Cyclin Dependent Kinase 5 is amplified and over-expressed in pancreatic cancer and activated by mutant K-Ras

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Statement of Translational Relevance.

We demonstrate that CDK5 or its co-activators p35 and p39 are significantly over-expressed in >90%, 94% and 75% of PDACs respectively compared to normal pancreases, in part due to genomic amplification, as genes for CDK5, p35, or p39 were amplified in 67% (n=39) of tumors analyzed. CDK5 is hyperactivated by signaling downstream of mutant K-Ras, resulting in increased tumor migration and invasive properties. CDK5 represents a novel and unexplored therapeutic target for both early and late stage PDACs, and should be investigated in solid tumors that harbor K-Ras mutations, including those of pancreas, lung and colon.
Abstract

Purpose: To evaluate the nature of CDK5 hyperactivity in pancreatic cancer progression.

Experimental Design: We utilized genetic, biochemical, and molecular biology methods to investigate the nature and function of overexpression of CDK5 and its activators p35 and p39 during the progression of pancreatic cancer.

Purpose: Investigate the expression and function of CDK5 and its co-activators p35 and p39 in pancreatic cancer.

Experimental Design: Determine the genetic and biochemical cause of CDK5 hyperactivity in pancreatic cancer.

Results: Amplification of the CDK5 gene or either of its main activators, p35 and p39, was observed in 67% of human pancreatic ductal adenocarcinoma (PDAC). CDK5, p35 and p39 were rarely expressed in pancreatic ducts, while greater than 90% of PDACs had increased levels of CDK5 and p35. Increased levels of CDK5, p35 and p39 protein were observed in several pancreatic cancer cell lines. Inhibition of CDK5 kinase activity using a CDK5 dominant negative mutant or the drug roscovitine significantly decreased the migration and invasion of pancreatic cancer cells in vitro. Increased CDK5 kinase activity was also observed in immortalized human pancreatic cells (HPNE) expressing a mutant form of K-Ras (G12D) compared to HPNE cells expressing native K-Ras. G12D K-Ras increased cleavage of p35 to p25, a stable and greater activator of CDK5, thus implicating a role for CDK5 in early progression of PDAC. Inhibition of the signaling cascade downstream of mutant K-Ras (G12D) that involves MEK, PI3K or CDK5 decreased p25 protein levels.
Conclusion: These results suggest that mutant K-Ras acts in concert with CDK5 and its activators to increase malignant progression, migration, and invasion of pancreatic cancer cells.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) are lethal tumors that result in a median survival of less than 6 months from time of diagnosis and a five-year survival of less than 5% \(^1\). The poor survival is largely attributed to aggressive growth, advanced stage at diagnosis, and local recurrence. Activating mutations in K-Ras are nearly universal in PDAC and common in precursor pancreatic intraepithelial neoplasia (PanIN) lesions (1). The identification of effectors downstream of K-Ras that modulate growth, cell migration, and invasion will help understand the process of disease progression and enable the development of novel therapeutic targets for patients with PDAC.

Perineural invasion (PNI) is a hallmark of pancreatic cancer (2, 3). Pancreatic innervation includes enteric nervous connections to spinal cord and brain that mediate pancreatic exocrine and endocrine functions (4). An extensive neural network surrounding the pancreas, coupled with the frequent PNI observed in pancreatic cancer led us to investigate molecules expressed in pancreatic cancer cells that are also involved in cellular migration during neural development. Cyclin Dependent Kinase 5 (CDK5) is necessary for proper cortical neuronal migration and patterning. CDK5 -/- (deficient) mice die in the perinatal period, because of the congenital loss of 4 of 6 cortical layers in the brain (5). CDK5-catalyzed phosphorylation of PAK1 and FAK is required for proper neuronal migration (6, 7). PAK1 and FAK are also implicated in regulating cytoskeletal organization during tumor cell migration (8-10). Recently, CDK5 kinase activity has been correlated with increased invasion in prostate carcinomas and glioblastoma multiforme (11, 12).

CDK5 is the only known cyclin dependent kinase to become activated through binding non-cyclin molecules (13). The best characterized CDK5 activators, p35 and p39, have C-terminal regions that contain cyclin box folds (CBF) that bind and activate CDK5 (14).

\(^1\) http://emedicine.medscape.com/article/280605-overview
neural cells, Calpain-mediated cleavage of p35 and p39 to p25 and p29, respectively, yields activators with greater stability, increased CDK5 kinase activation, and altered localization and substrate specificity as compared to the full length p35 and p39 forms (15, 16). In this report we investigated the expression and function of CDK5 and two of its activators, p35 and p39, during the pathogenesis of PDACs.

Results

CDK5, p35, and p39 expression in non-transformed pancreas

We determined the expression pattern of CDK5, p35, and p39 in nine different normal pancreatic tissue samples. Consistent with previous reports, CDK5, p35, and p39 were highly expressed in islet β cells (17, 18). We extend this finding to show that CDK5, p35, and p39 were rarely visualized in ductal cells (Fig 1A). Only two of nine samples expressed detectable CDK5 in a few ductal cells and weak staining in adjoining acinar cells. Ductal cells positive for CDK5 also expressed p35, which was independently expressed sporadically in over half of the normal samples; however, only 3 of 9 samples showed greater than 25% of the ducts staining positive for p35 (Table 1). p39 was only expressed in 38% of normal ducts, and only 1 sample had greater than 10% of the ducts staining positive for p39 (Table 1).

CDK5 and two of its activators, p35 and p39, are expressed in pancreatic adenocarcinoma cell lines, tumors, and metastases

CDK5, p25 (the cleavage product of p35 with higher capacity for activating CDK5), and p29 (the cleavage product of p39 with a higher capacity for activating CDK5) were expressed in 10/10 PDAC cell lines tested, ranging from poorly to well differentiated (Fig. 1B), all expressing mutant K-Ras except T3M4 cells (19-21). Thus, the predominant forms of p35 and p39 observed in these cell lines were their activated N-terminal cleavage products, p25 and p29 (15, 16).
Examination of CDK5, p35, and p39 expression in primary PDACs and liver and lung metastases by immunohistochemistry revealed CDK5 expression in greater than 90% of primary PDACs with similar expression of p35 and less for p39 (Fig. 1C, Table 2). CDK5, p35, and p39 were observed in all six examples of PNI that were examined (Fig. 1C, Table 2). CDK5 was significantly (p<0.0005) over-expressed in primary PDACs compared to normal pancreases (Tables 1-2). CDK5, p35, and p39 expression was especially evident at the leading front of tumors (Fig. 1C).

Genomic amplification of CDK5, p35, and p39 in PDACs

CDK5, p35, or p39 were individually amplified in approximately 33% of PDACs (n=39) (Fig. 1D). Interestingly, 67% of 39 pancreatic tumors examined exhibited amplification of at least one of these genes (Fig. 1D). A few deletions were noted (approximately 10%, data not shown); however, almost all (3/4) tumor samples with deletions in either p35 or p39 had a concomitant amplification in the reciprocal gene (p35 or 39).

K-Ras increased CDK5 kinase activity

We investigated the expression and activity of CDK5, p35, and p39 during the premalignant and malignant transformation of pancreatic epithelial cells by utilizing an immortalized human pancreatic ductal progenitor cell line (HPNE) (22), which was transduced independently and in combination with retroviruses expressing mutant active K-Ras (G12D) (kras), human papillomavirus E6 and E7 (E6/7) to inhibit Rb and p53, and SV40 small t antigen to inhibit protein phosphatase 2A (St) (22, 23). The utility of these cells for investigating relevant signaling pathways downstream of mutant K-Ras has been established (24). HPNE.kras cells had slightly higher p35 mRNA and protein levels compared to HPNE parental cells (Fig. 3, data not shown). K-Ras transformed cells showed significantly elevated levels of the p35 cleavage product, p25, (Fig. 2A) and enhanced CDK5 kinase activation, as evidenced by increased phosphorylation of the CDK5 substrates FAK at serine (S) 732 (6) and PAK1 at...
threonine (T) 212 (7) (Fig. 2A). Inhibiting CDK5 kinase activity with R-roscovitine (greater than four fold specificity for CDK5 compared to CDK2) (25) for 24 hrs in serum containing media abolished S732 FAK phosphorylation in HPNE.kras cells, and markedly reduced S732 phosphorylation in HPNE.kras.E6/7.St cells (Fig. 2B). Inhibition of CDK5 kinase activity also decreased S732 phosphorylation in S2-013, HPAF, tHPNE, and HPNE.kras cells when introduced under growth factor stimulation during a six hour stimulation with 10% FBS after an 18 hour serum starvation (Fig. 2D). We did not observe a difference in p29 (the cleavage product of p39) expression in concert with expression of mutant K-Ras (Fig. 2A); thus, we focused on the correlation between mutant K-Ras expression and the subsequent increased p25 expression and CDK5 kinase activity.

We investigated other signaling pathways that may regulate p35 cleavage to p25 in HPNE.kras cells. Inhibition (24 hours) of MEK (U0126), PI3K (LY290027), and CDK5 (roscovitine) inhibited p35 cleavage to p25 (Fig. 2C). Inhibition of Calpain had a minimal decrease in p35 cleavage to p25, while other protease inhibitors including MG132, Protease Inhibitor 1, and Lactacystin had no effect on p25 levels (Fig 2C). All HPNE cells with mutated K-Ras had similar levels of p35 protein, indicating that the changes in p25 protein expression are likely the result of proteolytic processing. Therefore, cleavage of p35 to p25 in the context of mutant active K-Ras is influenced by MEK, PI3K, and CDK5 signaling and regulatory pathways (Fig. 2C).

**CDK5 kinase activity increases cell migration**

S2-013 cells, a cloned pancreatic tumor cell line derived from a PDAC liver metastasis, displayed significantly reduced migration when treated with the CDK5 inhibitor roscovitine (Fig. 3A). S2-013 cells expressing the reverse tetracycline-controlled transactivator (rtTA), “tet-on,” and the CDK5DN construct under the tetracycline responsive element had significantly
reduced migration compared to the S2-013 control cell lines when expression was induced by treatment with doxycycline (Fig. 3A).

The effects of CDK5 kinase activity on cellular motility in the context of mutant K-Ras activity were investigated at the different stages of transformation in HPNE cells. Expression of mutant K-Ras in HPNE.kras and HPNE.kras.E6/7.St significantly increased migration compared to HPNE.E6/7.St cells lacking mutant K-Ras (Fig. 3B), thus confirming prior reports of mutant K-Ras increasing cellular migration (26, 27). CDK5 inhibition with roscovitine in HPNE.kras and HPNE.kras.E6/7.St cells resulted in a similar reduction of total migration, 18% of the total area, in both cell lines and correlated to a 38% and 35% decrease in total migration with CDK5 inhibition, respectively (Fig. 3B). HPNE.E6/7.St cells had significantly less migration than cells containing mutant K-Ras, and in these cells inhibition of CDK5 kinase with roscovitine did not significantly affect migration into the total area (3.6% reduction) as compared to both cell lines tested expressing mutant K-Ras (18% reduction) (Fig. 3B). The smaller decrease in HPNE.E6/7.St migration following roscovitine treatment is due to minimal CDK5 activity in these cells, which express p35 and very little p25 (Fig. 2A).

**CDK5 kinase activity increases invasion in vitro through a Matrigel-coated Boyden Chamber**

Inhibition of CDK5 kinase activity with roscovitine in PDAC cell lines S2-013, T3M4, FG, HPAF2, HPNE.kras.E6/7.St (tHPNE), and HPNE.kras cells reduced invasion through a Boyden Matrigel coated chamber by 60%, 37%, 49%, 60%, 47%, and 47% respectively (Fig. 4A).

Inhibition of CDK5 kinase alters cell morphology in HPNE cells expressing mutant K-Ras

We sought to investigate the role of CDK5 kinase activity on cell morphology in the context of mutant K-Ras expression in HPNE cells. Parental HPNE cells treated with
roscovitine for 3 days showed no observable changes in cellular morphology (Fig. 4B). HPNE.kras cells treated with roscovitine for 3 days displayed enhanced spreading upon culture in plastic, as evidenced by a more flattened appearance with shorter cellular processes compared to cells with the vehicle control (Fig. 4C). HPNE.kras cells transduced with the CDK5DN construct had a cellular phenotype similar to HPNE.kras cells treated with roscovitine (Fig. 4D). Thus, inhibition of CDK5 kinase activity increased spreading of HPNE cells but only in the context of mutant K-Ras.

Discussion

We report for the first time that CDK5 or its activators are overexpressed in PDACs (Fig. 1B-D, Table 2) compared to normal pancreatic ducts (Fig. 1A, Table 1) (p<0.0005). Primary human PDACs exhibit frequent amplification of the genes encoding p35, p39 or CDK5. Each of these genes is individually amplified in about 33% of tumors, and collectively one or more of these genes is amplified in approximately 67% of tumor samples (Fig. 1D, n=39). Results presented by Harada et al. (that did not address or discuss CDK5, p35, and p39 directly) are consistent with our results, except p39 was not as frequently amplified (28). Overexpression of any of these (p35, p39, or CDK5) is predicted to result in enhanced activation of CDK5 kinase activity. This supports the hypothesis that activation of CDK5, in part through gene amplification and commensurate overexpression of CDK5, p35 or p39, contributes to the progression of pancreatic cancer. Our results also shed light on the types of cells within the pancreas that express CDK5, which is widely reported to be "ubiquitously" expressed in all organs. Within the pancreas, expression of CDK5 is highest in islets, but we find that there is little or only sporadic expression of CDK5, p35 or p39 in normal acinar or ductal cells in the pancreas.

The known contribution of CDK5 to neuronal migration and patterning (5) suggested to us a link between CDK5 expression and perineural invasion (PNI), a prominent feature of
PDACs. Sustained activation of phospho Erk 1/2 was achieved through stimulation by nerve growth factor (NGF) in rat pheochromocytoma PC12 cells, which resulted in Egr1 binding and activation of the p35 promoter and a subsequent increase of p35 transcript and protein (29). NGF induced upregulation of p35 protein levels may explain in part the high p35 levels observed in PNI (Fig. 1C, Table 2), and we propose that the microenvironment around the nerves, where physiologically normal CDK5 expression is highest (30, 31), induces or selects for increased CDK5 and p39 levels in PDACs undergoing PNI.

We sought to determine if CDK5 activation contributed to pancreatic cancer progression in the context of known early transforming events (32). Analysis of variants of HPNE, a normal pancreatic cell line immortalized with hTert, that was subsequently transduced with retroviruses expressing mutant active K-RasG12D (HPNE.kras) showed higher p35 or p39 mRNA and protein levels in response to activated K-Ras, while CDK5 levels remained constant in HPNE.kras cells (Fig. 3, data not shown). Differences in CDK5 expression between normal pancreas (Fig. 1, Table 1) and immortalized but non-transformed HPNE cells (Fig. 2A) could be attributed to the expression of Nestin, which HPNE cells express, as Nestin expression has been correlated to CDK5 expression in differentiating myoblasts and in regenerating muscle tissue (33). Alternatively, CDK5 may be expressed in pancreatic ductal progenitor cells, which would also explain CDK5 expression in HPNE cells, which are derived from progenitors of ductal cells (22).

There were increased p25 levels in HPNE.kras cells, a stable cleavage product of p35 that is highly activating for CDK5 (Fig. 2A). p25 lacks the N-terminal myristoylation sequence of p35, which causes relocalization from the cell membrane to cytoplasm and potentiates its capacity to bind and activate CDK5 (13). p25 expression is likely responsible for increased CDK5 kinase activity in HPNE.kras cells compared to HPNE parental cells (Fig. 2A). Similar to SH-SY5Y neuroblastoma cells (34) and in contrast to normal neuronal cells (35), we found that
CDK5 kinase activity in the context of mutant K-Ras (HPNE.kras cells) increased p25 levels (Fig. 2B), while inhibition of CDK5 kinase had no effect on p25 levels in the context of the additional oncogenic effects of E6/7 and small t antigen (Fig. 2B). This supports the hypothesis that CDK5 hyperactivation contributes to tumor progression especially in the context of early mutant K-Ras expression. We show further that signaling through MEK and PI3K increased p25 expression (Fig. 2C). This leads us to propose that signaling from activated mutant K-Ras (G12D) through MEK and PI3K enhance p25 expression, which in turn increases CDK5 kinase activity (Fig. 3, Supplemental Fig. 1).

Our results complement and extend recently published findings by Feldman et al (36), which showed that inhibition of CDK5 in pancreatic cancer cell lines by the dominant negative construct decreased signaling through the RalA and RalB pathways that are downstream of activated Ras, and that commensurate inhibition of MEK and PI3K pathways reinforced these effects. The Ral pathways were shown to contribute to anchorage independent growth and tumorigenicity of these tumor cells lines and their results suggest that these effects are downstream of CDK5. Our results are consistent with these findings and extend them by demonstrating that K-Ras signaling enhances the activity of CDK5 by increasing the steady state levels of p25 upstream of the Ral pathways.

The influence of mutant K-Ras and CDK5 kinase activity on migration was investigated in HPNE cells bearing selected mutations and transforming insults. CDK5 is necessary for proper neuronal cortical layering, a process that requires CDK5-mediated phosphorylation of FAK at S732 and probably PAK1 at T212 (6, 7). We found that increased CDK5 activity and p25 expression in HPNE cells expressing mutant K-Ras increased phosphorylation of S732 FAK and T212 PAK1 (Fig. 2A). We propose that PDACs have appropriated these phosphorylation events to alter cell morphology and increase cellular migration. Inhibition of CDK5 with roscovitine or the CDK5 dominant negative construct enhanced spreading in
HPNE.kras cells, as evidenced by a more flattened appearance with shorter cellular processes compared to the respective controls (Fig. 4C, D), whereas there was no change in cellular morphology in parental HPNE cells with CDK5 inhibition (Fig. 4B). Our results support the proposal that CDK5 has a role in modulating cell morphology and cytoskeletal reorganization that has been presented by Mao and Hinds and others (37, 38), even though the precise morphological findings are not entirely congruent because they were obtained in different cell types and experimental systems that were evaluating morphological changes associated with senescence, which were not investigated in our experimental system.

Along with increased p25 expression and CDK5 kinase activity, mutant K-Ras increased migration of HPNE.kras and HPNE.kras.E6/7.St cells as compared to HPNE.E6/7.St cells lacking mutant K-Ras (Fig. 3B). The increase of migration in the HPNE.kras.E6/7.St cells relative to HPNE.kras cells suggested that the transforming insults introduced by E6/7 and small t antigen had little effect on migration (Fig. 3B). Inhibition of CDK5 kinase activity significantly reduced migration of HPNE cells expressing mutant K-Ras (HPNE.kras and HPNE.kras.E6/7.St cells) while it had little effect on cells lacking mutant K-Ras (HPNE.E6/7.St cells) (Fig. 3B). Inhibiting CDK5 kinase activity with a CDK5 dominant negative construct or roscovitine also significantly decreased migration of S2-013 cells (Fig. 3A).

CDK5-inhibition with roscovitine reduced invasion by 37-60% in S2-013, tHPNE, FG, HPAF2, and T3M4 PDAC cell lines (Fig. 4A). These results are consistent with previous findings in neuroblastoma, prostate cancer and other pancreatic cancer cell lines, where inhibition of CDK5 kinase activity reduced invasion in vitro (11, 12).

In summary, we demonstrate that CDK5 or its activators p35 and p39 are over-expressed in >90% PDACs (Table 2) compared to normal pancreases (Table 1). CDK5, p35, and p39 expression in PDACs can be attributed in part to genomic amplification, as CDK5, p35, or p39 were amplified in 67% (n=39) of tumors analyzed (Fig. 1D). Furthermore, CDK5 is
hyperactivated downstream of mutant K-Ras signaling, resulting in increased phosphorylation of CDK5 substrates, increased p35 mRNA and protein expression, increased p35 cleavage to p25 (which is dependent on MEK, PI3K, Calpain, and CDK5 signaling), decreased cell spreading, and increased cell migration (Fig. 3-5, S. Fig. 1, data not shown). We confirm prior reports that CDK5 inhibition can decrease migration and invasion in vitro (Fig. 4A).

CDK5 represents a novel and unexplored therapeutic target for both early and late stage PDACs, and should be investigated in other solid tumors that harbor K-Ras mutations, including those of lung and colon. R-Roscovitine (Selicilib) has been investigated in phase I clinical trials, with minimal toxicity at lower doses, supporting the feasibility of using a CDK5 inhibitor in the clinic (39). More potent CDK5 kinase inhibitors have been developed as potential therapies for Alzheimer’s Disease, in an attempt to downregulate hyperactive CDK5 kinase activity associated with p25 expression that is observed in that disease (15). We are currently investigating the use of such inhibitors in xenograft and other animal models of pancreatic cancer. It is also possible that CDK5 inhibitors may reduce pain in pancreatic cancer patients with PNI. CDK5 has previously been demonstrated to reduce thermal afferent nociceptive pain signaling (40). Type II Diabetes Mellitus (T2DM) is observed in the majority of PDACs (41), and increased CDK5 kinase activity is known to increase glucotoxicity and independently decrease insulin synthesis and secretion (42, 43). Thus, inhibiting CDK5 kinase activity with molecular inhibitors may also improve the associated T2DM in PDAC.

Materials and Methods

Cells, antibodies, and reagents. S2-013 cells are a cloned subline of a human pancreatic tumor cell line SUIT-2 derived from a liver metastasis (44). HPAF-II is a human pancreatic adenocarcinoma cell line derived from peritoneal ascitic fluid (19). T3M4 cells are a human carcinoembryonic antigen (CEA)-producing cell line established from a primary PDAC.
transplanted into nude mice and then cultured (19). FG cells are a fast growing clone established from the Colo-357 PDAC cell line (45). HPNE cells are an hTERT (active telomerase) immortalized human pancreatic Nestin expressing (HPNE) ductal progenitor cell line (22). tHPNE cells are HPNE cells transduced with mutant K-Ras (G12D), E6/E7, and SV40 small t antigen (22, 23, 46).

The rabbit pABs to p35, p39, and CDK5 (SCBT); p44/p42 MAPK mABs (Cell Signaling); both FAK, CDK5, and phospho PAK1 Abs (Abcam); mAB β-Actin (Sigma); mAB CDK5 (Upstate); and the HRP-conjugated goat α-mouse and α-rabbit (Pierce) were used at concentrations recommended by the manufacturer. The CDK5 inhibitor R-roscovitine was purchased from Calbiochem.

**Comparative Genomic Hybridization.** We selected FFPE blocks from pancreaticoduodenectomy specimens performed for PDA at UCSF between 1993 and 2004. Tissue blocks were reviewed by a gastrointestinal pathologist and confirmed to be PDA. Dissection was performed, genomic DNA was isolated by a proprietary phenol-chloroform based extraction procedure at Response Genetics (Los Angeles, CA). Extracted genomic DNA (70ng) was subjected to genome wide allele-specific copy number analysis on a molecular inversion probe (MIP) platform as described (47). MIP copy number was assessed by using the Nexus software (BioDiscovery, Los Angeles, CA).

**Cell Culture.** All cell lines were maintained in RPMI supplemented with 10% heat-inactivated FBS and ciprofloxacin. Stably transfected/ transduced cell lines were maintained with the appropriate selection as indicated in the ‘Plasmids and stable transfections and transductions’ section of the Materials and Methods.

**Plasmids and stable transfections and transductions.** The reverse tetracycline trans activator (rtta, ‘tet-on’, kindly provided by Kay Uwe Wagner, UNMC, Omaha, NE) (48) was transfected into S2-013 cells and selected with G418 at 600 μg/ml. After stable selection with
G418, the pBl (Clontech) vector with a zeocin resistant gene (kindly provided by Barry Nelkin, Johns Hopkins University School of Medicine, Baltimore, MD) was transfected and stable expressing cell lines were selected with zeocin at 200 μg/ml (11). Clones expressing the CDK5DN insert were selected if there was greater than a two fold increase in the CDK5 protein expression after addition of 3 μg/ml doxycycline, thus indicating induced expression of the CDK5DN. The clones were sorted for egfp to isolate a high level CDK5DN-expressing population.

All stable transfections utilized Genporter2 (Genlantis) per the manufacturer’s recommendations.

**Western blotting and immunoprecipitation.** Briefly and as previously described (49), cell lysate proteins were resolved on 12% or 4-20% Novex Tris-Glycine denaturing polyacrylamide gels (Invitrogen) in a 1x SDS-PAGE buffer. Blocking and primary AB incubation was carried out overnight (O/N) at 4 °C or for 1 hour (hr) at room temperature (RT). Secondary ABs were incubated for 1 hour at RT. Primary and secondary antibody incubations were followed with five three-minute washes in PBS. All incubations were carried out in PBS containing 3% dried milk.

**Immunohistochemistry (IHC)**. CDK5, p35, and p39 were investigated for frequency of expression in PDACs, metastases from PDACs, and normal pancreases. Briefly and as previously described (50), 5 μM thick paraffin embedded tissue sections were immunostained. The tissue sections in paraffin were rehydrated through incubations in xylene and then an alcohol gradient from 100 to 70% followed by a H2O incubation. Antigen retrieval using Antigen Unmasking Solution (Vector Labs) was performed according to the manufacturer’s instructions. The sections were blocked in Dako Blocking Solution (Dako) for 30’ at 24 °C. The primary antibodies were incubated for 45’ at 24 °C. CDK5 (Abcam), p35 (Scbt), p39 (Scbt), and rabbit IgG (Sigma) were used at 1:70, 1:100, 1:100, and 2 μg/ml, respectively and
the secondary goat anti-rabbit IgG (Dako) for 30’ at 24 °C with 3 PBS washes between each ab/staining step. Positive staining was visualized with a 3,3-diaminobenzidine substrate (Dab) solution (Vector Labs) for 2-5’ and then slides were counterstained in hematoxylin. All slides were analyzed by two individuals trained in IHC, and interpretations confirmed by a pathologist.

**Migration assay.** Cells were grown to 90% confluence on 6 well plates and placed in RPMI containing 0.5% FBS. 24 hrs later a scratch was made with either a p20 or p1000 pipet tip. Utilizing a colony marker, areas of similar cell density and scratch characteristics were marked and photographed. S2-013 cells were grown for 24 hrs and HPNE cells for 18 hrs and then were photographed again in the same field. The photographs were imported into Microsoft PowerPoint and analyzed for % migration into the scratch. The results are reported in fold migration relative to that of the control. TScratch (51) was used to analyze the total area of migration by HPNE cells. Results are reported in total area covered by migrated cells. Indicated cell treatments were initiated at time of making the scratch.

**Matrigel invasion assay.** In vitro analysis of invasion was measured by seeding cells on the upper chamber of a Boyden Matrigel coated chamber in serum free media with the lower chamber containing media with 10% FBS. T3M4, HPAF2, and S2-013 cells were seeded at 5 x 10^4 cells/filter. tHPNE and FG cells were seeded at 1 x 10^4 and 1 x 10^6 cells/filter, respectively. Roscovitine was added at 10 μM just prior to cell seeding. After a 24 hour incubation using 10%FBS as the chemoattractant in the lower chamber, the upper chamber was cleaned. The filter was stained with Diff-Quick staining kit (Allegiance) and the invading cells were counted.

**Statistical analysis.** Statistical analysis was performed using the paired student t-test or chi square test as indicated with p<0.05 being statistically significant. The data were analyzed with PROC GLIMMIX of SAS, version 9.2. Data entered directly as percents (migration) were evaluated with a regression model based on the beta distribution.
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**Figure 1. CDK5 and p35 Expression in Normal Pancreas and PDACs.** A. Most normal pancreatic ductal cells are negative for CDK5 and p35 in most ducts (Row 1). p35 detected within a pancreatic duct, while an adjacent duct is negative for p35 and the corresponding staining for CDK5 is negative in both ducts (Row 2). B. CDK5 and two of its activators, p35 and p39, and their cleavage products, p25 and p29, respectively, are expressed in multiple pancreatic ductal adenocarcinoma (PDAC) cell lines. Increased levels of S732 FAK (CDK5 phosphorylation site) were observed in the PDAC cell lines. C. Immunohistochemical analysis of primary pancreatic cancer samples shows expression of CDK5, p35, and p39 in a primary pancreatic cancer exhibiting PNI. D. Comparative genomic hybridization of PDACs (n=39) shows genomic amplification for CDK5, p35, and p39.

**Figure 2. Mutant K-Ras increases CDK5 kinase activity.** A. HPNE cells with the indicated oncogenic insults (K-Ras, E6/E7, small t) were examined for expression of CDK5, p35, p25, p29, phosphoT212PAK1, PAK1, phospho S732 FAK, FAK, and beta actin as a loading control. B. HPNE.kras and tHPNE (HPNE.kras.E6/7.St) cells were investigated for expression of CDK5, p25, phospho S732 FAK, FAK, p29, and β-actin with and without inhibition of CDK5 kinase activity by treatment with roscovitine for 24 hours in serum-containing media. C. CDK5, p35, p25, and β-actin expression in HPNE.kras cells after incubation for 24 hours with vehicle, CDK5 inhibitor (roscovitine), MEK inhibitor (U0126), PI3K inhibitor (LY290027), Caspase inhibitor, Protease inhibitors (MG132, Protease Inh 1, Lactacystin, ALLN), and Calpain inhibitors (Calpeptin, Calpain Inh 3, PD, EST) compared to HPNE cells without mutant active K-Ras. D. S2-013, HPAF, tHPNE, and HPNE.kras cells were serum starved for 18 hours followed by stimulation with 10% FBS for six hours with roscovitine or the vehicle control: the results show levels of phospho S732 FAK, FAK, p25, p35, CDK5, and β-actin expression as determined by western blotting.
**Figure 3. CDK5 kinase activity increases migration.** A. Inhibition of CDK5 in S2-013 cells, with roscovitine or under a doxycycline-inducible CDK5DN construct, significantly decreased cell migration as determined with scratch assays. B. Quantification of the total area of migration utilizing TScratch, a program that analyzes area occupied by cells, for each HPNE permutation and treatment to assess the differences in migration conveyed through mutant K-Ras and CDK5 kinase activity. Inhibition of CDK5 kinase activity, significantly reduced migration in HPNE.kras and tHPNE (HPNE.kras.E6/7.St) cells while showing minimal differences in cells lacking mutant K-Ras, HPNE.E6/7.St. (column 4 or 6 compared to 1). * p<0.005, ** p<0.02.

**Figure 4. CDK5 kinase activity increases in vitro invasion and enhances cell spreading in the context of mutant K-Ras.** A. Invasion in vitro through a Matrigel-coated Boyden Chamber by S2-013, tHPNE, FG, HPAF2, T3M4, and HPNE.kras cells following inhibition of CDK5 kinase activity with roscovitine compared to the vehicle control. B. HPNE cells incubated with roscovitine for 72 hours did not result in altered cellular morphology. C. HPNE.kras cells incubated with roscovitine for 72 hours resulted in enhanced spreading as evidenced by a more flattened appearance with shorter cellular processes. D. HPNE.kras cells transduced with a CDK5 dominant negative construct, HPNE.kras.CDK5DN, also displayed enhanced spreading compared to the control cell line, HPNE.kras.Control. * p<0.007, ** p<0.05, *** p<0.02.
**Fig. 1A**

- **H & E**
- **CDK5**
- **p35**
- **IgG**

Normal pancreas

Nontransformed Ducts

Nontransformed Ducts

Islet

Nontransformed Duct

Normal pancreas
Fig. 1B

Phospho S732 FAK

β-actin  CDK5  p39  p29  p35  p25  FAK

Capan1  Capan2  CFPAC1  COLO357  FG  HPAF  Panc1  S2-007  S2-013  T3M4  tHPNE  HPNE.kras  HPNE
Fig. 1C

- **H & E**
  - nerve

- **CDK5**
  - Invading PDAC
  - nerve

- **p35**
  - Invading PDAC
  - nerve

- **p39**
  - Invading PDAC
  - nerve
Fig. 1D

cdk5 Pathway Gene Amplification

% Change

Gene Amplified

- cdk5
- p35
- p39
- cdk5, p35, or p39

Research.
on May 28, 2017. © 2011 American Association for Cancer
Fig. 2B

HPNE.kras + vehicle  HPNE.kras + Roscovitine

CDK5  p25  Phospho S732 FAK

FAK  p29  β-actin

tHPNE + vehicle  tHPNE + Roscovitine
Fig. 2C

Protease Inhibitors  |  Calpain Inhibitors

- HPNE + Vehicle
- HPNE.kras + Vehicle
- HPNE.kras + Roscovitine
- HPNE.kras + U0126
- HPNE.kras + LY290027
- HPNE.kras + Caspase
- HPNE.kras + MG132
- HPNE.kras + Protease Inh 1
- HPNE.kras + Lactacystin
- HPNE.kras + ALLN
- HPNE.kras + Calpeptin
- HPNE.kras + Calpain Inh 3
- HPNE.kras + PD
- HPNE.kras + EST

- p35
- p25
- CDK5
- β-actin
Fig. 2D

Phospho S732 FAK

FAK

CDK5

β-actin
Fig. 3A

Experiment Migration/
Control Migration

S2-013 + Vehicle
S2-013 + Roscovitine
S2-013.pbi + 0 dox
S2-013.pbi + dox
S2-013.CDK5DN28 + 0 dox
S2-013.CDK5DN28 + dox

0.0 0.5 1.0 1.5

*
Fig. 3B

% Scratch Closure

HPNE.E6/7.St

HPNE.kras

HPNE

Vehicle

Roscovitine

*
Fig. 4A

# Cells Invading/ Filter

- **52013**
- **t-HNE**
- **FG**
- **HPAF2**
- **T3M4**
- **HPAF2;08**

![Bar chart](chart.png)

- Vehicle
- Roscovitine

Legend:
- *p < 0.05
- **p < 0.01
- ***p < 0.001
Fig. 4B

HPNE parental cells

Vehicle

Roscovitine (CDK5 Inhibitor)
Fig. 4C

HPNE.kras cells

Vehicle

Roscovitine
Fig. 4D

HPNE.kras.Control

HPNE.kras.CDK5DN
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<th>1-10%</th>
<th>10-25%</th>
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<td>44%</td>
<td>22%</td>
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Cyclin Dependent Kinase 5 is amplified and over-expressed in pancreatic cancer and activated by mutant K-Ras


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