required for transformed growth of pancreatic cancer cells *in vitro* and tumorigenesis *in vivo*. PKC ζ promotes transformed growth of pancreatic cancer cells through activation of a proliferative Rac1-MEK/ERK1/2 signaling pathway. Finally, we make the novel observation that inhibition of PKC ζ expression blocks PDAC tumor angiogenesis and metastasis *in vivo*. Taken together, these results strongly suggest that PKC ζ will be an effective target for pancreatic cancer chemotherapy.

Materials and Methods

Reagents and cell culture

Antibodies were obtained from the following sources: PKC ζ and Rac1 (BD Transduction Laboratories), PKC ζ , β -actin, phospho-ERK1/2 Thr202/Tyr204 (p-ERK) and p44/42 ERK (Cell Signaling Technologies), PAK-1 PBD agarose conjugate (Rac/cdc42) (Millipore), 5-bromo-2'-deoxyuridine (BrdUrd) and VEGF (DakoCytomation) and CD31 (PECAM-1) (Santa Cruz Biotechnology, Inc.). U0126 was obtained from Sigma and NSC23766 from Tocris. Human pancreatic cancer cell lines were obtained from ATCC and maintained in a 5% CO₂ humidified tissue culture incubator as recommended by ATCC. Retroviral vector encoding firefly luciferase (pSIN-Fluc) was described previously (12).

Patient samples

Biospecimens were obtained from the Mayo Clinic Tissue Registry under an approved Institutional review board protocol. RNA was isolated from a set of pancreatic adenocarcinoma patient samples for which frozen, paired tumor and non-tumor pancreas tissue was available. A second set of formalin-fixed pancreatic adenocarcinoma biospecimens were selected as described previously (13).

RNA isolation, quantitative real-time PCR and analysis

Hematoxylin and eosin (H&E)-stained sections of matched normal and pancreatic tumor tissues were analyzed to confirm the presence of tumor or normal pancreas and overall integrity of the frozen tissue samples. Total RNA was isolated using RNAqueous Isolation Kit (Ambion) according to the manufacturer's protocols. TaqMan® Gene Expression Assay primer and probe sets (Applied Biosystems) were used for real-time, quantitative PCR (qPCR) analysis of hGAPDH (Hs99999905_m1), hPKC ζ (Hs00177051_m1) and 18S (Hs99999901_s1). Forward and reverse primer and probe sequences were designed and synthesized for hPKC ζ (forward-5'-CGTTCCTCCGAAATGTTGATTG-3', reverse-5'-

TCCCCAGAAATATTTGGTTTAAAGG-3', probe-5'-6FAMTTGCTCCATCATATCC-3'). qPCR analysis was carried out using 10 ng of cDNA or 2 ng cDNA (18S) on an Applied Biosystems 7900 thermal cycler. Data was evaluated using the SDS 2.3 software package. Gene expression in primary pancreatic cancers and in pancreatic cancer cell lines was normalized to 18S and GAPDH, respectively. All data is expressed as $2^{-(CT(\text{target})-CT(\text{endogenous reference}))}$.

Immunohistochemistry and expression analysis

A second set of formalin-fixed pancreatic cancers (13) were analyzed by immunohistochemistry (IHC) for PKC ζ expression. A few of the matched normal and pancreatic tumor tissue pairs were also available as formalin-fixed tissues and were analyzed by IHC for PKC ζ expression. Tissues were processed for IHC as described previously (14). PKC ζ staining was visualized using the Envision Plus Anti-Mouse Labeled Polymer-HRP (Dako). p-ERK1/2 staining was visualized using the Envision Plus Anti-Rabbit Labeled Polymer-HRP (Dako). Images were captured and analyzed using Aperio and Spectrum software. PKC ζ expression was scored by a pathologist blinded to patient clinical parameters (TCS). Nuclear and cytoplasmic PKC ζ levels were scored on a scale of 0-3 and combined for a total cellular expression score of 0-6. Low PKC ζ was defined as a total expression score of ≤ 3 and high PKC ζ as a total expression score > 3 , yielding two groups consisting of approximately half of the evaluable cases (45 and 40, respectively). Staining with only secondary antibody served as negative controls.

Knock down and re-expression of human PKC ζ gene expression and immunoblot analysis

Lentiviral vectors carrying short hairpin RNA interference (RNAi) targeting human PKC ζ were generated and used to obtain stable transfectants as described previously (15). PKC ζ RNAi #1 construct targets a sequence in the 3' untranslated region of PKC ζ (GCCTGGATACAATTAACCATT) and PKC ζ RNAi #2 construct targets a sequence in the coding region of PKC ζ (CCTGAAGAACATGCCAGATTT). Cells were stably transfected with pBabe and pBabe-PKC ζ as described previously (16). PKC ζ and PKC ζ protein expression was determined by immunoblot analysis of total cell lysates.

Cell viability assay

Cell viability was assessed by MTT assay (CellTiter 96 AQueous One Solution, Promega), as recommended by the manufacturer. Pancreatic cancer cells (3×10^3 cells) were cultured for 1, 3, 5 and 7 days prior to viability assay.

Anchorage-independent growth assays

Panc-1 and MiaPaCa-2 cells (5×10^3) were plated in soft agar and assessed for anchorage-independent growth as described previously (17).

Rac1 activity assay and signaling analysis

Rac1 activity was assayed as described previously (5,18). Cells stably expressing PKC ζ RNAi constructs were co-transfected with LZRS vector or LZRS carrying myc-tagged, constitutively active Rac1 (RacV12) as described previously (15,16). Transfectants were harvested and subjected to immunoblot analysis as previously described (17).

Orthotopic tumor model

Panc-1 human pancreatic cancer cells carrying pSIN-Fluc and expressing NT or PKC ζ RNAi (1×10^6) were mixed with growth factor reduced Matrigel (Becton Dickinson) and injected into the proximal pancreas (n=15 and 16 mice/group respectively) of 4-6 week old male athymic

nude mice. For weekly imaging, mice were injected intraperitoneally (IP) with 150 mg/kg body weight D-Luciferin solution (Xenogen), anesthetized with isoflourane and imaged using a bioluminescence imaging system (IVIS Imaging Spectrum System). Bioluminescence was calculated using IVIS Imaging Spectrum software. One hour prior to sacrifice, mice were injected IP with 100 μ g/g BrdUrd. All of the animal experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Orthotopic tumor analysis

Formalin-fixed pancreatic tumors were analyzed for proliferation using BrdUrd incorporation as described previously (14,19,20). Orthotopic pancreatic tumors were evaluated for apoptosis by TdT-mediated dUTP-biotin nick end labeling (TUNEL) of fragmented DNA as described (20). Angiogenesis was characterized by quantitative analysis of IHC detection of CD31 (PECAM-1) expression as described (17,20,21). Expression of p-ERK1/2, ERK1/2, PKC ζ , VEGF and β -actin was evaluated by immunoblot analysis of total cell lysates from orthotopic tumors.

Cellular invasion assay

Cellular invasion was measured as described (6,15). The selective MEK inhibitor, U0126 (10 μ M), was included in the medium in the upper and lower chambers in some experiments, as noted.

Statistical analysis

Survival rates were calculated using Kaplan-Meier analysis. Differences in survival were analyzed by log-rank test, Fisher Exact test, univariate and multivariate Cox proportional hazard models using SAS 9.1.3 software. All tests were two-sided. One-way Analysis of Variance (ANOVA) and the Pairwise Multiple Comparison Procedures were used to evaluate the statistical significance of the results. *p* values <0.05 were considered statistically significant.

Results and Discussion

PKC ζ is highly expressed in human pancreatic cancer

To investigate the role of PKC ζ in pancreatic cancer, we first evaluated PKC ζ expression in a panel of 28 human pancreatic tumors and adjacent non-tumor (normal) pancreas mRNA samples (Figure 1A). PKC ζ mRNA was detected in all 28 primary pancreatic tumors analyzed (Figure 1A). PKC ζ overexpression, defined as tumor mRNA abundance greater than two standard deviations above the average PKC ζ mRNA abundance in adjacent non-tumor pancreas, was observed in 27/28 pancreatic tumors analyzed (Figure 1A). Pancreatic tumors exhibited an average 9 ± 2 fold increase in PKC ζ mRNA expression relative to matched non-tumor pancreas (*inset*, *p*<0.001 for paired samples). IHC analysis of PKC ζ expression was conducted on two matched pancreatic tumor/non-tumor pancreas pairs from this set for which formalin-fixed tissues were available. IHC confirmed increased expression of PKC ζ in both tumors compared to the matched non-tumor pancreas (Supplemental Figure 1). PKC ζ localized to both the nucleus and cytoplasm of the tumor cells, with little or no expression of PKC ζ in the surrounding stromal components (Figure 1B and Supplemental Figure 1). We next validated our analysis of PKC ζ expression in a second group of 85 pancreatic tumor tissues (13) for which formalin-fixed tissue, clinical follow-up and pathological features were available (Supplemental Table 1). Cases were segregated into high and low PKC ζ expression groups based on IHC staining intensity and subjected to survival analysis, as described in **Materials and Methods**, (Figure 1C and Supplemental Table 2). Patients whose tumors exhibit high PKC ζ expression had significantly shorter survival time (median survival time 492 days

for high PKC ζ expression, vs 681 days for low PKC ζ expression, $p=0.033$) and a reduced 5 year survival rate (10% vs 29.5% for low PKC ζ expression, $p=0.032$). Multivariate analysis adjusting for age, sex and tumor stage, demonstrates a significant association between high PKC ζ expression and poor survival of PDAC patients (Hazards Ratio, 1.670; 95% CI, 1.037-2.688, $p=0.035$).

PKC ζ is required for transformed growth of PDAC cells in vitro

The prevalence of PKC ζ overexpression in primary pancreatic tumors, and the association of high PKC ζ with poor clinical outcome strongly suggests a role for PKC ζ in the pathology of pancreatic cancer. To evaluate this possibility, we first assessed PKC ζ mRNA and protein expression in a panel of PDAC cell lines (Figure 2A). Whereas PKC ζ was abundantly expressed in all PDAC cell lines evaluated, PKC ζ expression did not directly correlate with known characteristics of the PDAC cell lines including differentiation status, or biological behavior such as soft agar growth, migration or invasion rate, or sensitivity to gemcitabine (22-24).

To directly assess the role of PKC ζ in the PDAC phenotype, we used lentiviral-mediated RNAi knock down (KD) to inhibit PKC ζ expression in two widely-utilized human PDAC cell lines, Panc-1 and MiaPaCa-2 (Figure 2B and C). Two PKC ζ -targeted RNAi constructs significantly inhibited PKC ζ mRNA and protein expression in both PDAC cell lines. These RNAi constructs had no effect on expression of the closely-related atypical PKC zeta (PKC ζ) isozyeme, demonstrating their specificity for PKC ζ (Figure 2B and C). Whereas PKC ζ KD had no significant effect on log-phase, adherent (non-transformed) cellular growth (Figure 2D) or cellular proliferation (Supplemental Figure 2) of Panc-1 and MiaPaCa-2 cells, PKC ζ KD significantly inhibited anchorage-independent growth in both PDAC cell lines (Figure 3A and B). While PKC ζ has been implicated in both pro- and anti-apoptotic signaling (25, 26), we did not observe any effect of PKC ζ KD on PDAC cell apoptosis (Supplemental Figure 3). These results are consistent with previous findings in intestinal epithelial, NSCLC and ovarian cancer cells (5, 15, 17, 27).

To confirm that PKC ζ RNAi-mediated inhibition of transformed growth was due to inhibition of PKC ζ expression, we expressed human PKC ζ as a transgene in Panc-1 NT and PKC ζ RNAi cells (Figure 3C). In this and all further experiments, the PKC ζ #1 RNAi construct was used to knock down PKC ζ expression because it targets the 3'UTR of the endogenous human PKC ζ mRNA, making it possible to reconstitute PKC ζ expression in PKC ζ RNAi cells using an exogenous human PKC ζ cDNA construct lacking the 3'UTR target sequence (15). Re-expression of PKC ζ reconstituted the anchorage-independent growth of Panc-1 PKC ζ RNAi cells demonstrating the specific requirement for PKC ζ in the transformed growth of PDAC cells (Figure 3D).

Rac1 is a critical effector of PKC ζ -mediated transformed growth in pancreatic cancer cells

We previously demonstrated that PKC ζ drives transformed growth of intestinal epithelial cells and NSCLC cells by activating a Rac1-MEK/ERK signaling pathway (15,17,28). Having established a requirement for PKC ζ for PDAC cell transformed growth, we evaluated the required signaling downstream of PKC ζ . We first assessed the requirement for Rac1 and MEK in Panc-1 cell transformed growth. Pharmacological inhibition of either Rac1 (NSC23766) or MEK (U0126) significantly inhibited Panc-1 cell soft agar colony formation (29) (Supplemental Figure 4), demonstrating the requirement for both Rac1 and MEK activity for Panc-1 cell transformed growth. We next assessed whether PKC ζ regulates Rac1 and MEK activity in PDAC cells. PKC ζ RNAi significantly reduced Rac1 activity (Figure 4A) and ERK phosphorylation in Panc-1 cells (Figure 4B and 4C). Similar results were observed in MiaPaCa-2 cells (data not shown). To determine whether Rac1 and MEK/ERK activity are required downstream of PKC ζ in the transformed growth of PDAC cells, we evaluated the

ability of myc-tagged constitutively active Rac1 (RacV12) to reconstitute transformed growth in PKC ζ RNAi Panc-1 cells. Expression of RacV12 restored the transformed growth of PKC ζ RNAi cells (Figure 4D) and ERK1/2 phosphorylation (Figure 4B and 4C), without affecting PKC ζ or ERK1/2 expression (Figure 4B). Furthermore, inhibition of MEK blocks RacV12-mediated reconstitution of transformed growth in PKC ζ KD cells (Figure 4D). Taken together, these data demonstrate that a PKC ζ -Rac1-MEK/ERK signaling pathway is required for transformed growth of Panc-1 cells.

PKC ζ plays a critical role in PDAC cell tumorigenesis

We next utilized an orthotopic pancreatic tumor model to evaluate the role of PKC ζ in PDAC tumor growth and metastasis *in vivo* (30). Panc-1 cells expressing the firefly luciferase gene (pSIN-Fluc) and either NT or PKC ζ RNAi were injected orthotopically into the pancreas of nude mice (31). Tumor growth was monitored by bioluminescence weekly over a 5 week time course (Figures 5A). Tumor formation was observed in all mice injected with either NT or PKC ζ RNAi-expressing Panc-1 cells. However, PKC ζ RNAi tumors grew at a slower rate than NT RNAi tumors, resulting in significantly smaller tumors (Figure 5A). We hypothesized that the smaller size of PKC ζ RNAi tumors was due to reduced proliferation of the tumor cells. As predicted, tumor cell proliferation, as detected by BrdUrd incorporation, was significantly inhibited in PKC ζ RNAi tumors when compared to NT RNAi tumors (Figure 5B). In contrast, PKC ζ KD had no effect on tumor apoptosis (Figure 5C). Thus, the reduced tumor volume of PKC ζ RNAi pancreatic tumors is due to decreased cellular proliferation of the tumor cells.

To investigate whether MEK/ERK1/2 activity is regulated by PKC ζ expression in Panc-1 cells *in vivo*, as observed *in vitro* (Figure 4), we evaluated the status of ERK1/2 phosphorylation in NT and PKC ζ RNAi tumors. IHC analysis revealed a dramatic decrease in p-ERK1/2 and PKC ζ in PKC ζ RNAi tumors when compared to NT RNAi tumors (Figure 5D). Immunoblot analysis confirmed reduced PKC ζ expression and reduced p-ERK1/2 in PKC ζ RNAi tumors compared to NT RNAi tumors (Figure 5D). These data strongly implicate PKC ζ -mediated activation of a Rac1-MEK/ERK1/2 proliferative signaling pathway in PDAC tumorigenesis *in vivo*. In this regard, we detect elevated ERK phosphorylation in our panel of human pancreatic tumors (Supplemental Figure 5) as previously described (32,33).

PKC ζ expression regulates angiogenesis, invasion and metastasis of PDAC orthotopic tumors

Angiogenesis plays an important role in tumor cell proliferation. We therefore evaluated the effect of PKC ζ KD on angiogenesis in orthotopic PDAC tumors by IHC detection of the endothelial cell marker CD31 in NT and PKC ζ RNAi tumors (Figure 6A). CD31 expression was significantly decreased in PKC ζ RNAi tumors (Figure 6A), indicating that PKC ζ in tumor cells regulates tumor angiogenesis. Vascular endothelial cell growth factor (VEGF) is a major pro-angiogenic factor expressed in tumor tissue. VEGF expression was considerably decreased in PKC ζ RNAi tumors (Figure 6A); supporting the conclusion that PKC ζ drives tumor angiogenesis by regulating VEGF expression in pancreatic tumors. Since ERK activity has been shown to regulate VEGF expression in PDAC cells (34), it is possible that PKC ζ regulates VEGF expression, at least in part, via regulation of ERK activity. The significantly higher level of tumor angiogenesis in NT RNAi tumors may contribute to the increased tumor proliferation observed in NT RNAi tumors compared to PKC ζ RNAi tumors.

Since tumor angiogenesis can be permissive for tumor metastasis, we determined the effect of PKC ζ RNAi on the metastatic capacity of Panc-1 orthotopic tumors *in vivo* (Figure 6B and 6C). As described previously (35, 36), Panc-1 cells not only form orthotopic tumors in the pancreas but also develop metastases in other organs. Metastases to the kidney, liver, diaphragm and mesentery were observed in more than 50% of the mice harboring NT RNAi

tumors (Figure 6B and 6C). In contrast, PKC ζ RNAi tumors exhibited significantly reduced metastases to all of these organ sites (Figure 6C). To investigate the mechanism by which PKC ζ regulates tumor metastasis, we evaluated the requirement for PKC ζ for cellular invasion (Figure 6D). Cellular invasion is significantly inhibited in PKC ζ RNAi Panc-1 cells (Figure 6D). Expression of exogenous PKC ζ reconstituted the PKC ζ KD phenotype, demonstrating the specific requirement for PKC ζ for invasion (Figure 6D). Since Rac1 and MEK activity are also required for Panc-1 cellular invasion (Supplemental Figure 6), we evaluated whether Rac1/MEK signaling is downstream of PKC ζ in cellular invasion (Figure 6D). Expression of RacV12 reconstituted cellular invasion in PKC ζ KD Panc-1 cells in a MEK-dependent manner (Figure 6D). These data reveal a novel required role for PKC ζ -Rac1-MEK/ERK signaling in cellular invasion *in vitro* and taken together with our *in vivo* data, suggest a role for PKC ζ -Rac1-MEK/ERK signaling in PDAC tumor cell invasion and metastasis.

Pancreatic cancer is a highly lethal disease with no effective therapeutic options. The overall goal of our research is to reduce this statistic by identifying and characterizing new molecular targets for more effective pancreatic cancer therapy. Our results demonstrate that PKC ζ is dispensable for adherent pancreatic cell growth, but is required for transformed growth of PDAC cells *in vitro* and tumorigenicity *in vivo*. This observation suggests that chemotherapeutic interventions targeting PKC ζ will specifically inhibit the growth of transformed pancreatic tumor cells while having little effect on non-transformed pancreatic epithelial cells. In this regard, we have identified and characterized a molecularly-targeted inhibitor of PKC ζ , aurothiomalate (18,21,37), which is currently in clinical trials for the treatment of advanced lung and ovarian cancers. Future studies will evaluate the ability of aurothiomalate to inhibit pancreatic tumor growth and metastasis *in vivo*. Interestingly, a small molecule (oncrasin-1) identified for its ability to selectively induce apoptosis in mutant K-ras-expressing cells requires not only mutant K-ras, but also expression of PKC ζ , for its cytotoxic effects (38). While the target of oncrasin-1 remains to be elucidated (it does not directly inhibit PKC ζ activity *in vitro*)(38), this study provides further support for the conclusion that PKC ζ plays a critical role in the transformed phenotype of oncogenic K-ras-mediated cancer.

Our study elucidates a critical molecular mechanism by which PKC ζ promotes transformed growth and cellular invasion of PDAC cells. PKC ζ regulated Rac1 and ERK1/2 activity, both of which are required for PDAC cell transformed growth and invasion. Specifically, PKC ζ KD in Panc-1 cells inhibits *in vitro* transformed growth and invasion which can be reconstituted by RacV12 in a MEK-dependent manner. These results are consistent with the role of PKC ζ in NSCLC cells (17). We further demonstrate a required role for PKC ζ in PDAC tumorigenesis *in vivo* and a novel role for PKC ζ in PDAC tumor metastasis. Since PKC ζ also regulates ERK activation *in vivo*, these data suggests that a Rac1-MEK/ERK1/2 signaling pathway plays a role in PKC ζ -dependent PDAC tumor cell proliferation and metastasis *in vivo*. Interestingly, similar to high PKC ζ expression, ERK phosphorylation predicts poor survival of pancreatic cancer patients (32,33).

We have recently demonstrated that PKC ζ is overexpressed in a majority of primary lung cancers, and that high PKC ζ expression predicts poor survival in NSCLC patients (39). In lung squamous cell cancers, as well as ovarian cancer, elevated PKC ζ expression is the result of tumor-specific amplification of the PKC ζ gene (27,39). As gene amplification frequently occurs in pancreatic cancer, this is a possible mechanism for increased PKC ζ in human pancreatic cancers. Future studies will address this possibility.

In summary, PKC ζ is highly overexpressed in the majority of primary pancreatic cancers and elevated PKC ζ expression correlates with decreased survival time. PKC ζ and its downstream effectors Rac1 and MEK/ERK1/2 are required for PDAC transformed growth and cellular invasion *in vitro* and PKC ζ is required for PDAC tumorigenicity and tumor cell proliferation

in vivo. Finally, we describe a previously unappreciated role for PKC ι in PDAC tumor angiogenesis and metastasis. These data identify PKC ι as an attractive therapeutic target for the treatment of pancreatic cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

PKC	protein kinase C
qPCR	quantitative real time polymerase chain reaction
BrdUrd	5-bromo-2'-deoxyuridine
H&E	hematoxylin and eosin
RNAi	RNA interference
KD	knock down

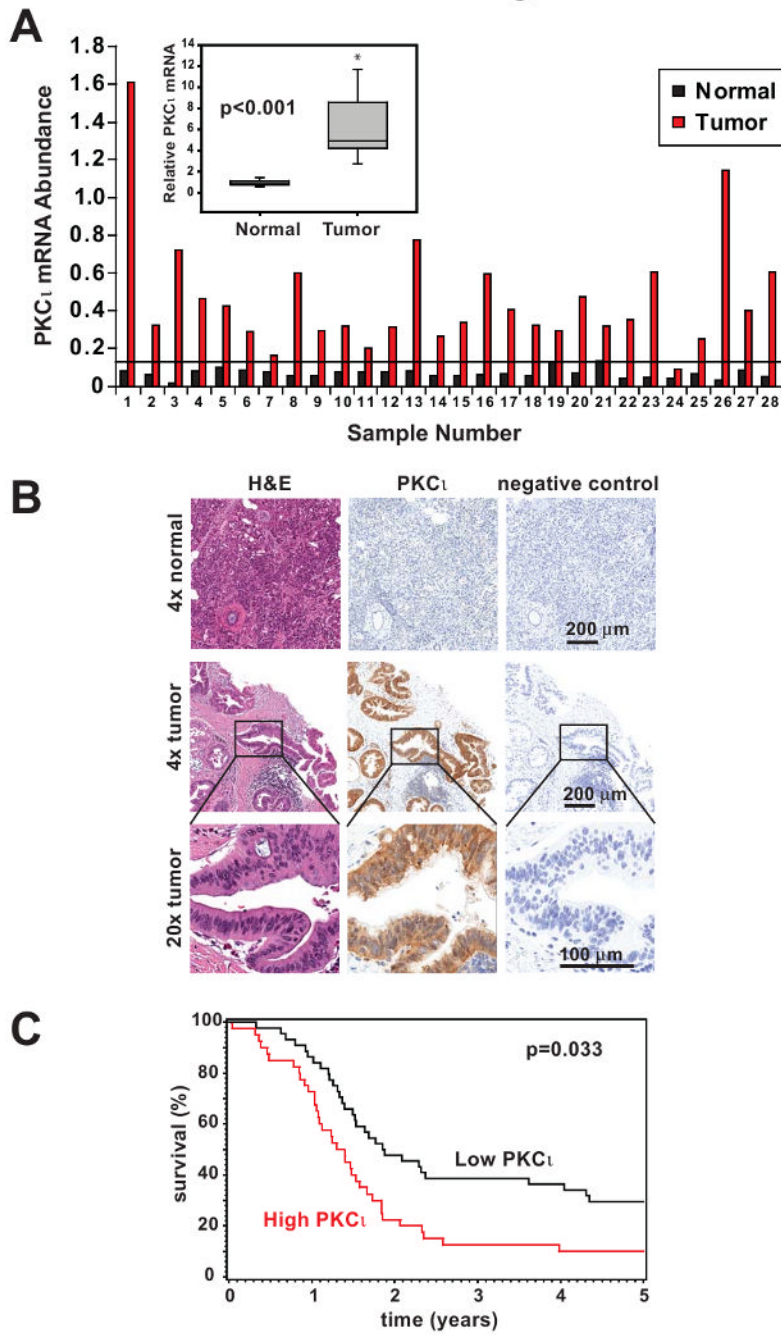


Figure 1. PKC ι is highly expressed in human pancreatic cancer and correlates with poor survival in PDAC patients

A) qPCR analysis of PKC ι mRNA expression in 28 matched human pancreatic tumor and adjacent non-tumor pancreas. Data were normalized to 18S RNA abundance ($\times 10^4$) to control for RNA concentration. Horizontal line indicates 2 standard deviations above the mean PKC ι mRNA abundance in adjacent non-tumor pancreas samples. Inset: PKC ι mRNA expression is significantly increased in tumors compared to matched non-tumor pancreas tissue. Average fold increase in PKC ι mRNA abundance in tumor/matched non-tumor is plotted. B) Representative images of IHC detection of PKC ι expression in formalin-fixed human pancreatic adenocarcinoma and normal pancreas. H&E staining and negative control

secondary antibody staining are also shown in serial sections. C) Kaplan-Meier survival curves. PDAC patient tumors were analyzed by IHC for PKC ζ expression and divided into high (red line) and low (black line) expression groups as described in **Materials and Methods**.

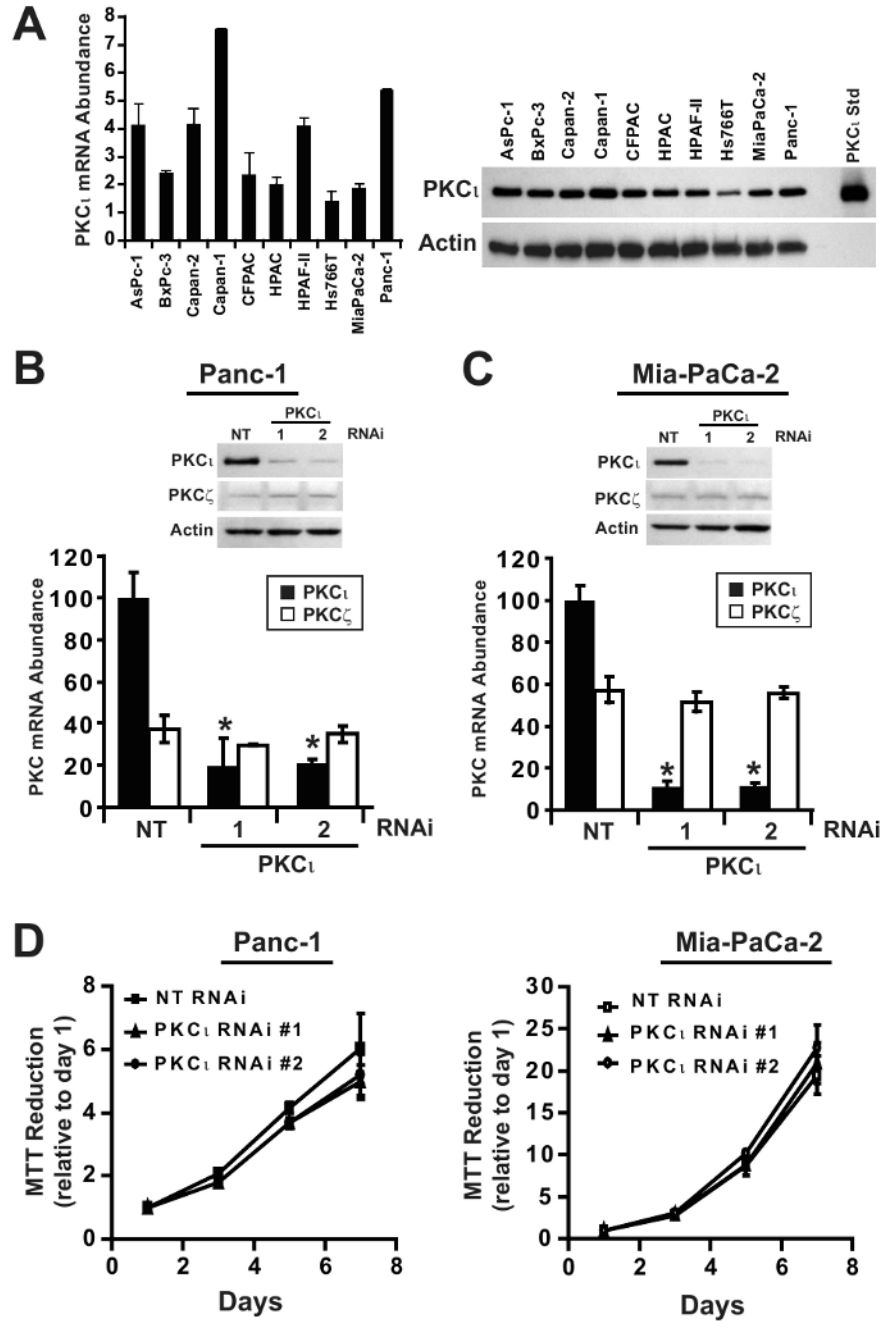


Figure 2. PKC ι is highly expressed in PDAC cell lines and is not required for anchorage-dependent (non-transformed) growth of PDAC cells

A) *Left*, qPCR analysis of PKC ι mRNA expression in ten human pancreatic cancer cell lines. mRNA abundance is normalized to GAPDH ($\times 10^2$), n=3. *Right*, Immunoblot analysis of ten human pancreatic cancer cell lines for expression of PKC ι and β -actin. qPCR analysis of PKC ι and PKC ζ mRNA expression in B) Panc-1 and C) MiaPaCa-2 stably carrying either non target (NT), PKC ι -specific RNAi constructs (PKC ι #1) or (PKC ι #2). PKC mRNA abundance is normalized to GAPDH and presented relative to PKC ι in NT RNAi cells. *Insets*, Immunoblot analysis of PKC ι , PKC ζ and β -actin protein expression in B) Panc-1 and C) MiaPaCa-2 NT or PKC ι -RNAi (PKC ι #1 and PKC ι #2) constructs. D) Anchorage-dependent growth in Panc-1

(*left*) and MiaPaCa-2 (*right*) stably carrying either NT or PKC ϵ -RNAi (PKC ϵ #1 and PKC ϵ #2) was determined by MTT colorimetric assay. Analysis was performed in triplicate and represents two independent experiments.

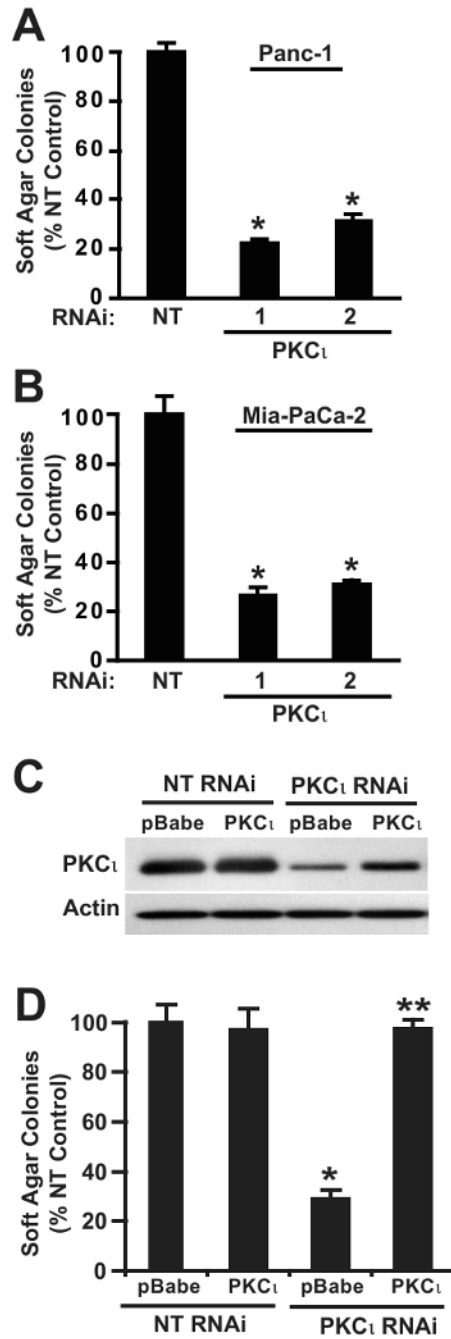


Figure 3. PKC ι is required for anchorage-independent growth of PDAC cells

Soft agar colony formation of A) Panc-1 and B) MiaPaCa-2 cells with NT or PKC ι -RNAi (PKC ι #1 and PKC ι #2) constructs. *= significantly different than NT. C) Immunoblot analysis of PKC ι expression in Panc-1 cells co-transfected with RNAi (NT or PKC ι) and control vector (pBabe) or vector expressing wild type PKC ι (PKC ι). D) Re-expression of PKC ι overcomes the inhibitory effect of PKC ι RNAi on soft agar colony formation. *= significantly different than control (NT & pBabe), **= significantly different than PKC ι RNAi & pBabe. Mean +/- SEM is plotted and represents two independent experiments.

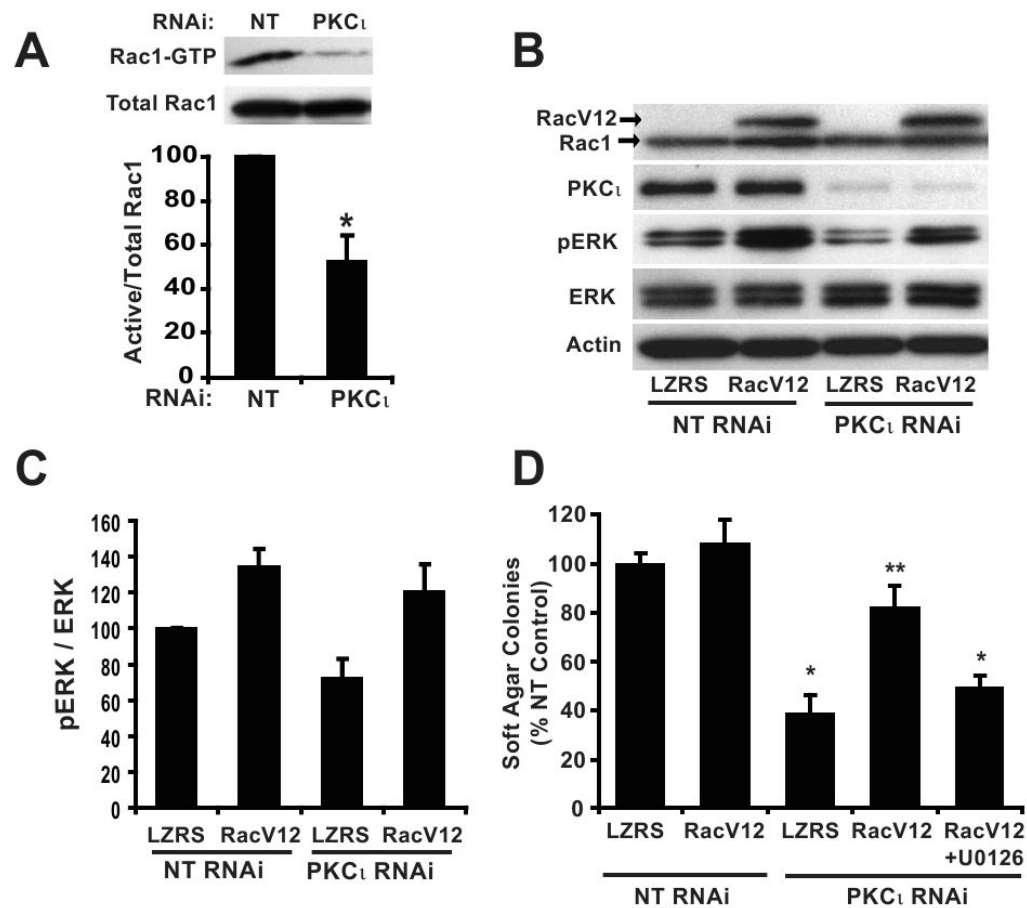


Figure 4. Constitutively active Rac1 (RacV12) recovers transformed growth of PKC ι RNAi PDAC cells in a MEK-dependent manner

A) Panc-1 cells stably expressing NT or PKC ι RNAi were assayed for Rac1 activity. *Top panel*, (Active) Rac1-GTP was precipitated from cell extracts with PAK-1 PBD agarose. Immunoblot analysis of precipitates and total cellular extracts (total Rac1) was performed using an anti-Rac1 antibody. *Bottom panel*, Quantitative, densitometric analysis of relative Rac1 activity (active Rac1/total Rac1). Mean \pm SEM is plotted, n=3. B) Panc-1 cells co-transfected with RNAi (NT or PKC ι) and control vector (LZRS) or vector expressing RacV12 were subject to immunoblot analysis for expression of Rac1, PKC ι , p-ERK1/2 (Thr202/Tyr204), ERK1/2 and actin as a loading control. Arrows indicate migration of endogenous Rac1 and slower migrating myc-tagged RacV12. C) Quantitation of densitometric analysis of relative p-ERK1/2 to ERK1/2 expression. Mean \pm SEM is plotted, n=3. D) Expression of RacV12 recovers the inhibitory effect of PKC ι RNAi on soft agar colony formation and requires MEK activity. +U0126= 10 μ M U0126 was included in the assay media and agar; *= significantly different than control (NT & LZRS) and RacV12 reconstituted (PKC ι RNAi & RacV12); **= significantly different than PKC ι KD (PKC ι RNAi & LZRS) and MEK-inhibited (PKC ι RNAi & RacV12+U0126). Mean \pm SEM is plotted and represents two independent experiments.

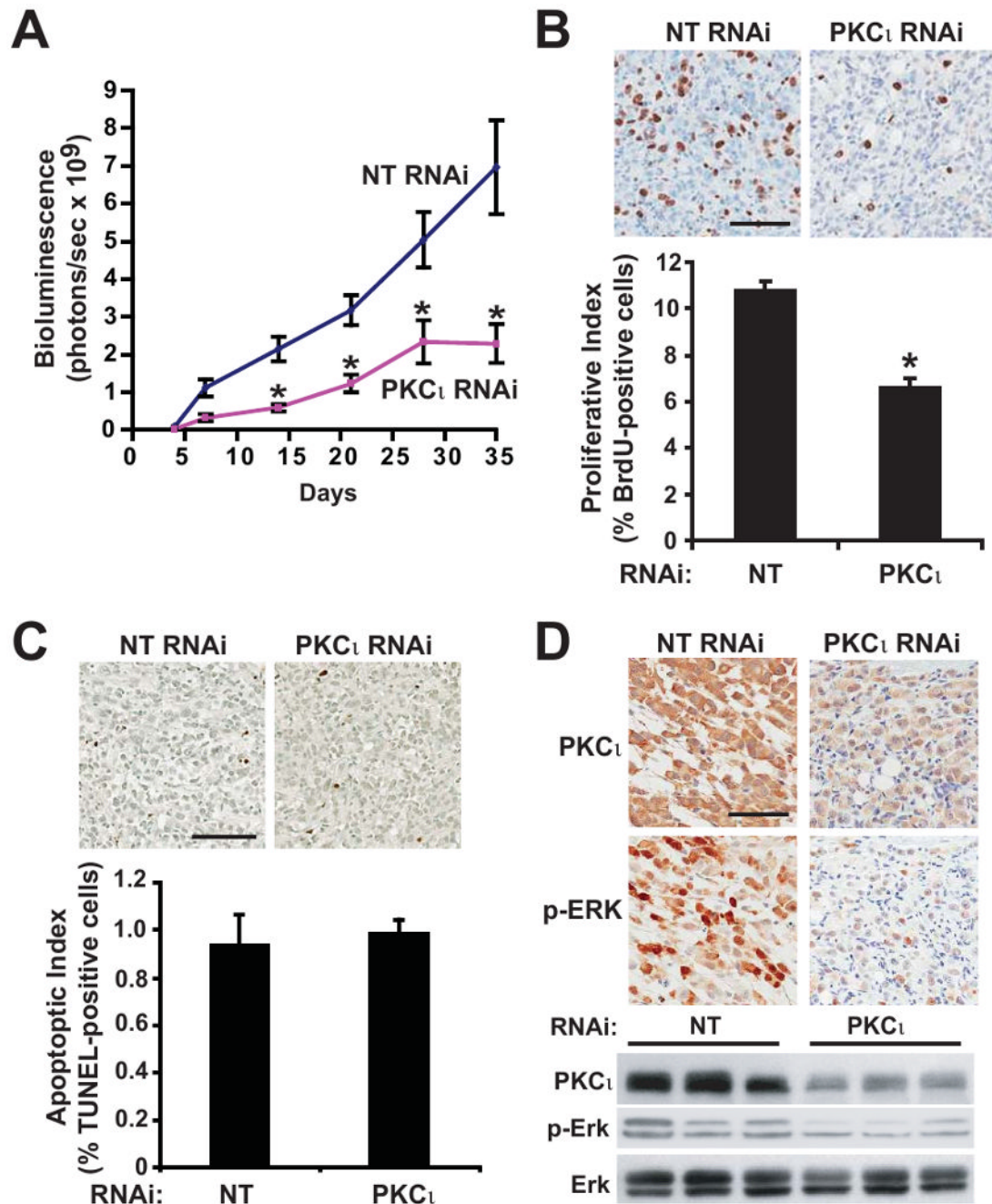


Figure 5. Inhibition of PKC_ι blocks orthotopic pancreatic tumor proliferation and proliferative signaling

A) Tumor growth was monitored by bioluminescence (total flux, photons/sec) detected by IVIS imaging of orthotopic Panc-1 NT versus Panc-1 PKC_ι RNAi pancreatic tumors in live, anesthetized mice at weekly intervals after tumor implantation. n=15, 16/group. *= significantly different than NT RNAi tumors. B) *Top*: Immunohistochemical analysis of BrdUrd incorporation. Bar=100μm. *Bottom*: Quantitative analysis of BrdUrd incorporation into Panc-1 tumors. Mean±SEM is plotted. C) *Top*: Immunohistochemical detection of TUNEL staining in representative tumors. Bar=100μm. *Bottom*: Quantitative analysis of TUNEL staining. Mean±SEM is plotted. D) Representative images of IHC detection of

PKC ζ and p-ERK1/2 (Thr 202/Tyr 204) in NT and PKC ζ RNAi tumors. Bar=100 μ m.
Representative immunoblot analysis of PKC ζ , p-ERK1/2 (Thr 202/Tyr 204) and ERK1/2 in Panc-1 NT and PKC ζ RNAi orthotopic pancreatic tumors. Equivalent amounts of protein from each tumor sample were analyzed.

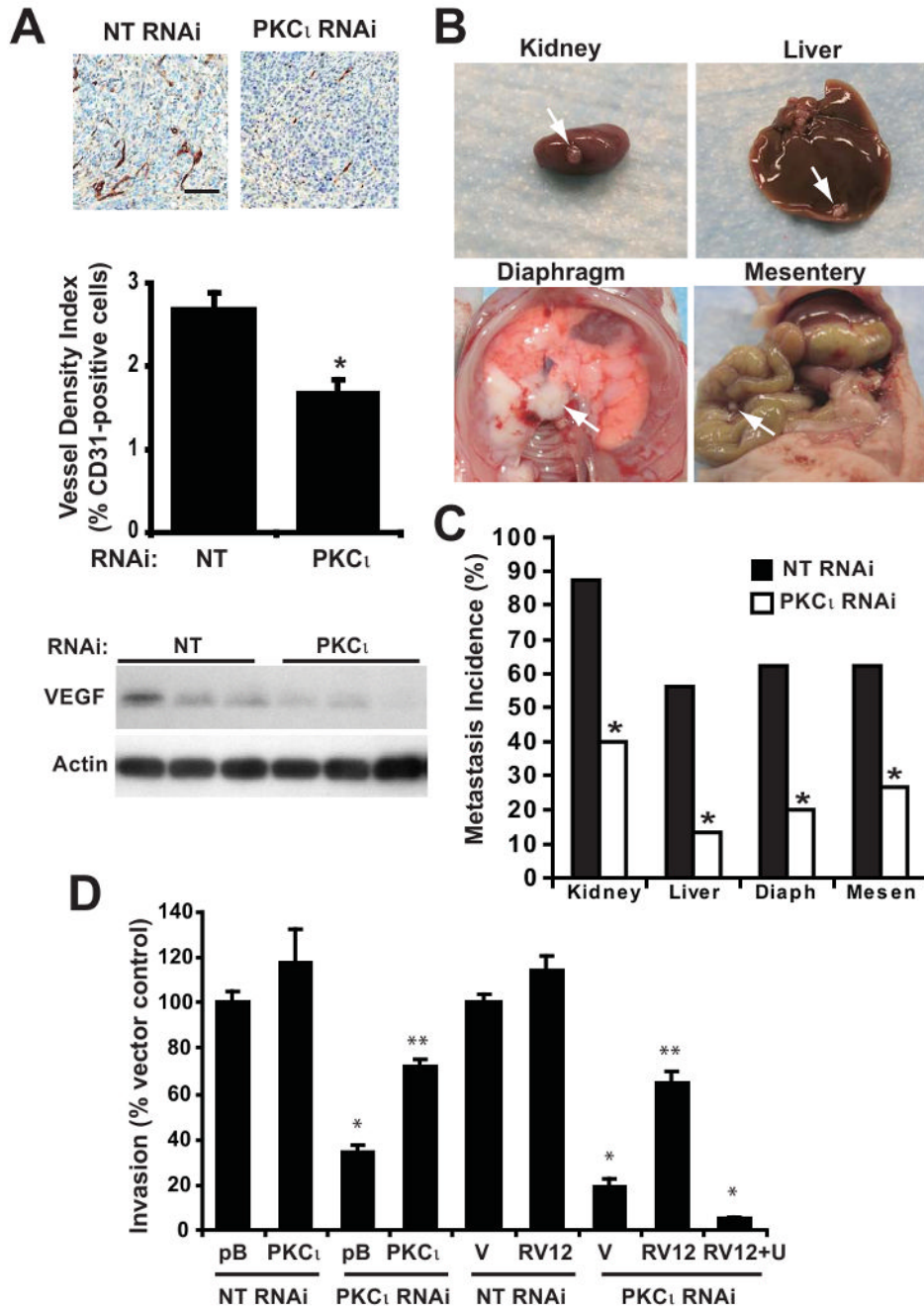


Figure 6. Inhibition of PKC ι blocks PDAC angiogenesis and metastasis

A) *Top*: Immunohistochemical detection of CD31 staining. Bar=100 μ m. *Middle*: Quantitative analysis of CD31 positive staining in Panc-1 tumors, calculated as the ratio of CD31-positive pixels to the sum of all pixels. Mean \pm SEM is plotted. *Bottom*: Representative immunoblot analysis of VEGF and actin in Panc-1 NT and PKC ι RNAi orthotopic pancreatic tumors. B) Representative images of tumor metastases to various organs. C) Percent of orthotopic Panc-1 NT and PKC ι RNAi pancreatic tumors that metastasized to various organs is plotted. *= significantly different than NT RNAi tumors. D) Panc-1 cells were assayed for cellular invasion through Matrigel-coated chambers as described in **Materials and Methods**. pB=pBABE control vector; V=LZRS control vector; RV12= RacV12; RV12+U= RacV12 treated with 10

μM U1026; *= significantly different than control (NT & pB) or (NT & V), **= significantly different than (PKC ζ RNAi & pB) or (PKC ζ RNAi & V) or (PKC ζ RNAi & RV12 +U). Mean \pm SEM is plotted and represents two independent experiments.