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The oncogenic kinase Pim-1 is modulated by K-Ras signaling and mediates transformed growth and radioresistance in human pancreatic ductal adenocarcinoma cells

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Oncogenic Pim-1 kinase is upregulated in multiple solid cancers, including human pancreatic ductal adenocarcinoma (PDAC), a highly lethal disease with few useful treatment options. Pim-1 is also transcriptionally induced upon oncogenic K-Ras-mediated transformation of the human pancreatic ductal epithelial (HPDE) cell model of PDAC. Given the near ubiquitous presence of mutant K-Ras in PDAC and its critical role in this disease, we wished to study the effects of oncogenic K-Ras signaling on Pim-1 expression, as well as the role of Pim-1 in growth transformation of PDAC cells. Pim-1 protein levels were upregulated in both PDAC cell lines and patient tumor tissues. Furthermore, ectopic oncogenic K-Ras increased Pim-1 expression in human pancreatic nestin-expressing (HPNE) cells, a distinct immortalized cell model of PDAC. Conversely, shRNA-mediated suppression of oncogenic K-Ras decreased Pim-1 protein in PDAC cell lines. These results indicate that oncogenic K-Ras regulates Pim-1 expression. The kinase activity of Pim-1 is constitutively active. Accordingly, shRNA-mediated suppression of Pim-1 in K-Ras-dependent PDAC cell lines decreased Pim-1 activity, as measured by decreased phosphorylation of the pro-apoptotic protein Bad and increased expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Biological consequences of inhibiting Pim-1 expression included decreases in both anchorage-dependent and -independent cell growth, invasion through Matrigel and radioresistance as measured by standard clonogenic assays. These results indicate that Pim-1 is required for PDAC cell growth, invasion and radioresistance downstream of oncogenic K-Ras. Overall, our studies help to elucidate the role of Pim-1 in PDAC growth transformation and validate Pim-1 kinase as a potential molecular marker for mutated K-Ras activity.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common cancer of the pancreas, comprising >85% of all cases. With an estimated 42 470 new cases and 35 240 deaths in 2009, PDAC ranks fourth in cancer-related deaths in the USA (1). PDAC has a relative 1-year survival rate of 20% and a 5-year survival rate of only 4% (2). Thus, to better combat this lethal and aggressive disease, it will be necessary to identify and validate novel molecular targets that are actively involved in the aberrant growth of PDAC cells.

Abbreviations: FBS, fetal bovine serum; HPDE, human pancreatic ductal epithelial; HPNE, human pancreatic nestin-expressing; PDAC, pancreatic ductal adenocarcinoma; Pim, Proviral Integration site for the Moloney murine leukemia virus.

One molecular target that has been extensively studied in PDAC is the oncoprotein K-Ras, which is mutated in >90% of PDAC (3,4). K-Ras normally functions as a regulated guanosine triphosphatase switch that is activated by a diverse spectrum of extracellular stimuli, transiently promoting normal cell growth and proliferation (3,4). In contrast, oncogenic K-Ras is constitutively active and results in persistent activation of a multitude of downstream effector pathways (3,4). Oncogenic K-Ras plays a large role in the development and progression of pancreatic cancer (5–9) but development of clinically effective K-Ras-directed cancer therapies has been unsuccessful. Instead, identification of novel molecular targets regulated by K-Ras signaling may provide a more useful therapeutic approach by indirectly targeting the consequences of K-Ras activity (4).

To identify genome-wide changes in gene expression induced by oncogenic K-Ras activation, Qian *et al.* (10) performed microarray analysis in immortalized human pancreatic ductal epithelial (HPDE) cells transformed by K-Ras. One of the 584 genes found to be upregulated in this *in vitro* model of PDAC was the oncogene Pim-1 kinase. Pim (Proviral Integration site for the Moloney murine leukemia virus) is categorized as a calmodulin-dependent protein kinase (11). Pim-1 is a member of the serine/threonine Pim kinase family and is a downstream effector of cytokine signaling through the signal transducer and activator of transcription signaling pathway (11,12). The Pim-1 gene locus has been mapped to the short arm of chromosome 6 (6p21) in the human genome and encodes a protein of 313 amino acids (13). Pim-1 occurs as two protein isoforms of 34 and 44 kD, each containing kinase domains with comparable *in vitro* kinase activity (13). Two other members of the Pim kinase family, Pim-2 and Pim-3, share strong sequence (~60% identity) and functional homology with Pim-1 (13) but are not transcriptionally upregulated by K-Ras activity. Pim-1 is constitutively activated when expressed and can be regulated at the transcriptional, posttranscriptional, translational and posttranslational levels (12,14). Pim kinases have been shown to phosphorylate substrates involved in numerous cellular functions including cell cycle progression and apoptosis (13). Two critical substrates mediating these activities include the cyclin-dependent kinase inhibitor $p27^{KIP1}$ and the pro-apoptotic BH3 family member Bad (15,16).

Although Pim-1 kinase was initially discovered in hematopoietic tissues and cancers, members of the Pim kinase family have also been shown to be expressed in a broad range of epithelial cancers, including breast, tongue, prostate, head and neck, gastric and pancreatic cancers (17–20). Initial studies by Li *et al.* (21) showed that the related kinase Pim-3 is aberrantly expressed in PDAC and phosphorylates Bad to inhibit Bad-mediated apoptosis in PDAC cell lines but did not explore Pim-1. Because Pim-1 but not Pim-3 was shown to be a transcriptional target of oncogenic K-Ras (10), we chose to focus on the role of Pim-1 in oncogenic K-Ras signaling and growth transformation of PDAC cells.

In this report, we demonstrate upregulation of Pim-1 protein expression in PDAC patient tumor tissues. We also show that oncogenic K-Ras signaling modulates Pim-1 expression in the K-Ras-dependent PDAC cell lines, MIA PaCa-2 and Capan-1. Downregulating Pim-1 via shRNA caused alterations in its downstream substrates Bad and p27, as well as decreased anchorage-dependent and -independent growth, invasion through Matrigel and radioresistance. Our results demonstrate additional roles for Pim-1 in K-Ras-mediated transformation and oncogenic properties of PDAC and help to validate Pim-1 as a novel molecular marker for K-Ras activity.

Materials and methods

Patient tissues

De-identified matched normal and primary pancreatic tumor samples from three PDAC patients from the University of North Carolina were obtained with institutional review board approval. Tissues were harvested in an NP-40-based

lysis buffer containing a cocktail of phosphatase and protease inhibitors (Sigma–Aldrich, St Louis, MO and Roche, Indianapolis, IN, respectively).

Cell culture

The human PDAC cell lines AsPC-1, BxPC-3, Capan-1, Capan-2, MIA PaCa-2, PANC-1 and T3M4 were obtained from the American Type Culture Collection and maintained in culture with RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Cellgro, Herndon, VA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. Lysates isolated from immortalized human pancreatic nestin-expressing (HPNE) cell lines overexpressing K-Ras^{12D} or vector were kindly provided by Dr Channing Der at University of North Carolina Lineberger Comprehensive Cancer Center.

Lentiviral shRNA and creation of stable cell lines

The shRNA constructs directed against Pim-1 kinase, K-Ras or scrambled sequences were purchased from Open Biosystems through University of North Carolina's Lentiviral Core. Lentiviral particles were generated using a three-plasmid system as described previously (22). Twenty-four hours after infection, cells were treated with 2 μg/ml puromycin for at least 4 days to eliminate uninfected cells and thus yield mass populations of puromycin-resistant cells expressing the shRNAs.

Preparation of cell lysates and western blotting analysis

Cells were collected from 10 cm plates and lysed in 200 μl freshly made lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1.5 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4, 1 mM NaF and 1 mM dithiothreitol) for 5 min on ice. Twenty-five micrograms of protein, as determined by a modified Bradford protein assay (Bio-Rad, Hercules, CA), were loaded per well onto a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. Protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Danvers, MA) and incubated with the following primary antibodies diluted according to the manufacturer's recommendation: β-actin (A5316) (Sigma–Aldrich); K-Ras (OP24) from Calbiochem (San Diego, CA); p34 isoform of Pim-1 (H-43) and Pim-3 (C-18) from Santa Cruz Biotechnology (Santa Cruz, CA); Bad (#9292), Phospho-Bad (Ser112) (#9291), Pim-2 (#4723), PARP [Poly (ADP-Ribose) Polymerase] (#9542), Caspase-3 (#9662) and p27^{Kip1} (#2552) from Cell Signaling Technology (Beverly, MA). After incubation with primary antibody, the membranes were washed in tris-buffered saline supplemented with 0.1% Tween 20 and incubated with the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) followed by development with the chemiluminescent substrate WestDura (Pierce Biotechnology, Rockford, IL) and exposure to X-ray film (Denville Scientific, Metuchen, NJ). After staining for Pim-1, membranes were stripped and reprobed for β-actin.

Cell proliferation assay in monolayer culture

Cell lines were trypsinized (0.5% trypsin/ethylenediaminetetraacetic acid) to generate single-cell suspensions. Between 1.0×10^3 and 2.0×10^3 cells per well (96-well plates) were seeded, and cells were counted using a cell hematocytometer daily for up to 6 days. Assays were performed a total of three times in triplicate, independently.

Soft agar colony formation assay

To study cellular transforming activity, 1.5×10^4 to 3.0×10^4 cells were mixed in 2.0 ml 0.3% agar/1 × RPMI 1640/10% FBS as the top agar and plated into six-well plates with 3.0 ml 0.6% agar/1 × RPMI 1640/10% FBS as the base agar. Plates were incubated at 37°C and checked every 3 days with addition of 0.5 ml of fresh complete RPMI 1640/10% FBS. Two to three weeks later, colonies were photographed (×10 magnification) and colony numbers were scored. Assays were performed a total of three times in triplicate, independently.

Cell cycle analysis

Stably infected cells were harvested and fixed in ice cold 70% ethanol at 4°C for at least 2 h. Fixed cells were washed in phosphate-buffered saline and then treated with a staining solution in phosphate-buffered saline composed of 20 μg/ml propidium iodide (Molecular Probes, Eugene, OR), 200 μg/ml RNase A and 0.1% Triton X-100. Samples were equilibrated for 30 min at room temperature prior to data collection at 575 nm on a FACSAria™ flow cytometer (Becton Dickinson, San Jose, CA). The cell cycle distributions in the collected sample data were determined by using ModFit LT Software (Verity Software House, Topsham, ME) following pulse width-based gating for doublet discrimination.

In vitro invasion assay

Cells growing in log phase were incubated in serum-free medium for 24 h. Matrigel invasion chambers (BD BioCoat BD Matrigel Invasion 24-well chamber, 8 μm pore, BD Biosciences) were rehydrated for 2 h at 37°C with serum-free medium. Immediately prior to the addition of dissociated cells to the upper chamber (5.0×10^3 cells per well), 10% vol/vol FBS was added to the lower chamber. After 48 h, Matrigel and non-migrating cells were removed from the upper chamber with a Q-tip. Invading cells on the bottom of the membrane were fixed in methanol and stained with 0.1% methylene blue. After drying overnight, stained cells were counted under light microscopy.

Clonogenic assay

MIA PaCa-2 cells stably expressing shRNAs were plated as single cells at low density. Approximately 18 h after plating, cells were irradiated with a single dose (2 Gy or 7 Gy) from a Cs137 irradiator (JL Shepherd, San Fernando, CA). After 2 weeks, cells were fixed in methanol:acetic acid (3:1, vol:vol) and stained with 1% crystal violet. Colonies were counted manually using a threshold of 50 cells as a minimum for a viable colony. Plating efficiency and surviving fraction were calculated as we have described previously (23).

Statistical analysis

All obtained data were calculated and expressed as mean ± standard deviation. The differences were analyzed statistically by a one-way analysis of variance based on the actual mean values. For all analyses, P value of <0.05 was considered statistically significant and displayed as $P < 0.05$ (*) and $P < 0.001$ (**).

Results

Pim-1 is aberrantly expressed in PDAC tumor tissues and cell lines

We initially compared Pim-1 expression in matched pairs of normal and tumor tissue samples of the pancreas. Lysates were prepared from PDAC tumor and adjacent normal tissues from three patients and analyzed by western blot analysis for Pim-1 protein expression. Densitometry was performed to quantitate band intensity of normal and tumor tissue samples. Pim-1 was overexpressed in all three tumor tissues compared with the matched normal tissues (Figure 1A). We then measured Pim-1 protein expression in a panel of PDAC cell lines compared with the immortalized HPNE epithelial cell line. We found constitutive expression of Pim-1 protein in all PDAC cell lines (Figure 1B). Thus, Pim-1 is overexpressed in PDAC tumor tissue and is constitutively expressed in PDAC cell lines.

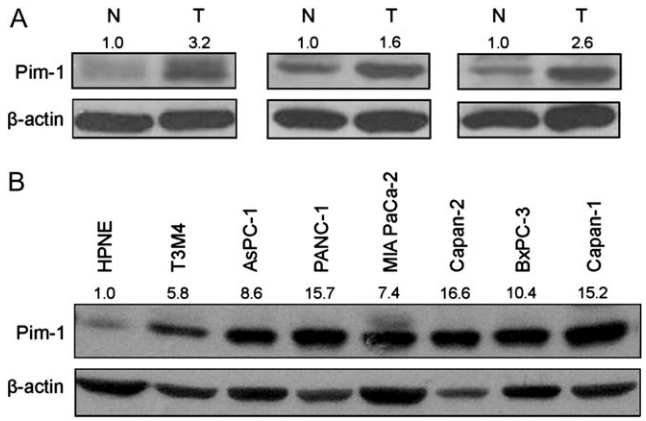

Fig. 1. Pim-1 protein is upregulated in human pancreatic cancer tissues and cell lines. (**A**) Lysates from three sets of matched normal (N) and tumor (T) pancreatic tissues were isolated and immunoblotted with an anti-Pim-1 antibody. The numerical values represent relative band intensities for Pim-1 expression normalized to β-actin. (**B**) Lysates were prepared from a panel of PDAC cell lines and immunoblotted for Pim-1 protein expression as in Panel A. Pim-1 expression was quantitated by densitometry and normalized to HPNE cells. Data shown are representative of three independent experiments.

Pim-1 expression is modulated by K-Ras signaling in PDAC cell lines

Qian *et al.* (10) demonstrated that overexpression of oncogenic K-Ras in immortalized HPDE cells induced expression of Pim-1 messenger RNA. However, the absence in HPDE cells of some *in vitro* phenotypes of K-Ras-mediated transformation, such as anchorage-independent growth (5), suggests that additional models of K-Ras-regulated genes in PDAC should be tested. In addition, we wished to determine whether the observed increase in messenger RNA also resulted in increased Pim-1 protein levels. Thus, we used a second immortalized and fully transformed HPNE epithelial cell line overexpressing oncogenic K-Ras to evaluate Pim protein expression (5). Consistent with the increase in Pim-1 messenger RNA seen in Ras-transformed HPDE cells, transformation of HPNE cells by ectopic oncogenic K-Ras^{12D} resulted in upregulation of Pim-1 protein compared with the control cells (Figure 2A). Additionally, we observed an increase in Pim-2 but not Pim-3 protein expression in the Ras-transformed cells. This suggests that oncogenic K-Ras is sufficient to upregulate two of the three Pim isoforms in this model system. To provide further support for Pim-1 regulation by K-Ras, we examined two human PDAC cell lines, MIA PaCa-2 and Capan-1, which possess K-Ras mutations (K-Ras^{12C} and K-Ras^{12V}, respectively) and are dependent on K-Ras signaling for growth transformation (6–9). Stable infection of these cells with lentivirus-containing shRNAs directed against mutated K-Ras resulted in both suppression of oncogenic K-Ras and decreased expression of Pim-1 (Figure 2B). In contrast, there were no significant changes in Pim-2 and Pim-3 protein expression, indicating that Pim-1 is the main Pim isoform regulated by oncogenic K-Ras signaling in these cells.

Suppression of endogenous Pim-1 kinase results in decreased phosphorylation of its target, the pro-apoptotic protein Bad, at Ser112.

We next wished to evaluate the requirement for Pim-1 in transformed growth and other properties known to be regulated by oncogenic K-Ras. To do so, we first stably knocked down Pim-1 in MIA PaCa-2 and Capan-1 using lentiviruses-expressing shRNAs targeting Pim-1 and confirmed suppression of the endogenous Pim-1 kinase in each cell line (Figure 3A and B). MacDonald *et al.* (24) showed previously that all members of the Pim kinase family phosphorylate the downstream target Bad selectively on Ser^{112}, a site whose phosphorylation status is key for its ability to promote apoptosis (15). We therefore evaluated the phosphorylation status of Bad and demonstrated that, as expected, suppression of Pim-1 resulted in decreased phospho-Ser^{112} Bad in each cell line compared with the scrambled shRNA controls (Figure 3A and B). The decrease in Pim-1 expression was the main contributing factor for the observed decrease in phosphorylated Bad, based on the fact that Pim-1 knockdown not only decreased total Bad but also decreased the ratio of phosphorylated to total Bad. These results are consistent with those of Li *et al.* (21), who demonstrated that inhibition of Pim-3 expression led to a similar reduction of phospho-Ser^{112} Bad in other PDAC cell lines. We also observed a modest increase in basal levels of apoptosis, as marked by modest increases in cleavage of PARP and caspase-3 (Figure 3C and D).

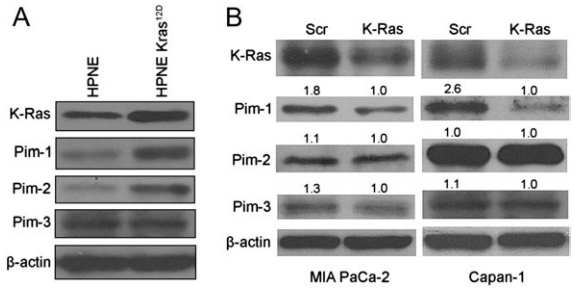

Fig. 2. Pim-1 expression is regulated by oncogenic K-Ras signaling in pancreatic cancer cell lines. (**A**) Overexpression of oncogenic K-Ras in HPNE^{Kras12D} cells results in upregulation of Pim-1 and Pim-2 but not Pim-3 protein compared with parental HPNE cells. Cell lysates were immunostained with anti-K-Ras, Pim-1, Pim-2, Pim-3 and β-actin antibodies. (**B**) Suppression of mutated K-Ras by shRNAs in MIA PaCa-2 and Capan-1 cells results in decreased expression of Pim-1 but not Pim-2 or Pim-3. Numerical values represent relative band intensities of each Pim family member normalized to those of β-actin in the corresponding cell lines. Data shown are representative of three independent experiments.

p27^{Kip1} protein levels increase upon knockdown of Pim-1.

The cyclin-dependent kinase inhibitor p27^{Kip1} is another known downstream target of Pim-1 and is responsible for regulating the G_1/S phase of the cell cycle (16,17). We therefore immunoblotted lysates for p27^{Kip1} protein collected from MIA PaCa-2 and Capan-1 cells stably expressing Pim-1 shRNAs. We observed a slight to modest increase in p27^{Kip1} protein expression in cell lines with decreased Pim-1 expression (Figure 4A and B). To determine if the observed p27^{Kip1} expression correlated with changes in the G_1/S phase of the cell cycle, we stained cells with propidium iodide and used flow cytometry to determine their cell cycle distribution. Neither cell line showed significant changes in the distribution of cells in the G_1/S phase of the cell cycle. However, MIA PaCa-2 but not Capan-1 cells had a 2-fold increase in the G_2/M fraction of the Pim-1 knockdown cells compared with control cells-expressing scrambled shRNA (Figure 4C and D). Thus, although suppression of endogenous Pim-1 increased p27^{Kip1} expression in both cell lines, effects on the distribution of cells was only observed in one of the cell lines at the G_2/M phase.

Pim-1 is required for anchorage-dependent and -independent growth of PDAC cells

Two key properties of growth transformation by oncogenic K-Ras are enhanced growth in monolayer culture and under anchorage-independent conditions. We first investigated the role of Pim-1 in anchorage-dependent growth of the MIA PaCa-2 and Capan-1 cell lines used in previous experiments. In each cell line, the efficacy of each shRNA to suppress Pim-1 correlated with cell numbers at day 6 of culture on plastic, with the most efficient shRNA knockdowns (see Figure 3A and B) producing the greatest decrease in cell growth (Figure 5A and B). These results indicate that Pim-1 plays a significant role in anchorage-dependent growth of these PDAC cells.

We also used a soft agar colony-forming assay to measure anchorage-independent growth, a property that has been shown to be predictive of tumor growth in xenograft mouse models (25). As with anchorage-dependent growth, suppression of endogenous Pim-1 also resulted in a significant decrease in anchorage-independent growth, with colonies that were both fewer and smaller in the knockdown cells compared with those in the control scramble groups (Figure 5C and D). Thus, Pim-1 is also important for anchorage-independent growth in these PDAC cells.

Pim-1 contributes to PDAC cell invasion

Pancreatic cancers tend to invade locally (1,2) and PDAC cell lines are often highly invasive through Matrigel matrices (5,26). K-Ras has been shown to play an important role in PDAC invasion (5,27), but the contribution of Pim-1 to this property has not been investigated. In order to determine if Pim-1 also plays a role in the invasive properties of PDAC cell lines, MIA PaCa-2 and Capan-1 cells stably expressing Pim-1 shRNAs were plated on Matrigel. Knockdown of Pim-1 resulted in a significant decrease in the number of cells able to invade through the gel matrix (Figure 6A and B). These results provide the first evidence that Pim-1 is required for PDAC cells to invade through Matrigel.

Pim-1 is important for radioresistance in PDAC cells

K-Ras is known to contribute to radioresistance (28–30), and pancreatic cancers are particularly resistant to radiation therapy (31–33). Moderate to high expression of Pim-1 kinase has been correlated with poor response to radiation therapy in patients with head and neck

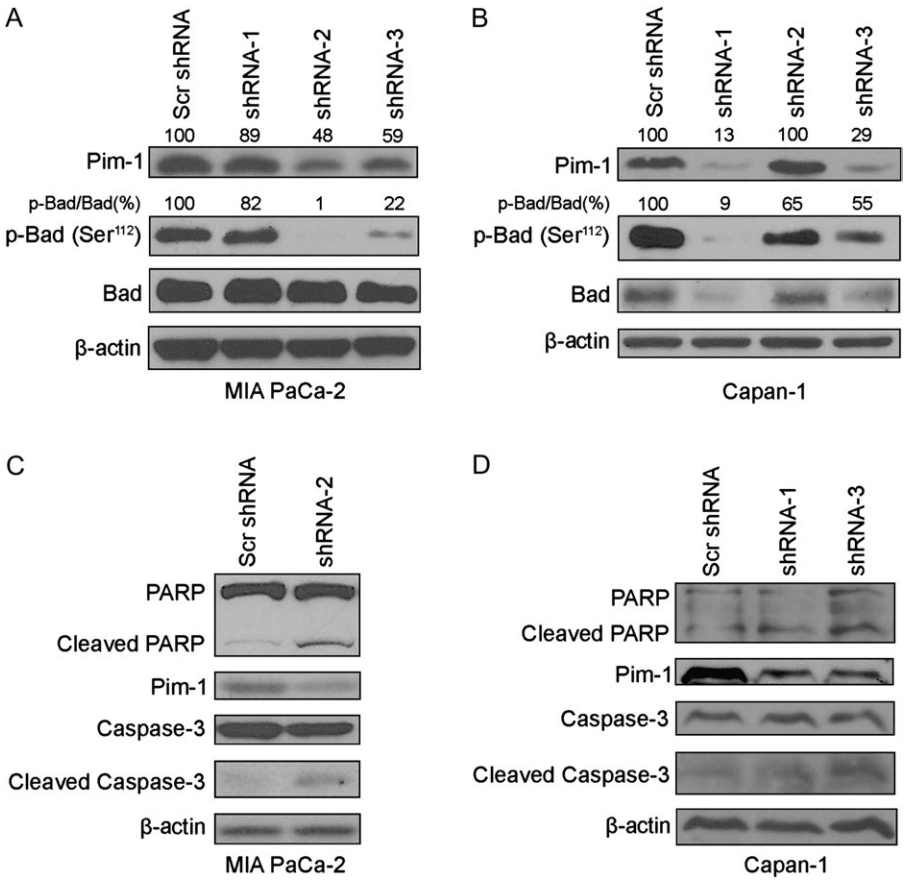

Fig. 3. Inhibition of endogenous Pim-1 protein downregulates phospho-Ser^{112} Bad and promotes apoptosis in PDAC cell lines. To evaluate the effect of downregulating Pim-1 on its immediate substrate, Bad, lysates from MIA PaCa-2 (**A**) or Capan-1 (**B**) cells stably expressing scramble (scr) or Pim-1 shRNAs were immunoblotted with antibodies to Pim-1, p-Bad (Ser^{112}) and total Bad. Numerical values for Pim-1 represent band intensities normalized to the scr shRNA protein lysates. Also, the ratio of phosphorylated Bad to total Bad was determined. To determine the consequences to apoptosis, PARP and caspase-3 antibodies were used to probe lysates of the MIA PaCa-2 (**C**) or Capan-1 (**D**) cells shown in panels A and B. β-Actin served as a loading control. Data shown are representative of three independent experiments.

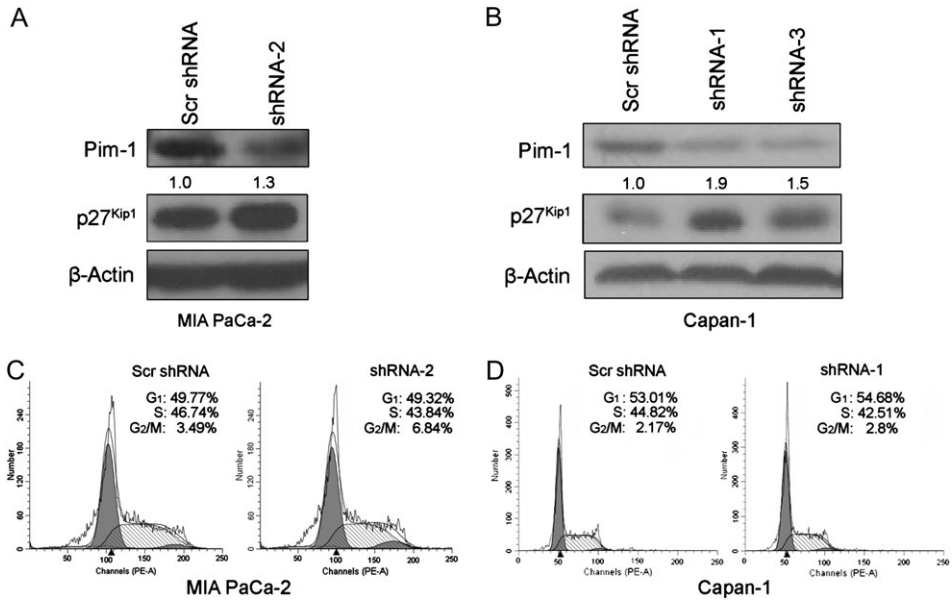

Fig. 4. Inhibition of endogenous Pim-1 protein increases cell cycle inhibitor p27^{Kip1} protein expression in PDAC cell lines. Cell lysates collected from MIA PaCa-2 (**A**) and Capan-1 (**B**) cells as in Figure 3 were immunoblotted with anti-Pim-1 and anti-p27^{Kip1}. Numerical values represent relative band intensities for p27^{Kip1} protein expression normalized to β-actin. Cell cycle distribution of MIA PaCa-2 (**C**) and Capan-1 (**D**) cells was analyzed by flow cytometry as described in Materials and Methods.

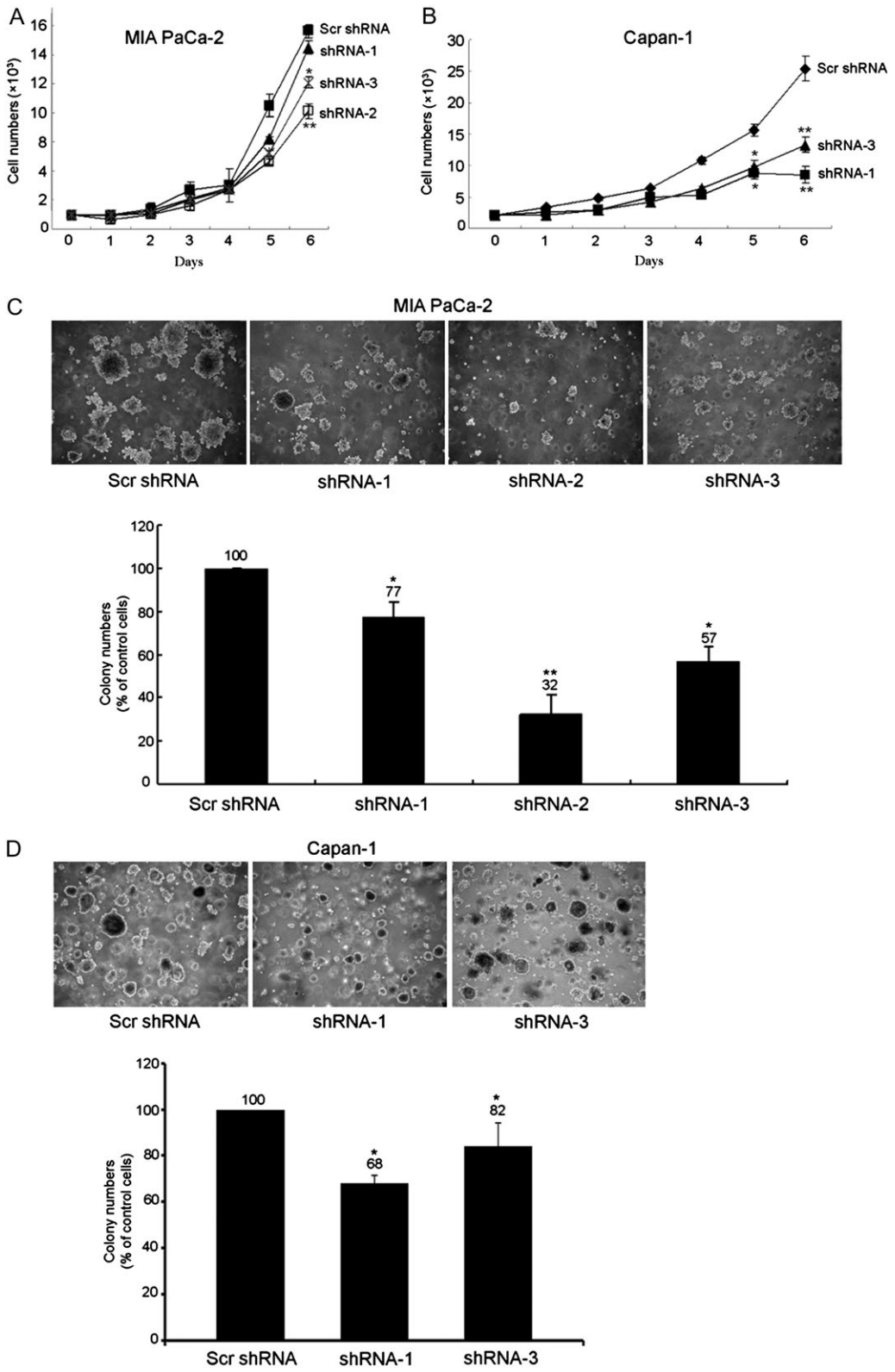

Fig. 5. Pim-1 is required for anchorage-dependent and -independent growth of pancreatic cancer cell lines. To evaluate anchorage-dependent growth, MIA PaCa-2 (**A**) and Capan-1 (**B**) cells stably expressing scramble (scr) or Pim-1 shRNAs were grown in 96-well microplates with the initial time point designated as Day 0. Cells were counted daily for a total of 6 days as described in Materials and Methods. Data are representative of at least three independent experiments and based on actual mean values. To examine anchorage-independent growth, MIA PaCa-2 (**C**) and Capan-1 (**D**) cells were evaluated for colony formation in a soft agar assay. Results collected from colony number counts were determined as described in Materials and Methods. All results are representative of three independent assays and normalized to scr shRNA-infected cells.

cancer (34), but such a correlation has not been examined in PDAC, and the role of Pim-1 in radioresponsiveness of PDAC is unknown. MIA PaCa-2 cells are radioresistant in part due to their expression of oncogenically mutated K-Ras^{12C} (33). We therefore performed standard clonogenic survival assays using MIA PaCa-2 cells in which Pim-1 was stably suppressed (shRNA-2) or not (Scr shRNA). Single cells were plated at low density, allowed to attach overnight and irradiated with either 2 Gy or 7 Gy using a cesium source. The fraction of MIA PaCa-2 cells surviving after 14 days was calculated and normalized to that of mock-irradiated cells (0 Gy) (Figure 6C).

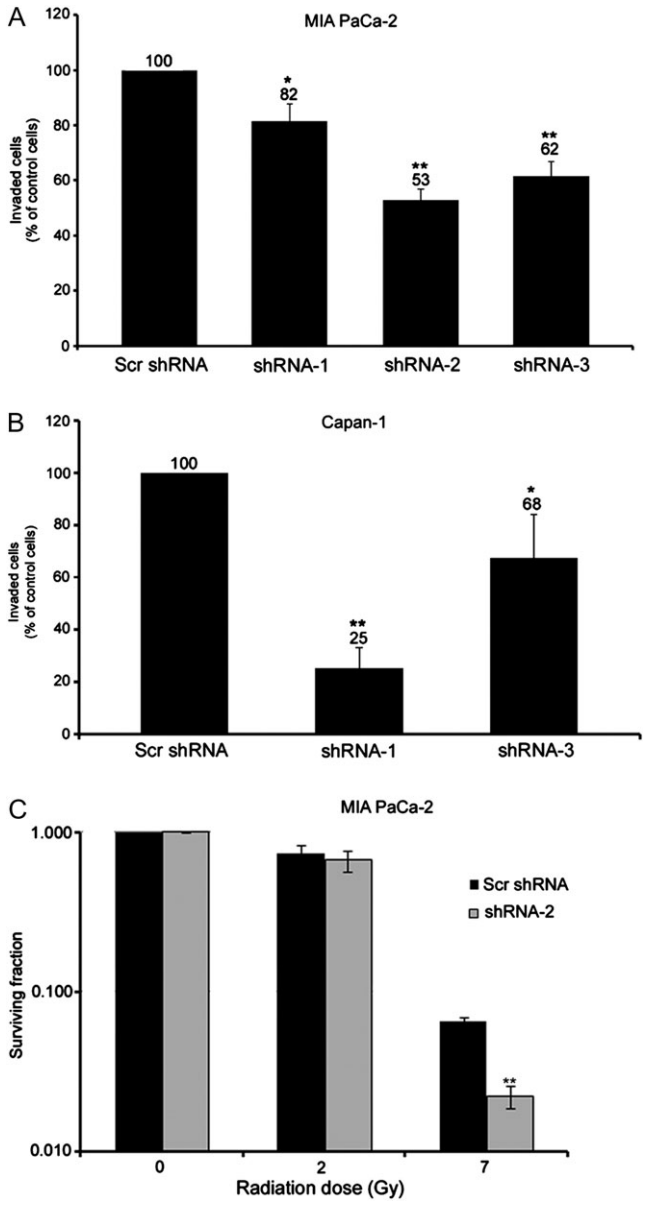

Fig. 6. Pim-1 is required for invasion and for radioresistance of pancreatic cancer cell lines. To determine the contribution of Pim-1 in invasion, MIA PaCa-2 (**A**) and Capan-1 (**B**) cells stably expressing scramble (scr) or Pim-1 shRNAs were plated in the Matrigel invasion assay as described in the Materials and Methods. Numerical values represent percentage of invasion of Pim-1 shRNA-expressing cells compared with scr shRNA control cells. Results are representative of three independent assays. To determine the contribution of Pim-1 to postradiation survival, MIA PaCa-2 (**C**) cells stably expressing scr or Pim-1 shRNA were subjected to a standard clonogenic survival assay as described in the Materials and Methods. Results shown are representative of three independent assays and the results were normalized to scr shRNA-infected cells.

Pim-1 knockdown resulted in lower but not significantly different clonogenic survival at 2 Gy (66% survival versus 74%). At 7 Gy, Pim-1 knockdown resulted in significantly fewer cells capable of surviving and forming colonies after gamma radiation (2.2 versus 6%). These results provide direct evidence to indicate that Pim-1 contributes to radioresistance of PDAC cells. Therefore, Pim-1 kinase inhibitors may be radiosensitizers in PDAC.

Discussion

K-Ras is oncogenically mutated in 90% of PDAC and has a critical role in the development and progression of this disease (3). Since no anti-Ras therapies have reached success in the clinic, current efforts are focused on identifying key signaling pathways of Ras as possible targets for drug intervention (8,9). Kinases are considered to be particularly tractable drug targets (4), so identifying downstream kinases required for K-Ras-mediated transforming properties in PDAC might be a particularly appealing approach to novel PDAC therapeutics. Members of the oncogene family of Pim serine/threonine kinases are upregulated in many cancers (13,17,20,21,34,35), have been shown to support cell growth and survival (13), and are the subjects of a hunt for novel molecularly targeted therapeutics (13). Our findings of Pim-1 protein expression being modulated by oncogenic K-Ras activity raise the possibility that Pim-1 levels may be a useful molecular marker of K-Ras activity in PDAC.

We show that Pim-1 protein is aberrantly expressed in a panel of PDAC cell lines as well as in tumors derived from PDAC patients compared with their matched normal tissues. Similarly, while our study was underway, a study conducted by Reiser-Erkan *et al.* (19) showed upregulation of Pim-1 expression in malignant PDAC compared with benign and precancerous conditions of the pancreas. Therefore, we investigated whether Pim-1 might also contribute to PDAC growth transformation. Using shRNA-mediated knockdown of Pim-1 expression, we show here a requirement for Pim-1 in anchorage-dependent cell growth of PDAC cell lines known to be dependent on K-Ras. Similarly, Li *et al.* (21) showed that suppression of endogenous Pim-3 resulted in decreased anchorage-dependent cell growth of PDAC cell lines. To further examine the mechanism of this growth inhibition in the PDAC cells infected with Pim-1 shRNAs, we analyzed the cell cycle and observed a 2-fold increase in the distribution of cells in G_2/M, suggesting cell cycle arrest as one mechanism for the differences in cell number. Additionally, we evaluated apoptosis as another potential explanation for the cell number differences in the Pim-1 knockdown cells. We observed modest increases in PARP and caspase-3 cleavage, suggesting that the differences could potentially reflect a greater susceptibility to apoptotic stimuli, which are supported by the decreased ratio of phosphorylated to total Bad protein (i.e. increased relative amounts of pro-apoptotic Bad protein in the presence of decreased Pim-1 expression). Mediators of this cell death other than Bad may be due to additional Pim-1 substrates such as apoptosis signaling kinase 1 (ASK1) or proline-rich Akt substrate 1 (PRAS1) (13). Studies have shown that Pim-1 can inhibit apoptosis by direct phosphorylation of these substrates. Additionally, studies have shown that Pim-1 is involved in the localization and stability of various substrates (13). Modulation of these processes may also lead to the susceptibility of apoptotic stimuli. Future studies should be conducted to evaluate how these various mediators of apoptosis can be regulated by Pim-1 expression and activity in PDAC.

To further consider the role of Pim-1 in PDAC growth transformation, we show here that suppression of endogenous Pim-1 expression decreased anchorage-independent growth of PDAC cells in the soft agar assay, which is routinely used to predict tumor growth in xenograft mouse models (25). This decrease in growth may also be contributed to effects on the cell cycle and increased susceptibility to apoptotic stimuli due to Pim-1 suppression as discussed above. Our results are in agreement with recent studies in which expression of a Pim-1 kinase-dominant negative decreased tumorigenicity and chemoresistance of a PDAC cell line in a murine xenograft model (18,20) and collectively suggest that high Pim-1 expression may be a marker

of poor prognosis. Additionally, recent studies have demonstrated a role for Pim-1 in cell motility, migration and invasion in several cancer types including leukemia, prostate and tongue carcinoma (36–38), but this has not been investigated in PDAC. We showed here that Pim-1 inhibition led to a significant decrease in PDAC cell invasion through Matrigel. Thus, Pim-1 contributes to invasive properties of K-Ras-dependent PDAC cell lines.

Finally, PDAC has been demonstrated to be insensitive to radiotherapy (31,32), and K-Ras is an important contributor to radioresistance in this (28–30,33) and other tumor types (30,34). Here, we observed that suppression of Pim-1 expression decreased the intrinsic radioresistance of PDAC cells that we have shown previously are rendered resistant in part due to K-Ras activity (33). These results are consistent with those of Peltola *et al.* (34), who found that high Pim-1 kinase expression predicts poor radiation response in head and neck cancers. Together, these findings suggest that novel Pim-1 inhibitors may serve as radiosensitizers of PDAC and help to improve outcomes.

PDAC is a highly aggressive and lethal disease with dismal survival rates. In this report, we have demonstrated that Pim-1 kinase is downstream of K-Ras signaling and may serve as a molecular marker of oncogenic K-Ras activity. Overall, our studies indicate that Pim-1 plays a significant role in growth transformation, invasion and radioresistance of PDAC and is potentially a useful target for therapeutics in this disease. To date, there have been >50 Pim kinase inhibitors developed by research groups in both industry and academia (13,39–41). One of the most potent of these, SGI-1776, impaired *in vitro* and *in vivo* growth of human leukemia cell lines and induced apoptosis in chronic lymphocytic leukemia and prostate cancer cell lines (42,43). Results from these studies have led to a Phase I clinical trial to explore the safety of SGI-1776 for the treatment of refractory non-Hodgkin lymphoma and prostate cancer patients (http://www.clinicaltrials.gov). Additionally, a recent study has identified 21 new substrates potentially targeted by Pim kinases (44). Future studies will help to better understand the molecular mechanisms involving oncogenic K-Ras and Pim-1 signaling that are responsible for the aberrant growth properties of PDAC.

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