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; therefore, there is a critical need for new molecular targets for pancreatic cancer chemotherapy. Mutational activation of the *KRAS* proto-oncogene occurs in >90% of PDAC. Oncogenic K-ras activates atypical protein kinase C iota (PKCι) and PKCι is required for oncogenic Ras-mediated transformed growth in lung cancer and intestinal epithelial cells. However, little is known about the role of PKCι in pancreatic cancer. In this study we evaluated the requirement for PKCι for the transformed growth and tumorigenicity of PDAC cells. Our results demonstrate that PKCι is significantly over-expressed in human pancreatic cancer and is required for PDAC cellular transformation *in vitro* and *in vivo*. Specifically, inhibition of PKCι expression blocks PDAC cell transformed growth *in vitro* and tumorigenicity *in vivo*. Analysis of PKCι downstream effectors implicates Rac1/(PAK)-MEK-ERK1/2 signaling in PKCι-mediated transformed growth. Inhibition of PKCι expression in orthotopic pancreatic tumors also significantly reduces tumor angiogenesis and metastasis. Taken together, our data demonstrate a required role for PKCι in the transformed growth of pancreatic cancer cells and documents a novel role for PKCι in pancreatic cancer cell metastasis and angiogenesis *in vivo*. These results strongly suggest that PKCι will be an effective target for pancreatic cancer therapy.

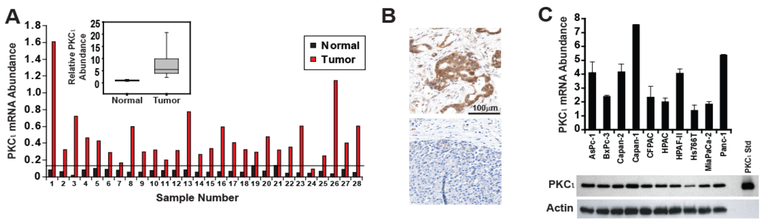

Figure 1: PKCι is highly expressed in human pancreatic cancer and PDAC cell lines.

A) qPCR analysis of PKCι mRNA expression in 28 matched human pancreatic tumor and adjacent non-tumor pancreas. mRNA abundance is normalized to 18S x 104. Horizontal line indicates the mean PKCι mRNA abundance in non-tumor matched tissues + 2*(SD). Inset: PKCι mRNA expression is significantly increased in tumors compared to normal pancreas tissue. The mean PKCι mRNA abundance in non-tumor matched tissues is set equivalent to 1, and average fold-increase is plotted for pancreatic tumors. B) A representative image of immunohistochemical detection of PKCι expression in a formalin-fixed primary pancreatic cancer reveals a high level of expression compared to normal adjacent human pancreas (bottom, right panel). C) qPCR analysis of PKCι mRNA expression in ten human pancreatic cancer cell lines (top). mRNA abundance is normalized to GAPDH x 100, n=3. Immunoblot analysis of total cell lysates from ten human pancreatic cancer cell lines for expression of PKCι and β-actin (bottom).

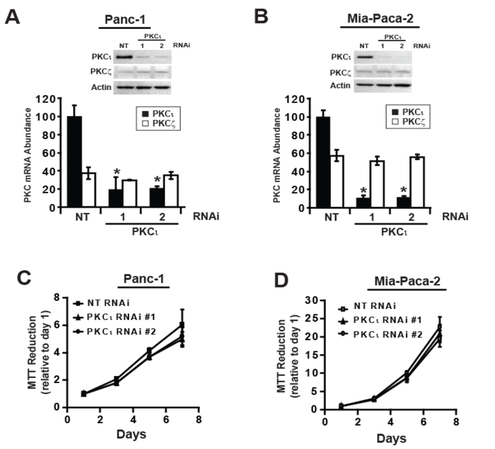

Figure 2: PKCι is not required for anchorage-dependent (non-transformed) growth of PDAC cells.

qPCR analysis of PKCι mRNA expression in A) Panc-1 and B) MiaPaCa-2 stably carrying either non target (NT), PKCι-specific RNAi constructs (PKCι #1) or (PKCι #2). Analysis was performed in triplicate and is representative of two independent experiments. Insets, Immunoblot analysis of PKCι, PKCζ and β-actin protein expression in A) Panc-1 and B) MiaPaCa-2 NT or PKCι-RNAi (PKCι#1 and PKCι#2) constructs. Anchorage-dependent growth in C) Panc-1 and D) MiaPaCa-2 stably carrying either NT or PKCι-RNAi (PKCι#1 and PKCι#2) was determined by MTT colorimetric assay.

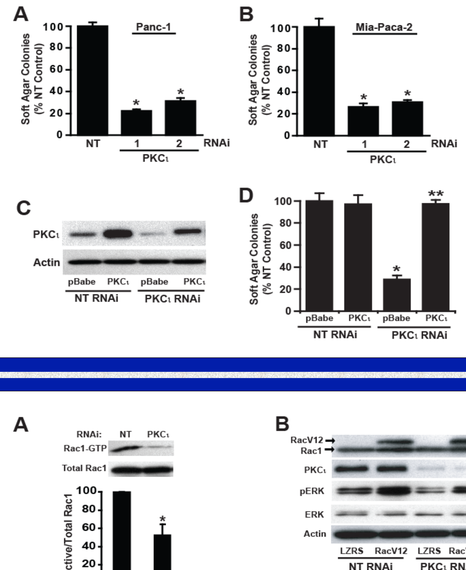

Figure 3: PKCι is required for anchorage-independent growth of PDAC cells.

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

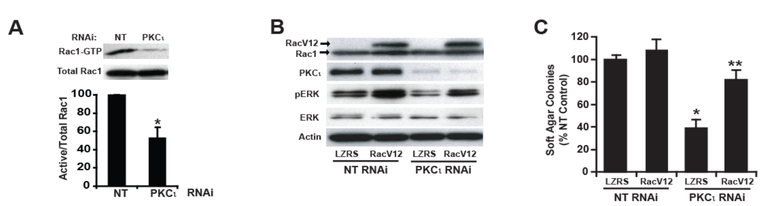

Figure 4: Constitutively active Rac1 recovers transformed growth of PKCι RNAi PDAC cells.

*A) Panc-1 cells stably expressing NT or PKCι 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 Ab. Bottom panel, Quantitative, densitometric analysis of relative Rac1 activity (active Rac1/total Rac1). Mean of three independent experiments +/- SEM is plotted. B) Panc-1 cells co-transfected with RNAi (NT or PKCι) and control vector (LZRS) or vector expressing constitutively active (ca) Rac1 (caRac) were subject to immunoblot analysis for expression of Rac1, PKCι, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 and actin as a loading control. C) Expression of caRac1 recovers the inhibitory effect of PKCι RNAi on soft agar colony formation. *= significantly different than control (NT & LZRS), **= significantly different than PKCι RNAi & LZRS. Mean +/- SEM is plotted and represents at least two independent experiments.*

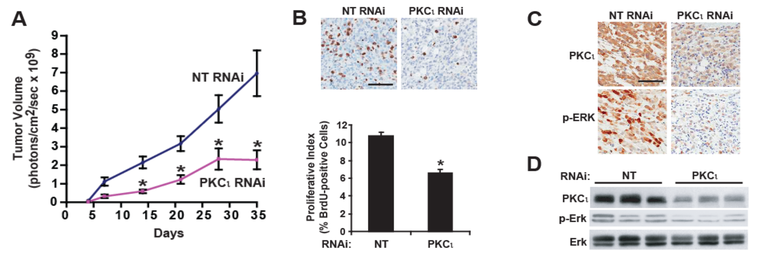

Figure 5: Inhibition of PKCι blocks orthotopic pancreatic tumor proliferation and proliferative signaling.

A) Tumor growth was monitored by bioluminescence (total flux, photons/sec) detected by IVIS imaging of orthotopic Panc-1 NT versus Panc-1 PKCι RNAi pancreatic tumors in live, anesthetized mice at weekly intervals after tumor implantation. *= significantly different than NT RNAi tumors. n=15-16/group. B) *Top:* Immunohistochemical analysis of BrdU incorporation. Bar=100μm. *Bottom*: Quantitative analysis of BrdU incorporation into Panc-1 tumors. Mean +/- SEM is plotted. C) Immunohistochemical detection of PKCι and phospho-ERK1/2 (Thr 202/ Tyr 204) (representative images). Bar=100μm. D) Representative immunoblot analysis of PKCι, phospho-ERK1/2 (Thr 202/ Tyr 204) and ERK1/2 in Panc-1 NT and PKCι RNAi orthotopic pancreatic tumors. Equivalent amounts of protein from each tumor sample were analyzed.

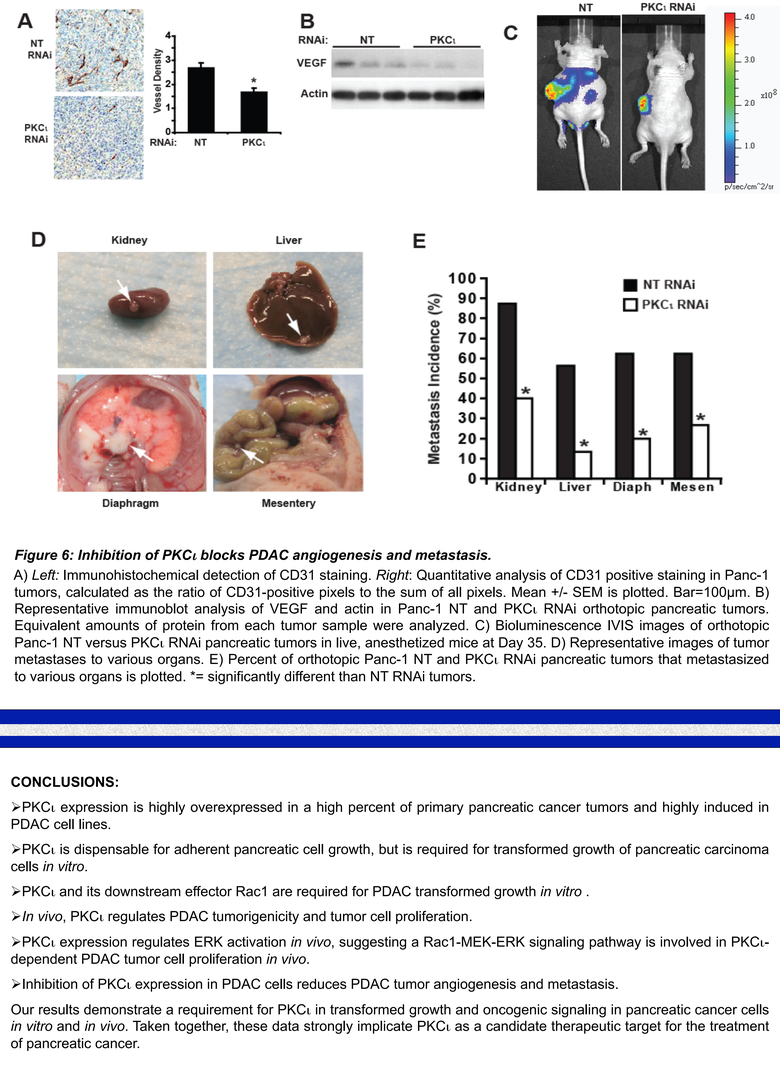

Figure 6: Inhibition of PKCι blocks PDAC angiogenesis and metastasis.

A) *Left:* Immunohistochemical detection of CD31 staining. *Right*: 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 +/- SEM is plotted. Bar=100μm. B) Representative immunoblot analysis of VEGF and actin in Panc-1 NT and PKCι RNAi orthotopic pancreatic tumors. Equivalent amounts of protein from each tumor sample were analyzed. C) Bioluminescence IVIS images of orthotopic Panc-1 NT versus PKCι RNAi pancreatic tumors in live, anesthetized mice at Day 35. D) Representative images of tumor metastases to various organs. E) Percent of orthotopic Panc-1 NT and PKCι RNAi pancreatic tumors that metastasized to various organs is plotted. *= significantly different than NT RNAi tumors.

CONCLUSIONS:

- PKCι expression is highly overexpressed in a high percent of primary pancreatic cancer tumors and highly induced in PDAC cell lines.
- PKCι is dispensable for adherent pancreatic cell growth, but is required for transformed growth of pancreatic carcinoma cells *in vitro*.
- PKCι and its downstream effector Rac1 are required for PDAC transformed growth *in vitro* .
- *In vivo*, PKCι regulates PDAC tumorigenicity and tumor cell proliferation.
- PKCι expression regulates ERK activation *in vivo*, suggesting a Rac1-MEK-ERK signaling pathway is involved in PKCι-dependent PDAC tumor cell proliferation *in vivo*.
- Inhibition of PKCι expression in PDAC cells reduces PDAC tumor angiogenesis and metastasis.

Our results demonstrate a requirement for PKCι in transformed growth and oncogenic signaling in pancreatic cancer cells *in vitro* and *in vivo*. Taken together, these data strongly implicate PKCι as a candidate therapeutic target for the treatment of pancreatic cancer.

ACKNOWLEDGEMENTS:

Grant Support: NIH grants CA12866 (N.R. Murray), Mayo Clinic SPORE in Pancreatic Cancer Career Development award P50 CA102701 (N.R. Murray), CA081436 (A.P. Fields), Daniel Foundation of Alabama Postdoctoral Fellowship (M.L. Scotti) and The Mayo Clinic Foundation. We thank Brandy Edenfield, Amanda Butler, Shelly R. Calcagno, Shuhua Li, Alyssa Kunz, and Lee Jamieson of the Mayo Clinic Jacksonville for excellent technical support. We thank Dr. Brian Rabinovich, Univ. Texas MD Anderson for providing the pSIN-LUC vector. We acknowledge the Mayo Clinic SPORE in Pancreatic Cancer (P50 CA102701 Gloria Petersen, PI) and the Lustgarten Foundation for providing the biospecimens used in this study.