

# Small interfering RNA-mediated knockdown of PRL phosphatases results in altered Akt phosphorylation and reduced clonogenicity of pancreatic cancer cells

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## Abstract

The PRL phosphatases have been implicated in cancer cell growth and metastasis in a variety of tumor types. Using cDNA microarray, we previously identified and reported PRL-1 as being highly up-regulated in pancreatic cancer cell lines. In this study, we sought to further evaluate the expression of all three PRL phosphatases in pancreatic cancer cell lines and extend our findings to *in situ* analysis of primary pancreatic tumors taken directly from patients. Additionally, we determine if small interfering RNA-mediated knockdown of relevant PRLs confers antitumor effects in pancreatic cancer cells. Using oligonucleotide expression arrays, mRNA levels of PRL-1 and PRL-2 but not PRL-3 were identified as up-regulated in pancreatic cancer cell lines and tumor samples taken directly from patients compared with those of normal pancreas. Focusing on PRL-1 and PRL-2, high levels of both proteins were detected in a subset of pancreatic cancer cell lines and tumor samples using Western blotting and immunohistochemistry, respectively. Small interfering RNA-mediated knockdown of PRL-1 and PRL-2 in combination resulted in a moderate reduction of cellular growth and migration in MIA PaCa-2 and PANC-1 cells. More importantly, knockdown of both PRL-1 and PRL-2 significantly inhibited colony formation of these cells in soft agar as well as serum-induced Akt phosphorylation. These data support the hypothesis that PRL phosphatases regulate key pathways involved in tumorigenesis and metastasis and that knockdown of both PRL-1 and PRL-2 is required to disrupt PRL phosphatase function in pancreatic cancer cells. [Mol Cancer Ther 2008;7(1):202–10]

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## Introduction

With a 5-year survival rate of ~5%, pancreatic cancer is the fourth leading cause of death from cancer in both men and women in the United States (1). It is estimated that in the year 2007 there will be 18,830 males and 18,340 females (aggregate of 37,170) diagnosed with pancreatic cancer and that 33,370 people will die from the disease in the United States alone (1). Currently, surgical resection is the only therapy that is considered to offer a cure; however, few patients are diagnosed early enough to have that option. Gemcitabine, which is the current frontline therapy for patients with advanced pancreatic cancer, only provides limited benefit. Therefore, new treatments as well as a better understanding of pancreatic cancer biology are urgently needed.

The PRL phosphatases (consisting of PRL-1, PRL-2, and PRL-3 in humans) are a subgroup of low-molecular-weight protein tyrosine phosphatases currently being investigated by our group and others as potential therapeutic targets in cancer (2). Of the PRL phosphatases, PRL-3 is most thoroughly investigated and high expression has been shown to correlate with disease progression in colorectal, gastric, ovarian, and breast tumors (3–7). The ongoing validation of these potential markers in clinical tumor tissues is critical to further characterize biomarker prevalence and possible utility as a therapeutic target. Several recent reports using RNA interference to knockdown endogenous PRL levels or expression vectors to ectopically express PRL-1 or PRL-3 protein provide accumulating evidence that PRL phosphatases might promote cancer cell motility and invasion and thus play a causal role in metastasis (8–12). Importantly, antimetastatic effects have been observed following RNA interference-mediated knockdown of PRL transcripts. More specifically, knockdown of PRL-1 or PRL-3 in DLD-1 colorectal cancer cells significantly impaired their ability to colonize in hepatic tissue, and inhibition of PRL-3 expression in SGC7901 gastric cancer cells and B16-BL6 mouse melanoma cells reduced metastases in mouse models (10, 13, 14). Although the exact biological function(s) of these phosphatases remains unclear, mechanistically PRL phosphatases have been linked to several pathways, including regulation of integrins, Erk, c-Src, Akt, and Rho family GTPases (15–19).

In the present study, we analyzed the expression of PRL proteins at both the RNA and protein level in pancreatic cancer cell lines and tumor samples taken directly from patients. We also evaluated the role of PRL-1 and PRL-2 in pancreatic cancer cells using PRL-1 and PRL-2-targeting small interfering RNA (siRNA). To our knowledge, this is the first report describing the effects of PRL-2 inhibition in cancer cells. The findings presented herein are unique

compared with other cancer types described in the literature in that we find both PRL-1 and PRL-2, but not PRL-3 to be up-regulated. In addition, knockdown of multiple PRL phosphatases was needed in pancreatic cancer cell lines to see a significant biological effect in our assays, suggesting that levels of all three PRL phosphatases should be taken into account when evaluating PRL function in cancer.

## Materials and Methods

### Cell Culture

All cells were grown in a humidified incubator at 37°C and 5% CO<sub>2</sub>. The cell line HPDE6 (an immortalized but not transformed human pancreatic epithelial cell line) was obtained from Dr. Ming-Sound Tsao (University of Toronto; ref. 20) and maintained in keratinocyte serum-free medium supplemented with 0.2 ng/mL epidermal growth factor and 30 µg/mL bovine pituitary extract (Invitrogen). All other cell lines were purchased from the American Type Culture Collection and maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine and 1% penicillin/streptomycin (Invitrogen).

### Human Tumor Samples

Patient samples used in these studies were provided to this study without patient identifiers through the Translational Genomics Research Institute Tissue Bank and the Molecular Profiling Institute. An Institutional Review Board exemption was obtained for use of the tissues.

### Oligonucleotide Microarray Analysis

Total RNA from pancreatic tumor samples, pancreatic cancer cell lines, and normal pancreas was isolated using TRIzol (Invitrogen). RNA (500 ng) was amplified and labeled with Cy3 (reference normal RNA) or Cy5 (sample RNA) dye using an Agilent fluorescent linear amplification kit. Labeled amplified RNAs were fragmented and hybridized on Agilent Human 1A(V2) Oligo Microarray slides using an Agilent *in situ* hybridization kit. Slides were scanned using an Agilent G2505B scanner, and Agilent feature extraction software (version 8.1) was used to calculate signal intensity. PRL-1, PRL-2, and PRL-3 intensities from each sample (Cy5 channel) were median normalized and set to log<sub>2</sub> values for evaluation.

### Cell Lysate Preparation and Western Blotting

Cells were rinsed with ice-cold PBS (1 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L orthovanadate) and lysed in ice-cold lysis buffer (1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 2 mmol/L orthovanadate, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). The lysates were cleared by centrifugation at 15,000 rpm for 10 min in a microcentrifuge, and protein concentrations were determined using the BCA protein assay kit (Pierce). Equal amounts of lysate were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the following antibodies: phospho-Erk<sup>T202,Y204</sup> (Cell Signaling; 9106), phospho-Akt<sup>S473</sup> (Cell Signaling; 9271), and PRL-1, PRL-2, and PRL-3 (Bethyl Laboratories). Western blots were stripped and reprobed with the appropriate antibodies [Erk

(Cell Signaling; 9102) and Akt (Cell Signaling; 9272)], and β-actin (Sigma).

### Tissue Microarray Construction and Immunohistochemical Analysis of Tissue Microarray Slides

Needle cores of 1.0 mm in diameter were extracted from regions of interest identified on the paraffin tissue blocks to include multiple (two or three) cores of infiltrating tumor as well as adjacent normal pancreas when available. The tissue microarray (TMA) block was constructed at TGen in the usual manner with cores arrayed in precise orientation, containing 53 tumors double punched. Only a subset of the tumors, 34 cases, contained adjacent normal pancreatic ductal tissue. The TMA master block was serially sectioned at 5 µm intervals onto standard charged glass using water floatation for TMA section transfer. The TMA slides were dipped in paraffin for uniform epitope preservation. Dewaxing, antigen retrieval, and all immunostaining steps were carried out with a Bond-MaX autostainer (Vision BioSystems) using the accompanying Bond Refine Polymer Detection Kit. PRL-1 antibody (Bethyl Laboratories) was used at a dilution of 1:250 with an incubation time of 20 min. PRL-2 (Bethyl Laboratories) was used at a dilution of 1:200 with an incubation time of 60 min. Staining (relative to background) was classified into four categories (0, negative; 1, weak; 2, moderate; or 3, strong) by a single pathologist (G.H.).

### Transient Transfection Using siRNAs

The PRL-1 targeting siRNAs used were as follows: PRL-1p (SMARTpool; Dharmacon), PRL-1a (target CCAACCAATGCGACCUUAAACAAAT), and PRL-1b (target TCAAAGATTCCAACGGTCATAGAAA). The PRL-2 targeting siRNAs used were as follows: PRL-2p (SMARTpool; Dharmacon), PRL-2a (target CGATTACGCTTCAGAGATA), and PRL-2b (target GCATTGCTGTGTTTCAGTAG). Pooled synthetic nontargeting siRNA was obtained through Dharmacon.

Cells were transiently transfected according to the manufacturer's protocol using LipofectAMINE 2000 (Invitrogen). Briefly, cells used in migration and soft agar assays were transfected in six-well plates as follows: (a) cells (0.3 × 10<sup>6</sup> per well) were plated in a six-well plate in medium without antibiotics 24 h before transfection, (b) siRNA (4 µL of a 20 µmol/L solution) was added to 100 µL Opti-MEM reduced serum medium (Invitrogen), (c) LipofectAMINE 2000 (3 µL) was added to 100 µL Opti-MEM reduced serum medium and incubated for 5 min at room temperature, (d) the siRNA and LipofectAMINE 2000 were mixed and incubated at room temperature for 20 min, and (e) the 200 µL mixture was added to the cells (2,000 µL total volume per well). For the cell proliferation assay, cells were plated in 96-well plates (2,500 per well) and transfected in a similar manner using 2 µL of a 2 µmol/L siRNA solution and 0.3 µL LipofectAMINE 2000 per well with a final volume of 100 µL/well. Cells used in phospho-Akt/phospho-Erk studies were plated in 100 mm dishes (1.25 × 10<sup>6</sup> per dish) and transfected using RNAiMAX (Invitrogen) transfection reagent according to the manufacturer's protocol with a final siRNA concentration of 20 nmol/L.

Total siRNA concentration in the experiments was kept constant; therefore, cells treated with both PRL-1 and PRL-2 siRNAs in combination were treated with the appropriate dilution of a PRL-1 and PRL-2 pooled siRNA.

#### Reverse Transcription-PCR

Total RNA from cell pellets was isolated using the NucleoSpin RNA II isolation kit (BD Biosciences). Total RNA (1  $\mu$ g) was used for reverse transcriptase reactions (20  $\mu$ L total volume), which were carried out using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). PCR amplification using 1  $\mu$ L reverse transcription product consisted of 25 cycles for PRL-1 and PRL-2 and 30 cycles for PRL-3. The sequences of specific primers were as follows: PRL-1 5'-CACAATCCAACCAATGCGAC-3' (forward) and 5'-GGAATCTTTGAAACGCAGCC-3' (reverse), PRL-2 5'-AGATCTCCTATGAGAACATGC-3' (forward) and 5'-TTGGAATTGAACGCTCCC-3' (reverse), and PRL-3 5'-CCCGGTGGAGGTGAGCTACAA-3' (forward) and 5'-TTGCTGTTGATGGCTCCGC-3' (reverse).

#### Cell Proliferation Assay

At 24, 48, and 72 h after siRNA transfection, cells were fixed with 10% trichloroacetic acid for 1 h at 4°C. Following fixation, cells were washed with water and then stained with a 0.04% sulforhodamine B solution for 1 h. Cells were then washed with a 1% acetic acid solution. When dry, 50 mmol/L Tris-HCl was added to each well and incubated for 15 min. Absorbance at 570 nm was quantified using a plate reader (BioTek). Three biological replicates (each done in triplicate) were done.

#### Migration Assays

Migration assays were done using the microliter-scale radial monolayer assay as described by Valster et al. (21). Briefly, 10-well Teflon-coated slides (CSM) were coated with 10  $\mu$ g/mL laminin and 0.1% bovine serum albumin. Cells were seeded through a cell sedimentation manifold (CSM) at 3,000 per well to establish a circular confluent monolayer at the center of the substrate-coated well. Six hours after seeding, a circle of best-fit that circumscribed the cells was drawn. The cells were allowed to migrate out over a 24-h period (microns per hour). Measurements are taken using a DM IRE2 inverted microscope (Leica Microsystems), digitized using a Spot camera (Diagnostic Instruments), and image analysis was carried out using Scion Image software (Scion).

#### Soft Agar Assay

Cells were treated with siRNA for 24 h, trypsinized, mixed with Difco agar (final concentration, 0.26%; BD Biosciences) and RPMI 1640 containing 10% fetal bovine serum, and overlaid onto an underlayer of 0.45% Difco agar containing the same medium in a 35-mm gridded Petri dish. Cells (3,000 per Petri dish) were seeded and allowed to grow for 14 days (MIA PaCa-2) or 21 days (PANC-1) before counting the number of colonies (defined as  $\geq 50$  cells).

#### Statistical Analysis

The  $\chi^2$  test with Yates' correction (two-tailed) was used to analyze the difference in PRL immunohistochemical staining between the pancreatic tumors and the normal

adjacent tissue. Other *P* values indicated in the figure legends were calculated using Student's *t* tests (two-tailed). *P* < 0.05 was considered statistically significant.

## Results

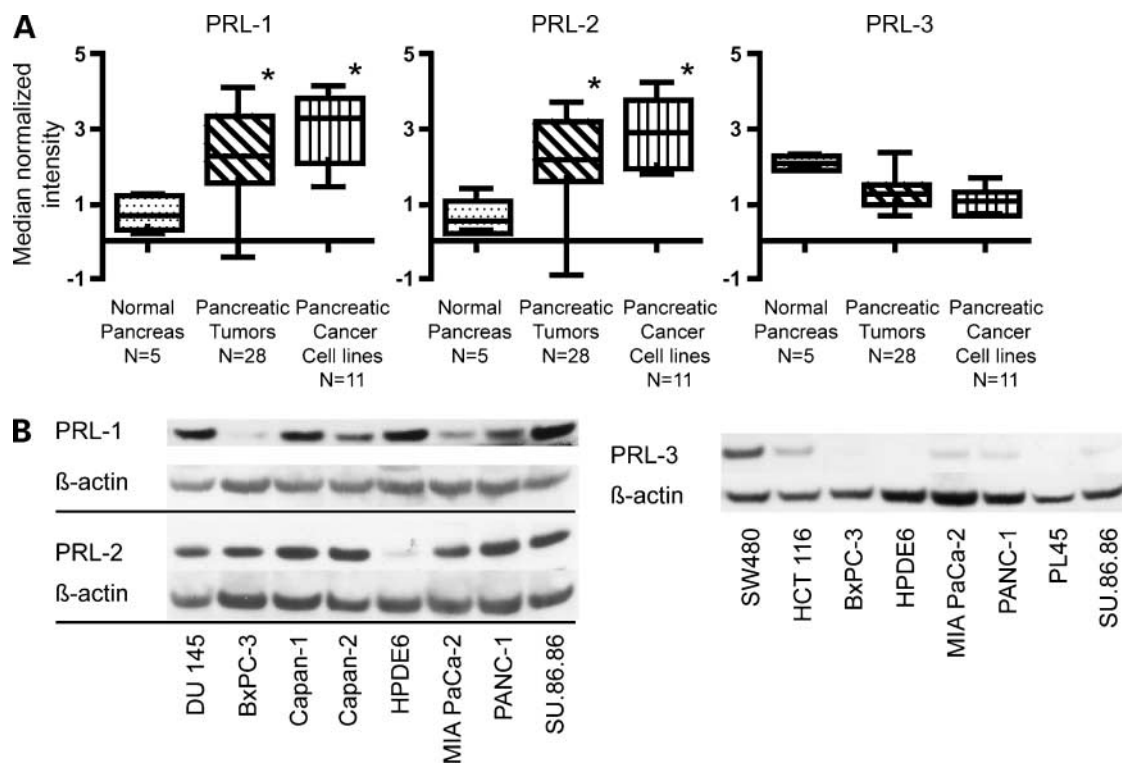
### PRL Expression in Pancreatic Tumor Samples and Cell Lines

Expression of PRL-1, PRL-2, and PRL-3 mRNA was analyzed in 5 normal pancreas samples, 28 tumor samples taken directly from patients, and 11 pancreatic adenocarcinoma cell lines using oligonucleotide expression arrays (Fig. 1A). For each sample, probe intensities for all three PRL genes were median normalized to the respective array, with the resulting relative expression converted to log<sub>2</sub> scale. Of the 28 patient tumor samples, 16 (57%) exhibited >2.5-fold expression of PRL-1 over the median expression of the normal pancreas samples. Likewise, PRL-2 was >2.5-fold overexpressed in 18 (64%) tumor samples. Moreover, it was determined that 13 (46%) tumor samples expressed both PRL-1 and PRL-2 >2.5-fold over the respective normal pancreas median values. PRL-3, however, was not found up-regulated in any of the 28 tumors samples when compared with the normal pancreas. Pancreatic cancer cell lines had a similar pattern of PRL expression, where PRL-1 and PRL-2 (but not PRL-3) were expressed at higher levels in comparison with normal pancreas.

To evaluate PRL expression at the protein level, Western blotting using antibodies against PRL-1, PRL-2, or PRL-3 was done with protein isolated from a panel of cancer cell lines. PRL-1 and PRL-2 were detectable in most of the pancreatic cell lines tested (Fig. 1B). The prostate cancer cell line DU145, described as having high mRNA levels of PRL-1 and PRL-2 (22, 23), exhibited similar levels of PRL-1 and PRL-2 to the pancreatic cancer cell lines tested. HPDE6 (an immortalized normal pancreatic ductal epithelial cell line) exhibited the lowest levels of PRL-2 but has relatively high level of PRL-1. Interestingly, PRL-2 was more uniformly expressed across this panel of cell lines in comparison with the varied expression of PRL-1. Relative PRL-3 protein levels in pancreatic cell lines (Fig. 1B) were compared with that of the colon cancer cell lines SW480, reported as having moderate levels of PRL-3 mRNA (10), and HCT116, which expresses low levels of PRL-3 protein (5). All pancreatic cancer cell lines had low to nondetectable levels of PRL-3 protein as predicted from our expression array data.

### PRL Protein Expression in Patient Tumor Samples

Because PRL-1 and PRL-2 transcript levels were differentially expressed in patients' tumor samples compared with normal pancreas, we therefore sought to evaluate PRL-1 and PRL-2 protein expression in patients' tumor samples. To compare protein levels of PRL-1 and PRL-2 between pancreatic tumors and normal pancreas tissue, immunostaining was done on pancreatic TMA slides. As described in Materials and Methods, TMA slides containing 34 tumor samples, with matched normal tissue arrayed adjacent to tumor, were stained and scored as negative (0), weak (1), moderate (2), or strong (3). Of the 34 tumor (with



**Figure 1.** Expression levels of PRL-1, PRL-2, and PRL-3 in pancreatic tissues and cell lines. **A**, PRL-1, PRL-2, and PRL-3 levels in 5 normal pancreas samples and 28 pancreatic tumor samples taken directly from patients. Intensities as determined by expression arrays were median normalized and set to  $\log_2$  values. Box and whiskers, median (middle line), 25th and 75th percentiles (boxed portion), and minimum and maximum of the normalized intensities. \*,  $P < 0.05$  versus normal pancreas samples. **B**, Western blot showing detectable PRL-1 and PRL-2 but not PRL-3 protein levels in cancer cell lines. HPDE6 represents a noncancerous pancreatic ductal cell line.  $\beta$ -Actin serves as a loading control.

matched normal) sets, 33 were evaluable for PRL-1 staining and 31 for PRL-2 staining. For further analysis, specimens were grouped into two categories. Those that exhibited moderate or strong staining were classified as positive, and those with weak or negative immunostaining results were classified as negative (Fig. 2A). In cases where the normal adjacent staining was negative, 11 of 33 (33%) patient tumor samples stained positive for PRL-1. Similarly, for PRL-2, 8 of 31 (26%) patient samples exhibited positive tumor staining, whereas the adjacent normal stained negative. Overall, staining was determined to be significantly stronger in the tumors than in the accompanying adjacent normal for both PRL-1 ( $P = 0.003$ ) and PRL-2 ( $P = 0.034$ ). Representative differential staining of PRL-1 is shown in Fig. 2B, where carcinoma and normal ducts are present in the same section. Regardless of which PRL antibody was used, PRL staining of tumor tissue was mainly restricted to the transformed ductal epithelium and not observed in adjacent stroma (Fig. 2B). Staining was cytoplasmic with occasional nuclear staining (data not shown). Strong staining of PRL-1 and PRL-2 was also observed in vascular wall smooth muscle (data not shown), similar to that which has been recently reported for PRL-1 and PRL-2 immunostaining in colonic adenocarcinoma (24).

#### PRL Targeting siRNAs Specifically Repress Respective PRL Transcript

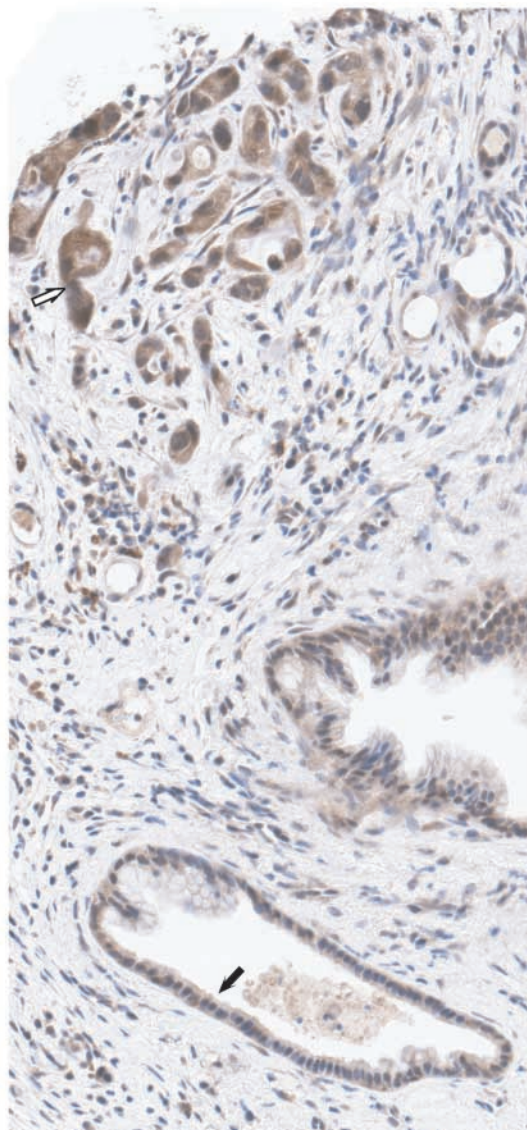
Transient knockdown of PRL-1 and/or PRL-2 in MIA PaCa-2 (Fig. 3) and PANC-1 (data not shown) cells was achieved using synthetic siRNAs. Pooled PRL-1 targeting siRNAs (PRL-1p) did not affect levels of the closely related PRL-2 and PRL-3 transcripts, and likewise pooled PRL-2 targeting siRNA (PRL-2p) did not disrupt PRL-1 and PRL-3 expression (Fig. 3A). PRL-1 and PRL-2 protein levels were reduced by 24 h and remained suppressed through 72 h (Fig. 3B). This result also showed the high specificity of the PRL-1 and PRL-2 antibodies used in this study.

#### PRL Targeting siRNAs Influence Cellular Proliferation, Migration, and Colony-Forming Efficiency

To determine if PRL knockdown has an effect on pancreatic cancer cell growth, a sulforhodamine B assay was used. Sulforhodamine B is a protein dye used to quantify total biomass as a measure for cell proliferation. siRNAs targeting either PRL-1 (PRL-1p) or PRL-2 (PRL-2p) had little effect on cellular proliferation; however, when PRL-1 and PRL-2 were knocked down in combination, 30% and 27% reductions in cell proliferation compared with nontargeting siRNA-treated cells were observed after 72 h in PANC-1 and MIA PaCa-2 cells, respectively (Fig. 4A).

**A** Summary of Pancreatic Cancer Tissue Staining

Staining intensity	PRL-1		PRL-2	
	Tumor	Normal	Tumor	Normal
Negative = 0	11	24	6	17
Weak = 1	9	7	14	11
Moderate = 2	10	1	9	3
Strong = 3	3	1	2	0
Total Negative	20	31	20	28
Total Positive	13	2	11	3
	PRL-1 (N=33)		PRL-2 (N=31)	

**B**

**Figure 2.** Protein levels of PRL-1 and PRL-2 in clinical tumor samples as determined by immunohistochemistry. **A**, summary of PRL-1 and PRL-2 staining intensities of pancreatic tumor samples with matched adjacent normal counterparts. **B**, representative PRL-1 staining of a section containing near-normal ductal epithelium (solid arrow) and invasive adenocarcinoma (hollow arrow) at  $\times 100$  magnification.

As stated previously, PRL-1 and PRL-3 have been shown to influence migration. Using a two-dimensional migration assay, as described in Materials and Methods, cell migration rates for MIA PaCa-2 and PANC-1 cells treated with PRL-1 and/or PRL-2 targeting siRNAs (PRL-1p and PRL-2p) were quantitatively compared with cells treated with nontargeting siRNA (Fig. 4B). A statistically significant difference in the rate of migration was observed in both cell lines when knockdown of both PRL-1 and PRL-2 in combination was compared with the nontargeting siRNA-treated cells.

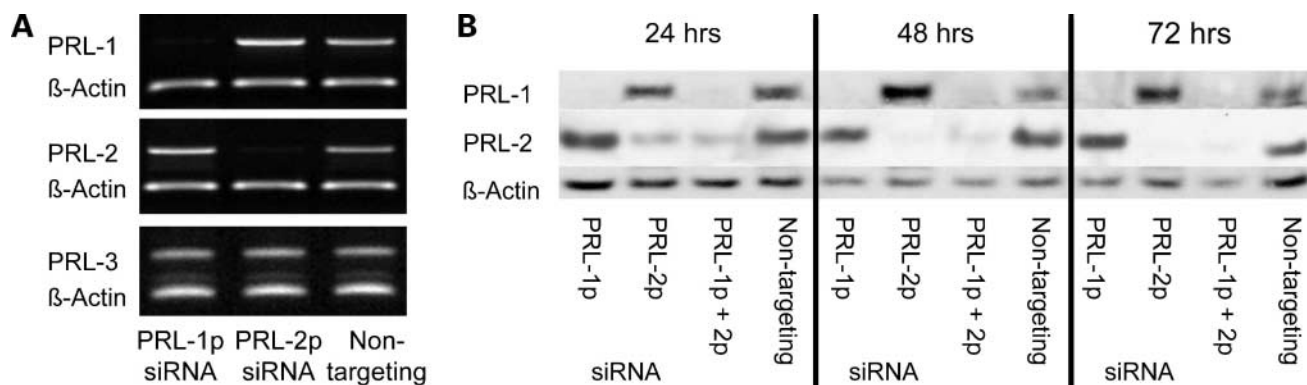
Finally, we examined whether PRL knockdown abrogated the ability of MIA PaCa-2 and PANC-1 cells to form colonies in soft agar. Cells treated with siRNAs targeting PRL-1, PRL-2, PRL-1 and PRL-2, or nontargeting siRNA were plated in soft agar and allowed to grow for 14 days (MIA PaCa-2) or 21 days (PANC-1) before counting the number of colonies ( $\geq 50$  cells). With both PRL-1 and PRL-2 knocked down in combination, colony-forming efficiency was reduced by 65% and 90% in MIA PaCa-2 and PANC-1 cells, respectively, when compared with nontargeting siRNA-treated cells (Fig. 4C).

#### PRL Knockdown Alters Akt and Erk Signaling in Pancreatic Cancer Cells

Given that PRL knockdown had the greatest affect on anchorage-independent growth in our assays, and phosphatidylinositol 3-kinase/Akt signaling has been reported as critical for anchorage-independent survival and growth (25), we evaluated the effect of PRL targeting siRNAs on Akt phosphorylation by Western blot. To verify that differences in Akt phosphorylation were not due to nonspecific effects of the pooled siRNA, individual combinations of siRNAs were used as indicated in Fig. 5. On pathway induction by addition of serum, PANC-1 and MIA PaCa-2 cells treated with PRL-1 and PRL-2 targeting siRNAs in combination exhibited reduced levels of phospho-Akt compared with nontargeting siRNA-treated cells (Fig. 5A). On quantification of the Western blots by densitometry, PRL inhibition affected Akt activation to a greater extent in PANC-1 cells than in MIA PaCa-2 cells (Fig. 5B). Additionally, knockdown of PRL-1 or PRL-2 alone was insufficient to inhibit activation of Akt in MIA PaCa-2 cells. In contrast to Akt activation, levels of phospho-Erk were increased in PANC-1 but decreased in MIA PaCa-2 cells with PRL knockdown.

#### Discussion

Comprehensive and multimodality experimental analysis using expression microarrays on pancreatic cancer cell lines, tumor samples taken directly from patients, and normal pancreas cells showed that PRL-1 and PRL-2 but not PRL-3 were expressed at higher levels in a large subset of pancreatic cancer cell lines and patient tumor samples than in normal pancreas. The PRL mRNA expression profiles of cell lines observed here are consistent with a previous report comparing PRL mRNA levels across a variety of cell lines (including a limited number of



**Figure 3.** Knockdown of PRL expression by specific siRNA oligonucleotides. MIA PaCa-2 cells were transfected with the siRNAs as described in Materials and Methods. **A**, reverse transcription-PCR detection of PRL mRNA levels 24 h after siRNA transfection in MIA PaCa-2 cells. **B**, Western blotting detection of PRL-1 and PRL-2 protein levels at 24, 48, and 72 h after siRNA treatment.

pancreatic cancer cell lines; ref. 26). Western blotting using PRL-1 and PRL-2 antibodies showed detectable levels of PRL-1 and PRL-2 protein in cell lines. The HPDE6 cell line, as a near-normal immortalized human pancreatic ductal epithelial cell line, expressed the lowest levels of PRL-2 but surprisingly contained high levels of PRL-1. HPDE6 cells were established through transduction of the HPV16-E6E7 genes into primary cultures of normal pancreatic duct epithelial cells (20). Consistent with the presence of E6 gene product, there is both low expression of wild-type p53 and lack of functional p53 in HPDE6 cells. Functional p53 levels have been shown to affect PRL-1 levels in cell culture systems and might be the cause of high expression in this cell line (27).

Immunostaining of tumor samples showed that a subset of pancreatic tumors expressed significantly higher levels of PRL-1 and/or PRL-2 in neoplastic ductal cells than in normal ductal cells by semiquantitative analysis, consistent with mRNA expression levels observed in patient tumors through microarray. Interestingly, of the 11 tumor samples that showed differential staining for PRL-1, 9 samples had evaluable staining for PRL-2, with differential expression for PRL-2 in 5 of the 9 samples. With the high level of sequence identity shared among the PRL phosphatases, it has been suggested that a polyclonal antibody (such as the ones used in this study) would most likely see total levels of PRL protein and not specific PRL proteins (28). The PRL-1 and PRL-2 antibodies used in this study showed high specificity in Western blotting (Fig. 3; Supplementary Fig. S1).<sup>5</sup> Although it is possible that the antibodies might not be entirely specific to their respective PRL proteins in the immunostaining of the TMA, we believe the correlation between the moderate overexpression of both PRL-1 and PRL-2 protein together in tumor samples is of biological significance due to similar supporting mRNA data from the oligonucleotide expression arrays, where pancreatic tumors

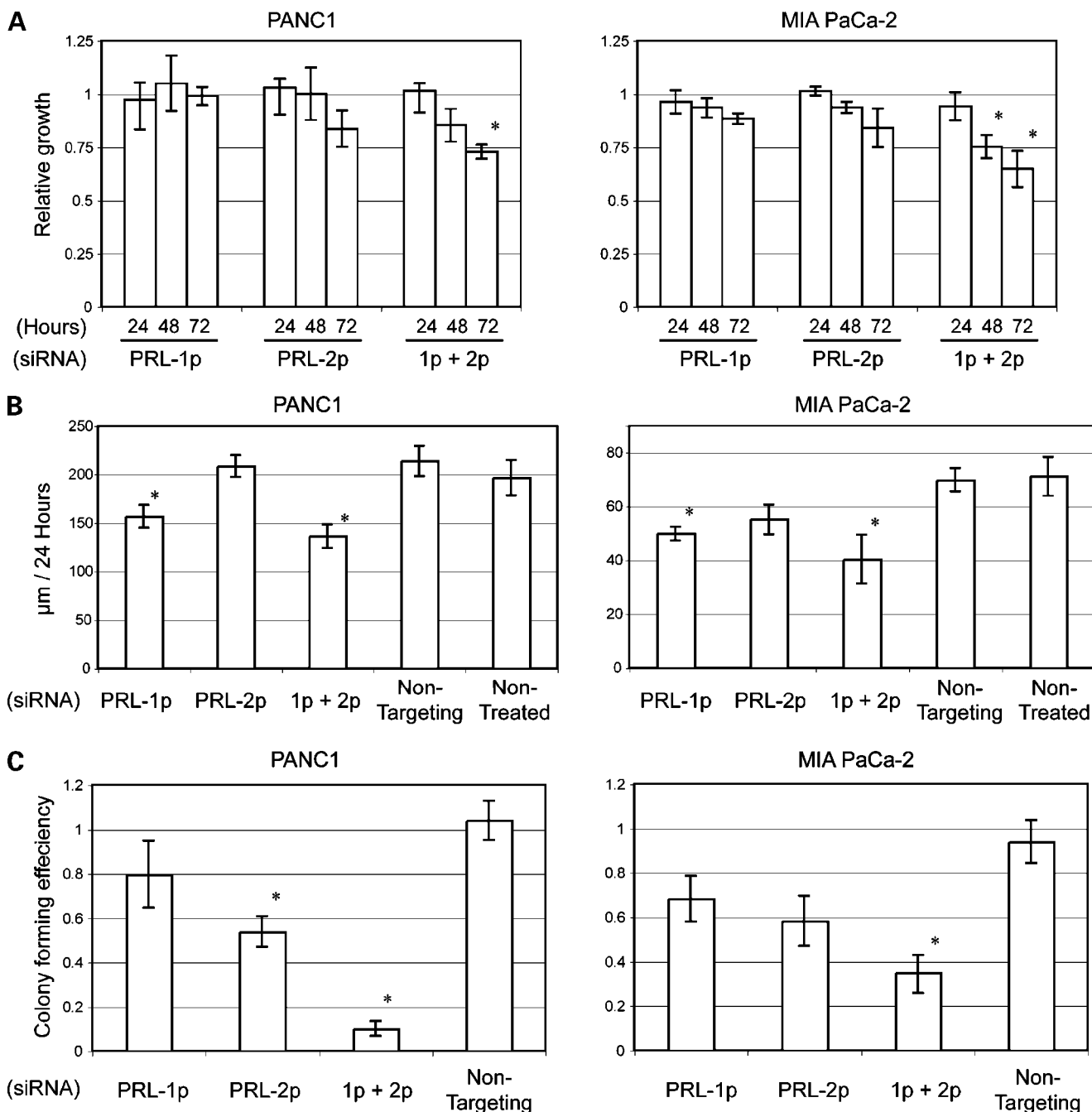
with high levels of PRL-1 transcript also had high levels of PRL-2. Regardless of the specificity of these antibodies with respect to immunohistochemistry, we conclude that PRL phosphatase expression at the protein level was elevated moderately to strongly in ~30% of the pancreatic tumors examined. Based on our results, PRL-3 (the PRL phosphatase most commonly associated with metastasis; refs. 3, 4, 10, 29) is not overexpressed in tumor samples compared with normal pancreas (mRNA) and expressed at low protein levels in pancreatic cancer cell lines derived from both primary and metastatic sites. It should be noted, however, that PRL-3 might be predominantly expressed in important tumor components that constitute a small percentage of the tumor (such as the vasculature; refs. 12, 30). Therefore, RNA isolated from total tissue, such as reported here, could underestimate the importance of PRL-3.

Because all three PRL proteins may be closely related by homology and function in neoplasia, it is possible that knockdown of an individual PRL protein would not manifest any effect on tumorigenicity, as a different PRL protein might compensate. We therefore hypothesized that siRNAs targeting both PRL-1 and PRL-2 in combination would have antitumorigenic effects on pancreatic adenocarcinoma cells. Using MIA PaCa-2 and PANC-1 pancreatic cancer cell lines, specific down-regulation of PRL-1 and/or PRL-2 was achieved with synthetic siRNA targeting PRL-1 or PRL-2. We found that double knockdown of both PRL-1 and PRL-2 in combination had a more significant effect on cellular growth and migration than knockdown of PRL-1 or PRL-2 alone, suggesting overlapping roles of PRL proteins. The effect of PRL levels of cellular proliferation appears to be cell line specific. Our data resemble those of Achiwa et al. (16), where moderate growth inhibition was observed in A549 non-small lung cancer cells expressing PRL-1 short hairpin interfering RNA. A more dramatic decrease in cellular growth following PRL-3 siRNA treatment has been observed in the ovarian cancer cell lines A2780, SKOV-3, and IGROV-1 (5). In contrast, B16-BL6 and DLD-1 cells show no difference in cell growth following relevant PRL knockdown (10, 14).

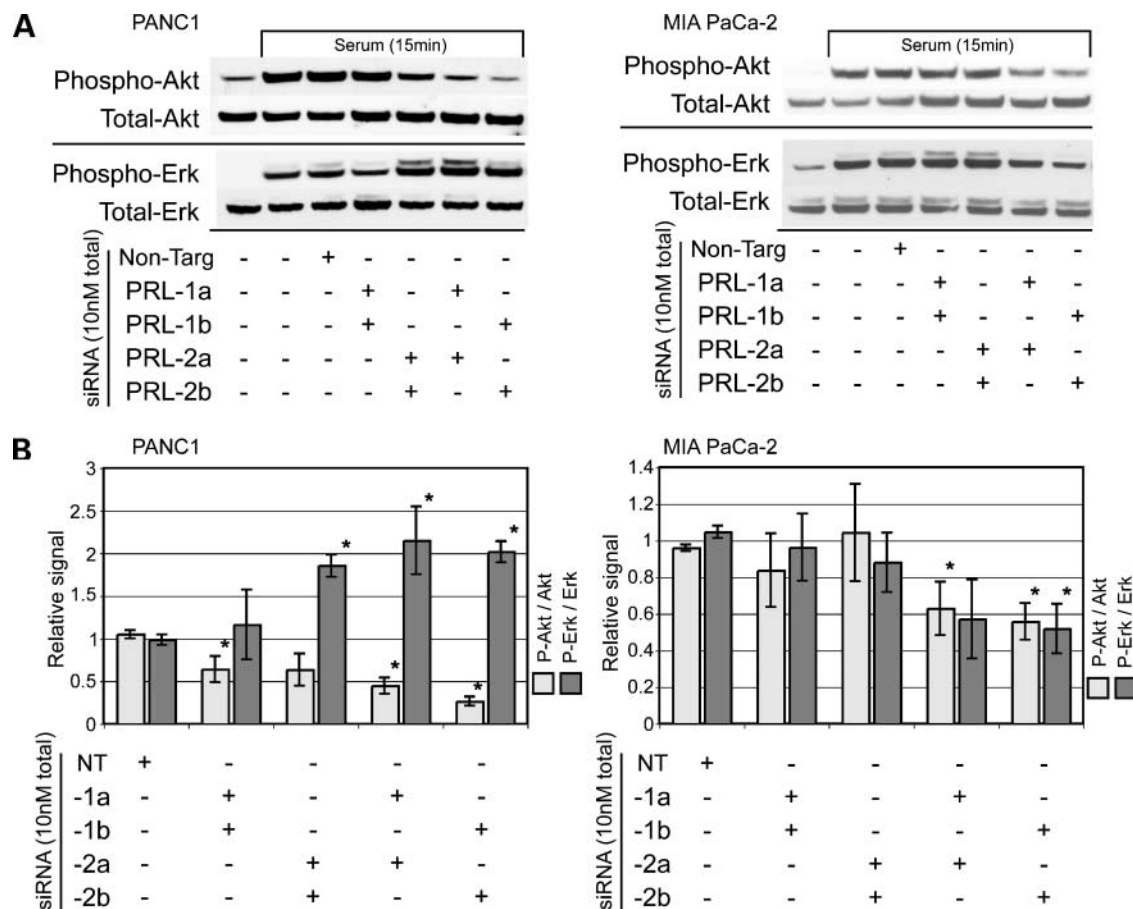
<sup>5</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

PRL-3 has been shown previously to play an important role in cancer cell colony formation in mouse models as well as in soft agar colony-forming assays (10, 13, 17). Our results show for the first time that PRL-1 and PRL-2 might

perform similar functions in pancreatic cancer cells, as siRNA knockdown of PRL-1 or PRL-2 and especially PRL-1 and PRL-2 together significantly reduced the ability of MIA PaCa-2 and PANC-1 cells to form colonies in soft agar.



**Figure 4.** PRL targeting siRNAs affect cellular growth, migration, and soft agar colony formation. **A**, MIA PaCa-2 and PANC-1 cells were treated with siRNAs targeting either PRL-1, PRL-2, or both for 24-, 48-, and 72-h periods as described in Materials and Methods. Growth as determined by sulforhodamine B assay was set relative to nontargeting siRNA-treated cells ( $n = 3$ ). \*,  $P \leq 0.05$ . **B**, two-dimensional migration rates on laminin-coated sides for MIA PaCa-2 and PANC-1 cells were determined as described in Materials and Methods ( $n = 3$ ). \*,  $P \leq 0.05$  versus nontargeting control. **C**, PRL knockdown affects anchorage-independent growth of MIA PaCa-2 and PANC-1 cells. Relative colony (> 50 cells) numbers per 35 mm<sup>2</sup> dish were set to those of nontreated control cells. The assay was done in biological triplicates (soft agar plating in duplicate for each biological replicate;  $n = 3$ ). \*,  $P < 0.05$  versus nontargeting control.



**Figure 5.** PRL targeting siRNAs inhibit Akt phosphorylation. **A**, cells were transfected as described in Materials and Methods and grown to 80% confluency (48 h). Cells were then serum starved for 12 h and Akt phosphorylation was induced by addition of serum (5% fetal bovine serum). Cell lysates and supernatants were collected as described in Materials and Methods. **B**, levels of phosphorylated signaling proteins were quantitated via densitometry and normalized to total expression of the respective proteins. Values expressed are relative to serum-induced nontargeting siRNA-treated cells ( $n = 3$ ). \*,  $P \leq 0.05$ .

Due to the importance of Akt signaling in anchorage-independent growth and survival, we hypothesized that PRL knockdown would affect levels of phospho-Akt. We then evaluated levels of phospho-Erk in pancreatic cancer cell lines, as phospho-Erk has been reported to be affected by PRL expression and inhibition in other cell types (15, 17). We find that knockdown of both PRL-1 and PRL-2 in combination results in decreased levels of serum-induced phospho-Akt in MIA PaCa-2 and, to a greater extent, in PANC-1 cells. It should be noted that the Akt antibodies used in this study recognize all three Akt isoforms (Akt1, Akt2, and Akt3) and that PANC-1 cells have amplified Akt2, with accompanying high levels of Akt2 mRNA and protein (31). Taken together, we suspect that PANC-1 cells might rely heavily on activated Akt2 under conditions of anchorage-independent growth and that the dramatic inhibition of colony formation by PRL targeting siRNAs in PANC-1 cells is at least partially due to suppressed levels of phospho-Akt. Interestingly, it has been shown that sustained Erk phosphorylation can be involved

in tumor growth inhibition (32). Therefore, it is possible that the increased Erk phosphorylation observed in PANC-1 cells might be part of the mechanism leading to the increased efficacy of PRL targeting siRNAs in soft agar colony formation of PANC-1 cells compared with MIA PaCa-2. During the preparation of this article, it was reported that increased levels of phospho-Akt (Ser<sup>473</sup>) were observed in DLD-1 cells ectopically expressing PRL-3 versus cells expressing catalytically inactive PRL-3 (19). This finding combined with our results suggests that all three PRL proteins might perform similar functions in Akt regulation.

Based on our results, PRL phosphatases, with additional characterization, will likely be found to play an important role in anchorage-independent growth in a subset of pancreatic tumors. Therefore, targeting PRLs therapeutically could potentially disrupt metastasis and influence survival. Interestingly, using a cell-based screening assay, MacKeigan et al. recently identified PRL-2 as a gene involved in cell survival (33). Ongoing research in our



laboratory includes evaluating PRL knockdown/inhibition as a possible mechanism to sensitize cancer cells to standard chemotherapeutic agents and biologics.

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