Protein Kinase Cι 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 Cι (PKCι) is required for the oncogenic Ras–mediated transformed growth of lung cancer and intestinal epithelial cells. However, little is known about the role of PKCι in pancreatic cancer. In this study, we evaluated the expression of PKCι in human pancreatic cancer and the requirement for PKCι for the transformed growth and tumorigenicity of PDAC cells. We find that PKCι is significantly overexpressed in human pancreatic cancer, and high PKCι expression correlates with poor patient survival. Inhibition of PKCι expression blocks PDAC cell transformed growth in vitro and tumorigenicity in vivo. Inhibition of PKCι expression in pancreatic tumors also significantly reduces tumor angiogenesis and metastasis. Analysis of downstream PKCι effector pathways implicates the Rac1-MEK/ERK1/2 signaling axis in PKCι-mediated transformed growth and cellular invasion. Taken together, our data show a required role for PKCι in the transformed growth of pancreatic cancer cells and reveal a novel role for PKCι in pancreatic cancer cell metastasis and angiogenesis in vivo. Our results strongly indicate that PKCι will be an effective target for pancreatic cancer therapy. Cancer Res; 70(5); 2064–74. ©2010 AACR.

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 a 5-year survival rate of only 20% (1). Due to the frequent failure of conventional therapies, there is an urgent 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). Antisense inhibition of oncogenic K-ras expression in pancreatic ductal adenocarcinoma (PDAC) cell lines blocks cellular transformation, showing 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 were introduced into the clinic to target mutant Ras, but have not been proven to be therapeutically effective in pancreatic cancer (reviewed in ref. 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 for pancreatic cancer.

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

Materials and Methods

Reagents and cell culture. Antibodies were obtained from the following sources: PKCι and Rac1 (BD Transduction...
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Laboratories); PKCζ, 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 vascular endothelial growth factor (VEGF; DakoCytonyme); and CD31 [platelet/endothelial cell adhesion molecule 1 (PECAM-1); Santa Cruz Biotechnology, Inc.]. U0126 was obtained from Sigma and NSC23766 from Tocris. Human pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in a 5% CO₂ humidified tissue culture incubator as recommended by ATCC. Retroviral capture and analyzed using Aperio Spectrum software. (Dako). p-ERK1/2 staining was visualized using the Envision using the Envision Plus Anti-Mouse Labeled Polymer-HRP described previously (14). PKCζ expression was scored by a pathologist blinded to patient clinical parameters (T.C.S.). Nuclear and cytoplasmic PKCζ levels were scored on a scale of 0 to 3 and combined for a total cellular expression score of 0 to 6. Low PKCζ was defined as a total expression score of ≤3 and high PKCζ as a total expression score >3, yielding two groups consisting of approximately half of the evaluable cases (45 and 40, respectively). Staining with only the secondary antibody served as a negative control.

**Knockdown and reexpression of human PKCζ gene and immunoblot analysis.** Lentivectors carrying short hairpin RNA interference (RNAi) targeting human PKCζ were generated and used to obtain stable transfectants as described previously (15). The PKCζ RNAi #1 construct targets a sequence in the 3′ untranslated region (UTR) of PKCζ (GCTGATGATCATCACGTTT), and the PKCζ RNAi #2 construct targets a sequence in the coding region of PKCζ (CCTGAGAATAGTCCCATGTT). Cells were stably transfected with pBabe and pBabe-PKCs as described previously (5). PKCζ and PKCζ 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 × 10⁴) were cultured for 1, 3, 5, and 7 d before the viability assay.

**Anchorage-independent growth assays.** Panc-1 and MiaPaCa-2 cells (5 × 10⁴) were plated in soft agar and assessed for anchorage-independent growth as described previously (16).

**Rac1 activity assay and signaling analysis.** Rac1 activity was assayed as described previously (5, 17). Cells stably expressing PKCζ RNAi constructs were cotransfected with LZRS vector or LZRS carrying myc-tagged, constitutively active Rac1 (RacV12) as described previously (5, 15). Transfectants were harvested and subjected to immunoblot analysis as previously described (16).

**Orthotopic tumor model.** Panc-1 human pancreatic cancer cells carrying pSIN-Fluc and expressing nontarget (NT) or PKCζ RNAi (1 × 10⁵) were mixed with growth factor–reduced Matrigel (Becton Dickinson) and injected into the proximal pancreas (n = 15 and 16 mice/group, respectively) of 4- to 6-wk-old male athymic nude mice. For weekly imaging, mice were injected i.p. with 150 mg/kg body weight D-luciferin solution (Xenogen), anesthetized with isofluorane, and imaged using a bioluminescence imaging system (IVIS Imaging Spectrum System). Bioluminescence was calculated using IVIS Imaging Spectrum software. One hour before sacrifice, mice were injected i.p. with 100 μg/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, 18, 19). Orthotopic pancreatic tumors were evaluated for apoptosis by TdT-mediated dUTP-biotin nick end labeling (TUNEL) of fragmented DNA as described (19). Angiogenesis was characterized by quantitative analysis of immunohistochemistry detection of CD31 (PECAM-1) expression as described (16, 19, 20). Expression of p-ERK1/2, ERK1/2, PKCs, VEGF, and β-actin was evaluated by immunoblot analysis of total cell lysates from orthotopic tumors.

**RNA isolation, quantitative real-time PCR, and analysis.** H&E-stained sections of matched normal and pancreatic tumor tissues were analyzed to confirm the presence of tumor or normal pancreas and the overall integrity of the frozen tissue samples. Total RNA was isolated using the RNAqueous 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 analysis of hGAPDH (Hs99999905_m1), hPKCζ (Hs00177051_m1), and 18S (Hs99999901_s1). Forward and reverse primer and probe sequences were designed and synthesized for hPKCζ (forward, 5′-CGTTCTTCCGAAATGTTGATTG-3′; reverse, 5′-TCCCGGAAGATTTGTTTAAAAGG-3′; probe, 5′-6FAMTTGCTCCATCATCATA3′-3′). Quantitative PCR analysis was carried out using 10 ng of cDNA or 2 ng of cDNA (18S) on an Applied Biosystems 7900 thermal cycler. Data were 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 are expressed as 2^-ΔΔCT(target) – ΔCT(endogenous reference).

**Immunohistochemistry and expression analysis.** A second set of formalin-fixed pancreatic cancers (13) were analyzed by immunohistochemistry for PKCζ expression. A few of the matched normal and pancreatic tumor tissue pairs were also available as formalin-fixed tissues and were analyzed by immunohistochemistry for PKCζ expression. Tissues were processed for immunohistochemistry as described previously (14). PKCζ 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 Spectrum software. PKCζ expression was scored by a pathologist blinded to patient clinical parameters (T.C.S.). Nuclear and cytoplasmic PKCζ levels were scored on a scale of 0 to 3 and combined for a total cellular expression score of 0 to 6. Low PKCζ was...
**Cellular invasion assay.** Cellular invasion was measured as described (6, 15). The selective MEK inhibitor U0126 (10 μmol/L) was included in the medium in the upper and lower chambers in some experiments, as indicated.

**Statistical analysis.** Survival rates were calculated using the Kaplan-Meier analysis. Differences in survival were analyzed by log-rank test, Fisher’s exact test, and univariate and multivariate Cox proportional hazard models using SAS 9.1.3 software. All tests were two-sided. One-way ANOVA and the pairwise multiple comparison procedures were used to evaluate the statistical significance of the results. *P < 0.05* was considered statistically significant.

**Results and Discussion**

**PKCι is highly expressed in human pancreatic cancer.** To investigate the role of PKCι in pancreatic cancer, we first evaluated PKCι expression in a panel of 28 human pancreatic tumors and adjacent nontumor (normal) pancreas mRNA samples (Fig. 1A). PKCι mRNA was detected in all 28 primary pancreatic tumors analyzed (Fig. 1A). PKCι overexpression, defined as tumor mRNA abundance greater than 2 SD above the average PKCι mRNA abundance in adjacent nontumor pancreas, was observed in 27 of 28 pancreatic tumors analyzed (Fig. 1A). Pancreatic tumors exhibited an average 9 ± 2-fold increase in PKCι mRNA expression relative to matched nontumor pancreas (inset; *P < 0.001* for paired samples). Immunohistochemical analysis of PKCι expression was conducted on two matched pancreatic tumor/nontumor pancreas pairs from this set for which formalin-fixed tissues were available. Immunohistochemistry confirmed increased expression of PKCι in both tumors compared with the matched nontumor pancreas (Supplementary Fig. S1). PKCι localized to both the nucleus and the cytoplasm of the tumor cells, with little or no expression of PKCι in the surrounding stromal components (Fig. 1B; Supplementary Fig. S1). We next validated our analysis of PKCι expression in a second group of 85 pancreatic tumor tissues (13) for which formalin-fixed tissues were available (Supplementary Table S1). Cases were segregated into high and low PKCι expression groups based on immunohistochemical staining intensity and subjected to survival analysis, as described in Materials and Methods (Fig. 1C; Supplementary Table S2). Patients whose tumors exhibit high PKCι expression had significantly shorter survival time (median survival time of 492 days for high PKCι expression versus 681 days for low PKCι expression, *P = 0.033*) and a reduced 5-year survival rate (10% versus 29.5% for low PKCι expression, *P = 0.032*). Multivariate analysis adjusting for age, sex, and tumor stage shows a significant association between high PKCι expression and poor survival of PDAC patients (hazards ratio, 1.670; 95% confidence interval, 1.037–2.688; *P = 0.035*).

**PKCι is required for the transformed growth of PDAC cells in vitro.** The prevalence of PKCι overexpression in primary pancreatic tumors and the association of high PKCι with poor clinical outcome strongly suggest a role for PKCι in the pathology of pancreatic cancer. To evaluate this possibility, we first assessed PKCι mRNA and protein expression in a panel of PDAC cell lines (Fig. 2A). Whereas PKCι was abundantly expressed in all PDAC cell lines evaluated, PKCι expression did not directly correlate with known characteristics of the PDAC cell lines, including differentiation status and biological behavior such as soft agar growth, migration or invasion rate, or sensitivity to gemcitabine (21–23).

To directly assess the role of PKCι in the PDAC phenotype, we used lentiviral-mediated RNAi knockdown (KD) to inhibit PKCι expression in two widely used human PDAC cell lines, Panc-1 and MiaPaCa-2 (Fig. 2B and C). Two PKCι-targeted RNAi constructs significantly inhibited PKCι mRNA and protein expression in both PDAC cell lines. These RNAi constructs had no effect on the expression of the closely related atypical PKCζ isozyme, showing their specificity for PKCι (Fig. 2B and C). Although PKCι KD had no significant effect on log-phase, adherent (nontransformed) cellular growth (Fig. 2D) or cellular proliferation (Supplementary Fig. S2) of Panc-1 and MiaPaCa-2 cells, PKCι KD significantly inhibited anchorage-independent growth in both PDAC cell lines (Fig. 3A and B). Whereas PKCι has been implicated in both proapoptotic and antiapoptotic signaling (24, 25), we did not observe any effect of PKCι KD on PDAC cell apoptosis (Supplementary Fig. S3). These results are consistent with previous findings in intestinal epithelial cells, non–small cell lung carcinoma (NSCLC) cells, and ovarian cancer cells (5, 15, 16, 26).

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

**Rac1 is a critical effector of PKCι-mediated transformed growth in pancreatic cancer cells.** We previously showed that PKCι drives the transformed growth of intestinal epithelial cells and NSCLC cells by activating the Rac1-MEK/ERK signaling pathway (15, 16, 27). Having established a requirement for Rac1 and MEK in Panc-1 cell transformed growth, we evaluated the required signaling downstream of PKCι. We first assessed the requirement for Rac1 and MEK in Panc-1 cell transformed growth. Pharmacologic inhibition of either Rac1 (NSC23766) or MEK (U0126) significantly inhibited Panc-1 cell soft-agar colony formation (ref. 28; Supplementary Fig. S4), showing the requirement for both Rac1 and MEK activities for Panc-1 cell transformed growth. We next assessed whether PKCι regulates Rac1 and MEK activities in PDAC cells. PKCι RNAi significantly reduced Rac1 activity (Fig. 4A) and ERK phosphorylation in Panc-1 cells (Fig. 4B and C). Similar results were observed in MiaPaCa-2 cells (data not shown). To determine whether Rac1 and MEK/ERK...
PKCι is highly expressed in human pancreatic cancer and correlates with poor survival in PDAC patients. A, quantitative PCR analysis of PKCι mRNA expression in 28 matched human pancreatic tumor and adjacent nontumor pancreas. Data were normalized to 18S RNA abundance (×10^4) to control for RNA concentration. Horizontal line, 2 SD above the mean PKCι mRNA abundance in adjacent nontumor pancreas samples. Inset, PKCι mRNA expression is significantly increased in tumors compared with matched nontumor pancreas tissue. Columns, average fold increase in PKCι mRNA abundance in tumor/matched nontumor pancreas. B, representative images of immunohistochemistry detection of PKCι 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 immunohistochemistry for PKCι expression and divided into high (red line) and low (black line) expression groups as described in Materials and Methods.
activities are required downstream of PKCι in the transformed growth of PDAC cells, we evaluated the ability of myc-tagged, constitutively active Rac1 (RacV12) to reconstitute transformed growth in PKCι RNAi Panc-1 cells. Expression of RacV12 restored the transformed growth of PKCι RNAi cells (Fig. 4D) and ERK1/2 phosphorylation (Fig. 4B and C) without affecting PKCι or ERK1/2 expression (Fig. 4B). Furthermore, inhibition of MEK blocks RacV12-mediated reconstitution of transformed growth in PKCι KD cells (Fig. 4D). Taken together, these data show that the PKCι-Rac1-MEK/ERK signaling pathway is required for the transformed growth of Panc-1 cells.

**PKCι plays a critical role in PDAC cell tumorigenesis.**
We next used an orthotopic pancreatic tumor model to...
evaluate the role of PKCι in PDAC tumor growth and metastasis in vivo (29). Panc-1 cells expressing the firefly luciferase gene (pSIN-Fluc) and either NT or PKCι RNAi were injected orthotopically into the pancreas of nude mice (30). Tumor growth was monitored by bioluminescence weekly over a 5-week time course (Fig. 5A). Tumor formation was observed in all mice injected with either NT RNAi– or PKCι RNAi–expressing Panc-1 cells. However, PKCι RNAi tumors grew at a slower rate than NT RNAi tumors, resulting in significantly smaller tumors (Fig. 5A). We hypothesized that the smaller size of PKCι 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 PKCι RNAi tumors when compared with NT RNAi tumors (Fig. 5B). In contrast, PKCι KD had no effect on tumor apoptosis (Fig. 5C). Thus, the reduced tumor volume of PKCι RNAi pancreatic tumors is due to decreased cellular proliferation of the tumor cells.

To investigate whether MEK/ERK1/2 activity is regulated by PKCι expression in Panc-1 cells in vivo, as observed in vitro (Fig. 4), we evaluated the status of ERK1/2 phosphorylation in NT and PKCι RNAi tumors. Immunohistochemistry analysis revealed a dramatic decrease in p-ERK1/2 and PKCι in PKCι RNAi tumors when compared with NT RNAi tumors (Fig. 5D). Immunoblot analysis confirmed reduced PKCι expression and reduced p-ERK1/2 in PKCι RNAi tumors compared with NT RNAi tumors (Fig. 5D). These data strongly implicate PKCι-mediated activation of the Rac1-MEK/ERK1/2 proliferative signaling pathway in PDAC tumorigenesis in vivo. In this regard, we detected elevated ERK phosphorylation in our panel of human pancreatic tumors (Supplementary Fig. S5) as previously described (31, 32).

**PKCι expression regulates the angiogenesis, invasion, and metastasis of PDAC orthotopic tumors.** Angiogenesis plays an important role in tumor cell proliferation. We therefore evaluated the effect of PKCι KD on angiogenesis in orthotopic PDAC tumors by immunohistochemical detection of the endothelial cell marker CD31 in NT and PKCι RNAi tumors (Fig. 6A). CD31 expression was significantly decreased in PKCι RNAi tumors (Fig. 6A), indicating that PKCι in tumor cells regulates tumor angiogenesis. Vascular endothelial cell growth factor (VEGF) is a major proangiogenic factor expressed in tumor tissue. VEGF expression was considerably decreased in PKCι RNAi tumors (Fig. 6A), supporting the conclusion that PKCι drives tumor angiogenesis by regulating VEGF expression in pancreatic tumors. Because ERK activity has been shown to regulate VEGF expression in PDAC cells (33), it is possible that PKCι regulates VEGF expression, at least in part, through 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 with PKCι RNAi tumors.

Because tumor angiogenesis can be permissive for tumor metastasis, we determined the effect of PKCι RNAi on the metastatic capacity of Panc-1 orthotopic tumors in vivo (Fig. 6B and C). As described previously (34, 35), 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 (Fig. 6B and C). In contrast, PKCι RNAi tumors exhibited significantly
reduced metastases to all of these organ sites (Fig. 6C). To investigate the mechanism by which PKCι regulates tumor metastasis, we evaluated the requirement for PKCι for cellular invasion (Fig. 6D). Cellular invasion is significantly inhibited in PKCι RNAi Panc-1 cells (Fig. 6D). Expression of exogenous PKCι reconstituted the PKCι KD phenotype, showing the specific requirement for PKCι for invasion (Fig. 6D). Because Rac1 and MEK activities are also required for Panc-1 cellular invasion (Supplementary Fig. S6), we evaluated whether Rac1/MEK signaling is downstream of PKCι in cellular invasion (Fig. 6D). Expression of Racv12 reconstituted cellular invasion in PKCι KD Panc-1 cells in a MEK-dependent manner (Fig. 6D). These data reveal a novel required role for PKCι-Rac1-MEK/ERK signaling in cellular invasion in vitro and, taken together with our in vivo data, suggest a role for PKCι-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 show that PKCι is dispensable for adherent pancreatic cancer cell growth, but is required for PDAC cell transformed growth in vitro and tumorigenicity in vivo. This observation suggests that chemotherapeutic interventions targeting PKCι will specifically inhibit the growth of transformed pancreatic tumor cells while having little effect on nontransformed pancreatic epithelial cells. In this regard, we have identified and characterized a molecularly targeted inhibitor of PKCι, aurothiomalate (17, 20, 36), 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 the expression of PKCι for its cytotoxic effects (37). Although the target of oncrasin-1 remains to be...
elucidated (it does not directly inhibit PKC\(\text{\(\iota\)}\) activity in vitro; ref. 37), this study provides further support for the conclusion that PKC\(\text{\(\iota\)}\) plays a critical role in the transformed phenotype of oncogenic K-ras-mediated cancer.

Our study elucidates a critical molecular mechanism by which PKC\(\text{\(\iota\)}\) promotes the transformed growth and cellular invasion of PDAC cells. PKC\(\text{\(\iota\)}\) regulates Rac1 and ERK1/2 activities, both of which are required for PDAC cell transformed growth and invasion. Specifically, PKC\(\text{\(\iota\)}\) 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\(\text{\(\iota\)}\) in NSCLC cells (16). We further show a required role for PKC\(\text{\(\iota\)}\) in PDAC tumorigenesis in vivo and its novel role in PDAC tumor metastasis. Because PKC\(\text{\(\iota\)}\) also regulates ERK activation in vivo, these data suggest that the Rac1-MEK/ERK1/2 signaling pathway plays a role in PKC\(\text{\(\iota\)}\)-dependent PDAC tumor cell proliferation and metastasis in vivo. Interestingly, similar to high PKC\(\text{\(\iota\)}\) expression, ERK phosphorylation predicts poor survival of pancreatic cancer patients (31, 32).

We have recently shown that PKC\(\text{\(\iota\)}\) is overexpressed in the majority of primary lung cancers and that high PKC\(\text{\(\iota\)}\) expression predicts poor survival in NSCLC patients (38). In lung squamous cell cancers, as well as ovarian cancer, elevated PKC\(\text{\(\iota\)}\) expression is the result of tumor-specific amplification of the PKC\(\text{\(\iota\)}\) gene (26, 38). Because gene amplification frequently occurs in pancreatic cancer, this is a possible mechanism for increased PKC\(\text{\(\iota\)}\) in human pancreatic cancers. Future studies will address this possibility.

In summary, PKC\(\text{\(\iota\)}\) is highly overexpressed in the majority of primary pancreatic cancers, and elevated PKC\(\text{\(\iota\)}\) expression correlates with decreased survival time. PKC\(\text{\(\iota\)}\) and its downstream effectors Rac1 and MEK/ERK1/2 are required for PDAC transformed growth and cellular invasion in vitro, and PKC\(\text{\(\iota\)}\) is required for PDAC tumorigenicity and tumor cell proliferation in vivo. Finally, we describe a previously
Figure 6. Inhibition of PKCι blocks PDAC angiogenesis and metastasis. A, top, immunohistochemical detection of CD31 staining. Bar, 100 μm. 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. Columns, mean; bars, SEM. Bottom, representative immunoblot analysis of VEGF and actin in Panc-1 NT and PKCι RNAi orthotopic pancreatic tumors. B, representative images of tumor metastases to various organs. C, percent of orthotopic Panc-1 NT and PKCι 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, L2RS control vector; RV12, RacV12; RV12 + U, RacV12 treated with 10 μmol/L U1026; **, significantly different than control NT and pB or NT and V; ***, significantly different than PKCι RNAi and pB, PKCι RNAi and V, or PKCι RNAi and RV12 +U. Columns, mean; bars, SEM. Representative of two independent experiments.
unappreciated role for PKCι in PDAC tumor angiogenesis and metastasis. These data identify PKCι as an attractive therapeutic target for the treatment of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

Mayo Clinic has filed a provisional patent application for the technology associated with this research. This technology is not licensed.

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12. Hasegawa K, Nakamura T, Harvey M, et al. The use of a tropism-associate with this research. This technology is not licensed.