PR55α Subunit of Protein Phosphatase 2A Supports the Tumorigenic and Metastatic Potential of Pancreatic Cancer Cells by Sustaining Hyperactive Oncogenic Signaling

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Abstract

The protein phosphatase 2 (PP2A) holoenzyme consists of a catalytic subunit, a scaffold subunit, and a regulatory subunit. Based on loss-of-function analysis using PP2A catalytic inhibitors or inhibition via tumor viral antigens, limited studies suggest that PP2A is a putative tumor suppressor. However, PP2A has also been shown to facilitate the activation of oncogenic signaling pathways when associated with specific regulatory subunits. In this study, we investigated the possible oncogenic role of PP2A in pancreatic cancer. We found a striking increase in the expression of PR55α (PPP2R2A), a PP2A regulatory subunit, in pancreatic cancer cells compared with normal pancreatic epithelial cells. Consistently, PR55α expression was markedly elevated in pancreatic ductal adenocarcinoma tissues compared with adjacent normal pancreatic tissues (P < 0.0001) and correlated with poor survival of pancreatic cancer patients (P < 0.0003). RNAi-mediated depletion of PR55α in pancreatic cancer cell lines resulted in diminished phosphorylation of both AKT and ERK1/2 (MAPK3/1) and decreased protein levels of β-catenin (CTNNB1). Accordingly, pancreatic cancer cells with reduced PR55α expression exhibited significantly impaired properties of transformation, including attenuated cell growth, clonogenicity, mobility, and anchorage-independent growth. Moreover, orthotopic implantation of PR55α-depleted pancreatic cancer cells into nude mice resulted in markedly reduced tumorigenicity (P < 0.001) and distant metastases. Together, these results suggest that PR55α promotes pancreatic cancer development by sustaining hyperactivity of multiple oncogenic signaling pathways, including AKT, ERK, and Wnt. These studies also provide a basis for exploring PR55α as a diagnostic or therapeutic target in pancreatic cancer. Cancer Res; 76(8); 2243–53. ©2016 AACR.

Introduction

PP2A, a family of heterotrimeric holoenzymes, accounts for the majority of serine/threonine phosphatase activity in human cells (1, 2). Each PP2A holoenzyme consists of one catalytic subunit (C), one scaffolding subunit (A), and one regulatory subunit (1, 2). There are two PP2A catalytic subunits, Cα and Cβ, and two scaffold subunits, Aα/Aβ. Cα and Cβ share 97% sequence identity, while Aα and Aβ share 87% sequence identity (3). The PP2A regulatory subunits are classified into four distinct families, B/PR55, B'/PR61, B''/PR72, and PTP/PR53, and each family contains at least four members (1). Despite their recognition of similar sequence motifs within the A subunits, there is no sequence homology among the PP2A regulatory subunits (3). The specific function of each PP2A holoenzyme is determined by its regulatory subunit, which controls the substrate specificity, cellular localization, and enzymatic activity of the holoenzyme (1).

PP2A plays essential roles in many fundamental cellular functions, including cell proliferation, migration, and survival (1, 2). Evidence supports an important role for PP2A in cellular transformation and tumorigenesis: (i) several viral oncoproteins can function as PP2A regulatory subunits to induce transformation (4–6), (ii) PP2A regulates several oncogenic signaling cascades, including AKT, ERK1/2, and Wnt/β-catenin (1), and (iii) PP2A regulates the tumor suppressing signaling pathway ARF/MDM2/p53, which plays a key role in blocking transformation and tumorigenesis of many cancer types (2). However, due to the structural complexity of PP2A holoenzymes and the wide range of substrates that PP2A acts on, the role of PP2A in oncogenic transformation remains largely undefined. Based on loss-of-function analysis using PP2A catalytic inhibitors (e.g., okadaic acid) or viral oncoproteins that inhibit PP2A activity, a very limited number of studies have described PP2A as a putative tumor suppressor.
suppressor (4–8). However, several different studies indicate that PP2A can also facilitate activation of oncogenic signaling pathways when associated with specific regulatory subunits (9–11).

The PR55 family of PP2A regulatory subunits consists of 4 isoforms (α/β/γ/δ). While PR55β and PR55γ are primarily expressed in brain tissues, PR55α and, to a lesser extent, PR55δ are expressed almost ubiquitously (12–14). PP2A/PR55 associates regulates several core-signaling pathways, including ARF/MDM2/p53, PKCz/Par3/Par6, PI3K/akt, Raf/MEK/ERK, TGFBR1/TGF-β, and Wnt/β-catenin (1, 15). While dereligation of these pathways contributes critically to cellular transformation (16, 17), the role of PP2A/PR55 in the pathogenesis remains largely undefined.

PP2A/PR55α has been reported to promote ERK1/2 phosphorylation/activation through dephosphorylation of KRAS-Ser392, Raf-Ser259, and Raf-Ser295 (9, 18). KSR is a scaffold that facilitates the Ras/Raf/MEK/ERK cascade, and Raf kinase is an upstream activator of ERK1/2. While dephosphorylation of KRAS-Ser392 and Raf-Ser295 prevents the sequestration/inhibition of these proteins by 14–3-3 (9), dephosphorylation of Raf-Ser259 by PP2A/PR55α directly activates Raf (18). Therefore, through activation of KSR and Raf, PP2A/PR55α positively regulates ERK1/2 phosphorylation. On the other hand, PP2A/PR55γ shows the opposite effect on the Raf/MEK/ERK cascade via dephosphorylation/inhibition of c-Src that activates Raf (19).

PR55c is involved in the regulation of AKT activation, which requires phosphorylation of AKT-Thr308/Ser473 (20). In FL5.12 murine pro-B-cell lymphoid cells, PP2A/PR55c has been shown to inhibit AKT by dephosphorylation of AKT-Thr308 but not AKT-Ser473 (21). PR55c is also reported to positively regulate the Wnt/β-catenin pathway. Beta-catenin is a key component of the Wnt signaling pathway, and its intracellular level is primarily regulated by ubiquitination/proteasome degradation. Upon phosphorylation by casein kinase I and GSK3α/β at Ser33/Ser37/Thr41 residues, β-catenin forms a complex with E3-ubiquitin ligase β-TrCP, leading to proteasome-mediated degradation (22). PP2A/PR55x dephosphorylates β-catenin-Ser33/Ser37/Thr41, stabilizing β-catenin protein and activating Wnt signaling (10, 23).

In this study, we examined PR55α expression in human and murine pancreatic cancer cells/tissues and investigated the influence of PP2A/PR55α on the oncogenic signalings mediated by AKT, ERK, and β-catenin in pancreatic cancer cells. We also examined the impact of PR55α on transformed phenotypes, tumorigenicity, and metastatic potential of pancreatic cancer cells. Our results demonstrate that PP2A/PR55α is required for sustaining the hyperactivities of multiple oncogenic signaling pathways and maintaining the malignant properties of pancreatic cancer cells.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines AsPC-1, Capan-1, CD-18/HPAF, and L3.6 were recently obtained from the ATCC and resuscitated from early-passage liquid nitrogen stocks. Cells were cultured for less than 2 months before reinstituting culture and routinely inspected microscopically for stable phenotype. ATCC uses morphology, karyotyping, and PCR-based approaches to confirm the identity of human cell lines. HPNE cells are primary human pancreatic ductal cells immortalized using human telomerase and contain no transformed properties (24). Aliquots of HPNE cells were stored at −150°C to ensure that cells used for experiments were passaged for fewer than 6 months. The cells were routinely inspected for identity by morphology and growth curve analysis and validated to be mycoplasma free.

Antibodies

Antibodies are listed in Supplementary Materials and Methods.

Tissue microarray and immunohistochemistry

The clinical specimen for IHC analysis was a commercial tissue microarray (TMA: OD-CT-DgPan01-006; US Biomax). The TMA included 72 cases of human pancreatic ductal adenocarcinoma (PDAC) and matched normal adjacent tissues (NAT). Among those, 38 cases were documented with patients’ survival data. The TMA was analyzed for PR55α expression by IHC using a PR55α-specific antibody (14–27 or 100C1) at 1:100 dilution, as described previously (25). The PR55α immunostaining intensity was evaluated by a University of Nebraska Medical Center (Omaha, Nebraska) pathologist who was blinded to the clinical information.

Immunoblotting

Immunoblotting was performed as described previously (26).

siRNA transfection

siRNA duplexes were purchased from Dharmacon. Nontargeting control-siRNA were designed by the manufacturer to contain at least 4 mismatches to any human, rat, or mouse genes. SMARTpool siRNA targeting PPP2R2A (PR55α) contains four siRNA targeting multiple sites on PR55α. Cells were transfected with 100 nmol/L siRNA using DharmaFECT-1 (Dharmacon). The siRNA sequences are included in Supplementary Materials and Methods.

Growth kinetics

Growth kinetics was performed as described previously (27).

Clonogenic survival assay

Clonogenic survival assay was performed as described previously (28).

Wound-healing assay

Wound-healing assay was performed as described previously (29).

shRNA lentiviral vectors

Lentiviral vectors (pGIPZ) expressing nonsilencing control-shRNA or PR55α-shRNA were obtained from Dharmacon. Four PR55α-shRNAs targeting various regions of the PR55α gene were used for study. The shRNA sequences and lentiviral infection are described in Supplementary Materials and Methods.

Soft-agar assay

Soft-agar assay was performed as described previously (27).

Animal models

All in vivo experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Genetically engineered mouse models of PDAC. The triple-transgenic KPC mice (KrasG12D;Trp53R172H+/Pdx1-Cre) were...
originally acquired from the NCI Mouse Models of Human Cancers Consortium (Frederick, MD). The composite mouse strain with targeted expression of mutant KrasG12D and Trp53R172H in the mouse pancreas was generated and maintained by exploiting pancreas-specific Pdx1-Cre (KPC), which develops spontaneous PDAC.

Xenograft model of human pancreatic cancer. Six-week-old female athymic mice (Harlan) were divided into four groups (n = 5 per group): a control group, which bore Control-shRNA-transduced tumor cells, and three examining groups, which bore PR55α-shRNA-transduced with tumor cells. Additional routine details are given in Supplementary Materials and Methods.

Statistical analysis

Histo-score was evaluated by multiplying the values of the IHC-staining intensity and the percentage of immunoreactive cells. The survival period was defined as the duration from the time of surgery until the last clinical follow-up or date of death. Survival curves were generated by the Kaplan–Meier method using Prism Graphpad. All other statistical analyses were done using SigmaPlot.

Results

PR55α is overexpressed in human pancreatic cancer cells

Aberrant activation of oncogenic signaling pathways contributes essentially to tumorigenesis and changes in protein phosphorylations have been a major cause for this activation. Because PP2A is a major cellular serine/threonine phosphatase, we investigated its contribution to the activation of oncogenic pathways in pancreatic cancer cells.

We analyzed the expression of PP2A subunits in a panel of human pancreatic cancer cell lines along with normal human pancreatic cells as controls. As shown in Fig. 1A, with an antibody recognizing all four isoforms (α, β, γ, δ) of the PR55 regulatory subunit, immunoblotting detected an average of 6-fold increase in PR55 expression in the pancreatic cancer cells compared with HPNE cells, which are normal human pancreatic ductal cells immortalized with telomerase (30). We also examined the expression of the catalytic and scaffold subunits of PP2A, as well as another two PP2A regulatory subunits PR61α and PR61γ. The latter were suggested as tumor suppressors by previous studies (31–33). As shown in Fig. 1A, expressions of these PP2A subunits were not particularly associated with the malignant potential of these pancreatic cells.

Among PR55 isoforms, the α and, to a lesser extent, the δ isoforms were reported to be expressed almost ubiquitously (6), whereas the β and γ isoforms are primarily expressed only in brain tissues (12–14). We therefore analyzed the expression of PR55α and δ in the indicated pancreatic cancer cells. As shown in Fig. 1B, the PR55α expression pattern in these cells was the same as detected with the antibody recognizing all PR55 isoforms (PR55α vs. PR55). In contrast, no PR55δ expression was detected in the panel of pancreatic cancer lines, while PR55δ expression was detected in mouse brain tissue, used as a positive control (Fig. 1B). These results suggest that only the PR55α is expressed in these pancreatic cell lines.

PR55α overexpression in pancreatic cancer cells is associated with overall increases in the activities of oncogenic signaling pathways

We next assessed the phosphorylation of AKT and ERK1/2, which are known targets of PP2A, in the human pancreatic cell lines. Results in Fig. 1C showed an overall increase in phosphorylation of AKT-S473 and ERK1/2-T202/Y204, indicative of activation of these proteins (34, 35), in the pancreatic cancer cells compared with normal HPNE control. These increases in protein

Figure 1.

Analysis of PP2A subunits and PP2A downstream targets in normal and malignant pancreatic cells. A, expressions of PP2A-regulatory subunits (PR55, PR61α, and PR61γ), scaffold subunit (PP2A-A), and catalytic subunit (PP2A-C) were assessed by immunoblotting in the indicated cell lines. B, expressions of PR55α and PR55δ were determined by immunoblotting. The level of GAPDH was assessed for protein loading control. 1, mouse brain lysate. C, cells were analyzed for phosphorylation and total protein of AKT, ERK1/2, and β-catenin by immunoblotting. 2, HEK293 cell lysate.
phosphorylation were not accompanied by changes in total levels of the corresponding proteins (Fig. 1C).

Because a full activation of AKT requires phosphorylation of both Ser473 and Thr308 (20), we also analyzed the panel for AKT-
T308 phosphorylation. Compared with normal HPNE control, AKT-Thr308 phosphorylation was higher in Capan-1 and L3.6 cells, but was lower in AsPC-1 and CD18/HPAF cells (Fig. 1C), implicating a lack of correlation between PR55α expression and AKT-Thr308 phosphorylation in pancreatic cancer cells.

We also analyzed β-catenin protein in these cells. β-Catenin is a PP2A target and plays a key role in Wnt signaling (1). As shown in Fig. 1C, compared with HPNE control, a large increase in β-catenin expression was detected in CD-18/HPAF cells, while only a subtle increase of the β-catenin level was detected in L3.6 cells. On the other hand, β-catenin expression in AsPC-1 and Capan-1 cells was found to be similar to that of normal HPNE cells. Because previous studies described a negative regulation of β-catenin protein stability through a phosphorylation of β-catenin-S33/S37/T41 (22), we analyzed this phosphorylation in these cells, using HEK293 cells as a positive control (36). Figure 1C revealed an elevated β-catenin-S33/S37/T41 phosphorylation in Capan-1, CD-18/HPAF, and L3.6 pancreatic cancer cells compared with that found in HPNE control. Collectively, these data suggest that PR55α overexpression in pancreatic cancer cells is correlated with an overall increase in the activities of oncogenic signaling pathways, including ERK1/2, AKT, and Wnt.

**PR55α is overexpressed in human PDAC tissues**

To validate the detection of PR55α overexpression in pancreatic cancer cells by immunoblotting, we assessed PR55α expression by IHC in a TMA of 72 cases of human PDAC with NAT specimens. As shown in Fig. 2A–E, IHC detected specific staining for PR55α with moderate to intense staining in the cytoplasm of the majority of PDAC tissues, whereas the corresponding NAT were negative or weakly positive for PR55α staining. Among TMA samples, 67% (48/72) of cases of PDAC tissues had intense to moderately high levels of PR55α expression, whereas weak or no PR55α expression was detected in their normal counterparts. The analysis also showed that 19% (14/72) of cases were negative or showed a similar pattern of PR55α expression in the tumor and corresponding NAT and its matching tumor tissues. In addition, 14% (10/72) of cases showed much higher PR55α expression in normal tissues than in PDAC tissues. The higher PR55α level in these NATs was in part due to the involvement of the neoplastic transformation process, as they are obtained from areas close to tumors. Overall, IHC analysis indicates that there is a significant increase in PR55α expression in pancreatic tumor samples compared with their paired normal tissue samples (P < 0.0001).

To understand the clinical significance of PR55α expression, we performed a Kaplan–Meier survival analysis on 38 cases of PDAC, for which patient survival data were available. The clinical characteristics of these cases are described in Supplementary Table S1. In the 38 tumor samples, the H score for PR55α expression ranges from 0 to 2.7, while the mean H score for their matched NATs is 0.2 (Fig. 2E). To assess the relationship between PR55α expression and overall patient survival in these samples, we used the mean H score of NATs (0.2) as a threshold to divide the tumor samples into low and high PR55α-expressing cohorts, as described previously (37). As shown in Fig. 2F, a high PR55α expression level (H score of PR55α expression ≥0.2) was significantly associated with an overall poor survival of pancreatic cancer patients (P < 0.0003). The median survival for patients with high PR55α expression was only 11 months, whereas the median survival for patients with low PR55α expression was 48 months (Fig. 2F). This result suggests that a high level of PR55α expression is predictive of poor survival in patients with pancreatic cancer.

**PR55α overexpression is detected in mouse PDAC**

To verify the association of PR55α expression with pancreatic cancer development, we analyzed PR55α protein expression in pancreata of KPC triple-transgenic mice (KrasG12D; Tp53R172H/+, Pdx1-Cre). The KPC mice is a well-validated and clinically relevant spontaneous murine model of PDAC (38, 39). At about 10 weeks of age, KPC mice start to develop a spectrum of premalignant lesions named pancreatic intraepithelial neoplasias (PanIN) that ultimately progress to carcinomas with a moderate to well-differentiated ductal morphology, which is similar to that commonly observed in human PDAC (38). As a littermate control, LSL-KrasG12D mice that do not develop PDAC were included in the studies. Results of this study are consistent with those obtained from the human pancreatic TMA analysis described in Fig. 2A–E, revealing a marked increase in PR55α expression in PanINs developed in KPC mice at both 10 and 25 weeks of age compared with the pancreatic ducts of littermate control mice (Fig. 2G).

**Decrease of PR55α expression by siRNA diminishes oncogenic signalings in pancreatic cancer cells**

Using specific siRNA, we examined the impact of PR55α expression on the activities of oncogenic signalings targeted by PP2A.

As shown in Fig. 3A, PR55α-siRNA–transfected CD-18/HPAF cells exhibited a marked decrease in total PR55α protein expression compared with control-siRNA–transfected cells. Associating with this decrease in PR55α levels in the PR55α-siRNA–transfected cells, phosphorylation of AKT-S473 and ERK1/2-T202/Y204 was markedly reduced, indicative of decreased AKT and ERK1/2 activities (Fig. 3A). On day 3 after transfection, there was an increase in phosphorylation of ERK2, but not ERK1, in both control- and PR55α-siRNA–transfected cells, which is likely a nonspecific effect of the transient transfection method (Fig. 3A). To validate the inhibition of AKT and ERK1/2 signaling in these cells, we analyzed their common downstream target BAD, a proapoptotic protein whose stability is normally inhibited by the AKT and ERK1/2 pathways (40, 41). As shown in Fig. 3A and Supplementary Fig. S1A, BAD protein expression was upregulated by 2- to 3-fold in PR55α-siRNA–transfected cells compared with control cells.

We also assessed the effect of PR55α on AKT-T308 phosphorylation, which is also involved in AKT activation (35). Figure 3B showed that transfection of PR55α-siRNA resulted in a decrease in AKT-T308 phosphorylation in both Capan-1 and CD-18/HPAF cells.

We next examined the influence of PR55α expression on β-catenin. As demonstrated previously, β-catenin level is mainly regulated through proteasomal degradation that is generally facilitated by the phosphorylation of β-catenin-Ser33/Ser37/Thr41 (22). As shown in Fig. 3C and Supplementary Fig. S1B, decrease of PR55α expression by siRNA in CD-18/HPAF cells led to an increase in phosphorylation β-catenin-Ser33/...
Ser37/Thr41 and a concomitant decrease in β-catenin protein expression.

Together, these results provide direct evidence suggesting a vital role for PR55α in the maintenance of hyperactive AKT, ERK1/2, and Wnt signalings in pancreatic cancer cells.

Decrease of PR55α expression in pancreatic cancer cells suppresses cell growth

We tested the effect of PR55α on growth kinetics of pancreatic cancer cells using siRNA. As shown in Fig. 4A, transfection of PR55α-siRNA significantly decreased PR55α expression in both ...
Decrease of PR55α expression impedes migration of pancreatic cancer cells

PP2A plays an essential role in the regulation of cell mobility and invasion (42). We therefore tested the effect of PR55α on mobility of pancreatic cancer cells using a wound-healing assay (29). As shown in Fig. 4D and Supplementary Fig. S3, transfection of PR55α-siRNA markedly reduced the mobility of CD-18/HPAF, Capan-1, and AsPC-1 cells, suggesting that PR55α is essential for the migratory and invasive advantages of pancreatic cancer cells.

Decrease of PR55α expression inhibits anchorage-independent growth of pancreatic cancer cells

Anchorage-independent growth capability, the most important property of tumor cells, is conjointly driven by multiple oncogenic signaling pathways, including AKT, ERK1/2, and Wnt (43). As Fig. 3 showed that PR55α is required for maintaining the hyperactivities of these signaling pathways in pancreatic cancer cells, we assessed the effect of PR55α on anchorage-independent growth of pancreatic cancer cells.

For this study, we developed a series of pancreatic cancer cells (CD-18/HPAF) stably expressing shRNA targeting four different regions of PR55α. As a control, cells stably transduced with non-silencing control-shRNA were included in the study. As shown in Fig. 5A, transduction with each PR55α-shRNA markedly reduced PR55α expression in CD-18/HPAF cells. In contrast, control-shRNA–transduced cells showed no effect on PR55α expression in CD-18/HPAF cells compared with untransduced cells (Fig. 5A).

We next analyzed the effect of PR55α-shRNA on AKT, ERK1/2, and β-catenin. Consistent with the effect of PR55α-siRNA shown in Fig. 3A, decrease of PR55α expression in CD-18/HPAF cells by each of the four PR55α-shRNAs resulted in a marked reduction in phospho-AKT-S473 and phospho-ERK1/2-T202/Y204 with no noticeable change in protein levels (Fig. 5A). The stable knockdown of PR55α also markedly reduced the steady-state levels of β-catenin protein, along with minor increases in β-catenin-S33/S37/T41 phosphorylation in these cells. In contrast, none of these changes were observed in control-shRNA transduced cells (Fig. 5A).

Using a soft-agar assay (27), we tested the effect of PR55α on anchorage-independent growth. As shown in Fig. 5B, decrease of PR55α expression by each of the PR55α-shRNAs almost completely inhibited the ability of CD-18/HPAF cells to form colonies in soft agar. These results suggest a requirement for PR55α for anchorage-independent growth of pancreatic cancer cells.

Knockdown of PR55α expression by shRNA impairs pancreatic cancer cell growth and metastasis in vivo

Because decrease of PR55α expression using siRNA or shRNA resulted in suppression of signaling pathways that are normally required for hyperproliferative, survival, migratory and anchorage-independent properties of pancreatic cancer cells (Figs. 3–5), we further evaluated the effect of PR55α on the tumorigenic and metastatic potential of pancreatic cancer cells in vivo. Nude mice were orthotopically implanted with CD-18/HPAF cells stably expressing control- and PR55α-shRNAs and monitored for tumor growth for 5 consecutive weeks using in vivo bioluminescence imaging. As shown in Fig. 6A and Supplementary Fig. S4A, PR55α knockdown tumor cells grew much slower than control cells in the pancreata of mice. Based on the weights of tumor xenografts obtained at 5 weeks after implantation, there was a marked reduction in the size of tumor formed by PR55α-silenced
pancreatic cancer cells compared with control cells (Fig. 6B; Supplementary Fig. S4A). We also analyzed xenograft tissues by IHC/hematoxylin and eosin (H&E) staining and confirmed the persistent silencing of PR55α in tumor cells expressing PR55α-shRNA (Fig. 6C; Supplementary Fig. S4B). Remarkably, the tumor growth inhibitory effect was paralleled with a decreased metastatic potential in PR55α-knockdown cells. Compared with the control group, mice implanted with PR55α-knockdown cells showed a marked reduction in the incidence of metastasis to distant organs (liver, spleen, small intestine, diaphragm, peritoneum, cecum, and mesenteric lymph node; Table 1). For instance, a significant decline in liver metastasis was observed in mice implanted with PR55α-knockdown cells compared with those implanted with control cells (P < 0.02). These observations are consistent with the in vitro data showing a suppression of AKT/ERK/Wnt signalings in pancreatic cancer cells by PR55α knockdown (Figs. 3 and 5) and suggest an essential role for PR55α in tumor growth and metastasis of pancreatic cancer.

Figure 4. Effect of PR55α expression on growth kinetics and mobility of pancreatic cancer cells. A, indicated cells were transfected with control-siRNA or PR55α-siRNA and analyzed for PR55α and actin levels by immunoblotting. B, control-siRNA- or PR55α-siRNA-transfected cells were incubated for the indicated times and counted. Results depict the relative number of cells in each sample and represent the mean ± SD of two sets of experiments in duplicate. C, cells (3 × 10⁶) were transfected with control-siRNA or PR55α-siRNA and incubated for 10 days in growth medium to form colonies. The colonies were visualized by crystal violet staining and quantified using the ImageJ analytical program. The results are shown as mean ± SD of two set of experiments in duplicate samples. *, P < 0.001 (n = 4), significant difference between the PR55α-siRNA-transfected and control-siRNA-transfected cells. D, cells were transfected with control-siRNA or PR55α-siRNA, incubated for 48 hours and examined for mobility using wound-healing motility assay (29). Wounds were photographed and measured at 0, 24, and 48 hours after wounding. Cell migration was assessed as a percentage of wound coverage over time. The line graphs depict the percentage wound coverage and are shown as mean ± SD of two sets of experiments in duplicate samples. *, P < 0.001 (n = 4), significant difference between control-siRNA-transfected and PR55α-siRNA-transfected cells.
several regions of PR55a cells. CD-18/HPAF cells were stably transduced with shRNA targeting PP2A and suppresses anchorage-independent growth of pancreatic cancer cells. shRNA knockdown on anchorage-independent growth was examined using a soft-agar assay. Top, representative images of soft-agar assay. Bottom, colonies in soft agar were counted and are shown as mean ± SD of two separate experiments in duplicate.

**Figure 5.**

Discussion

The role of PP2A in tumorigenesis remains largely undefined, and much controversial information has been obtained on this function of PP2A. PP2A was initially suggested as a putative tumor suppressor, based on loss-of-function analyses using PP2A catalytic inhibitors or viral oncoproteins that inhibit PP2A (4–8). Furthermore, studies focusing on the PR61 family of PP2A regulatory subunits further supported PP2A as a tumor suppressor (1), indicating that PP2A/PR61 can inhibit Wnt signaling, stabilize p53 protein, and suppress BCL2 antiapoptotic activity (1). On the other hand, different studies also challenged the notion that PP2A holoenzymes are all acting as tumor suppressors. These studies show that PP2A, when associated with specific regulatory subunits, can facilitate activation of oncogenic signaling pathways (1). Among those, PP2A/PR55α has been demonstrated to activate ERK1/2 and Wnt pathways (9, 10, 23). Consistently, two cancer-related PP2A-Az point mutants, E64G in breast cancer and E64D in lung cancer, are defective in binding to PR61, but can bind normally to PR55α (44, 45). These paradoxical observations re-emphasize the complexity and diversity of PP2A functions, which are attributed to the large number of non-conserved PP2A regulatory subunits (1). Thus, the evidence now indicates that PP2A not only can function as a tumor suppressor, but can also act as a tumor promoter, depending on its regulatory subunit.

This report shows that the expression of PP2A regulatory subunit PR55α is detected at much higher levels in pancreatic cancer cell lines relative to normal pancreatic ductal cells (see Fig. 1). Consistently, PR55α expression is also markedly increased in both human and murine PDAC tissues compared with normal pancreatic tissues (see Fig. 2). Furthermore, Kaplan–Meier analysis reveals a significant inverse correlation between PR55α protein expression and overall survival of pancreatic cancer patients, suggesting that high PR55α expression in pancreatic cancer denotes a trend in poor survival of pancreatic cancer patients. Supporting this suggestion, the *in vitro* and *in vivo* studies in this report reveal that PR55α is required for sustaining hyperactivities of oncogenic signaling pathways, including AKT, ERK1/2, and Wnt, and maintaining both transformed phenotypes and malignant potential of pancreatic cancer cells (see Figs. 3–6 and Table 1).

PP2A/PR55α has been reported to promote ERK1/2 phosphorylation/activation through the dephosphorylation of KSR-Ser392, Raf-Ser259, and Raf-Ser295 (9, 18). While dephosphorylation of KSR-Ser392 and Raf-Ser295 releases these proteins from sequestration/inhibition by 14-3-3 (9), dephosphorylation of Raf-Ser259 directly activates Raf (18). Results in this report are consistent with those of previous reports showing that PR55α expression is required for sustaining ERK1/2 activation in pancreatic cancer cells (see Fig. 3 and 5).

The mechanism by which PP2A/PR55α regulates AKT still remains largely unclear; this is due to the complexity of AKT regulatory mechanisms. AKT can be activated through both PI3K-dependent and PI3K-independent mechanisms (46). With the PI3K-dependent mechanism, AKT is first recruited to the plasma membrane by PIP3, and subsequently phosphorylated by mTOR/Rictor (mTORC2) at Ser473 and then by PDK1 at Thr308, leading to the full activation of AKT (20, 46). Through PI3K-independent mechanisms, AKT can be directly regulated by numerous kinases, including PKA, ACHK1, TNK2, IKKε, TBK1, Src, PTK6, ATM, and DNA-PK, as well as several phosphatases, including PP2A, PTEN, PHLPPs, and INPP4B (46, 47). A previous study in FL5.12 murine pro–B-cell lymphoid cells shows that PP2A/PR55α can directly dephosphorylate AKT-Thr308 but not AKT-Ser473 (21). However, results in this report show that PR55α is apparently required for the maintenance of both AKT-Ser473 and AKT-Thr308 phosphorylation in human pancreatic cancer cells (see Figs. 3A–B and 5A).

The discordance between our result and previous observations might be due to difference in cell type or tumor type. It also suggests that the regulation of AKT phosphorylation involves multiple signaling mechanisms, which are differentially targeted by the PR55α linked PP2A.

Because PP2A/PR55α has been shown to increase β-catenin protein stability by dephosphorylating β-catenin-Ser33/Ser37/
Thr41 (10, 23), we have examined this regulation in pancreatic cancer cells using both siRNA (transient knockdown) and shRNA (stable knockdown). While both transient and stable knockdown of PR55α result in a marked decrease in β-catenin protein level in pancreatic cancer cells, a better correlation between the change in PR55α expression and β-catenin-S33/S37/T41 phosphorylation is observed in cells with PR55α transient knockdown (see Fig. 3C vs. Fig. 5). In PR55α stable knockdown cells, we observed only a

Figure 6.
Decrease of PR55α expression in pancreatic cancer cells suppresses tumor growth and metastases. Luciferase-expressing CD-18/HPAF cells (CD-18/HPAF-Luc; 5 × 10⁶), which had been stably transduced with control-shRNA and PR55α-shRNA (#2–#4), were orthotopically implanted into the pancreata of athymic mice and monitored for tumor growth and metastases for 5 weeks using a bioluminescent imaging system. A, images of tumor-bearing mice at the indicated days after implantation. B, box plot depicts the average pancreas weight of mice implanted with control- or PR55α-shRNA-transduced cells. *, P < 0.001 (n = 5); significant reduction of pancreas weight in the groups of mice implanted with PR55α-shRNA-transduced cells compared with the control group. C, PR55α expression in pancreatic tumor xenograft tissues was analyzed by IHC. Arrows, positive IHC staining for PR55α expression. Scale bar, 50 μm.

Table 1. Incidence of metastasis to various organs developed by the implanted CD-18/HPAF-Luc cells transduced with control-shRNA or PR55α-shRNA was analyzed and quantified in sacrificed mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Site of metastasis</th>
<th>Liver</th>
<th>Spleen</th>
<th>Intestine</th>
<th>Diaphragm</th>
<th>Peritoneum</th>
<th>Cecum</th>
<th>Mesenteric lymph node</th>
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<tr>
<td>Control shRNA</td>
<td></td>
<td>4/5 (80%)</td>
<td>3/5 (60%)</td>
<td>1/5 (20%)</td>
<td>1/5 (20%)</td>
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<tr>
<td>PR55α shRNA#2</td>
<td></td>
<td>0/5*</td>
<td>1/5 (20%)</td>
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<tr>
<td>PR55α shRNA#3</td>
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<td>0/5*</td>
<td>2/5 (40%)</td>
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<tr>
<td>PR55α shRNA#4</td>
<td></td>
<td>0/5*</td>
<td>2/5 (40%)</td>
<td>1/5 (20%)</td>
<td>0/5</td>
<td>0/5</td>
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</table>

NOTE: *, P < 0.02, significant difference from the control-shRNA-transduced cells.
subtle, if any, increase in β-catenin-S33/S37/T41 phosphorylation compared with control cells. The minimal change in β-catenin-S33/S37/T41 phosphorylation in stable knockdown cells can probably be attributed to the adaptation of these cells to the low level of PR55α, which has kept β-catenin at a very low steady-state level in the cells.

While PR55α has been demonstrated by in vitro studies to have a modulatory effect on the phosphorylation of key signaling players such as ERK1/2, AKT, and Wnt (21, 23, 48), its in vitro function in many cancers, including pancreatic cancer, remains poorly understood. Using an orthotopic mouse model of pancreatic cancer, the present study has demonstrated that inhibition of PR55α expression leads to a significant suppression in tumor growth and metastasis (see Fig. 6, Table 1 and Supplementary Fig. S4). Thus, these in vitro–obtained results are consistent with the in vitro modulatory effects of PR55α on the proliferation, survival, motility, and anchorage independence observed in pancreatic cancer cells (see Figs. 3–5), suggesting a critical role for PR55α in pancreatic tumor growth and metastasis. Future studies will be needed to determine whether the level of PR55α expression is associated with the staging and prognosis of pancreatic cancer. Additionally, a global genetic approach is needed to identify additional targets of PR55α that are involved in pancreatic cancer development and maintenance. Nevertheless, studies in this report provide direct evidence that PR55α is an attractive therapeutic target for pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.L. Hein, P. Seshacharyulu, Y.M. Sheinin, M.M. Ouellette, M.P. Ponnusamy, Y. Yan
Writing, review, and/or revision of the manuscript: A.L. Hein, P. Seshacharyulu, M.M. Ouellette, S.K. Batra, Y. Yan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.L. Hein, M.P. Ponnusamy, S.K. Batra, Y. Yan
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PR55α Subunit of Protein Phosphatase 2A Supports the Tumorigenic and Metastatic Potential of Pancreatic Cancer Cells by Sustaining Hyperactive Oncogenic Signaling

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