Periostin, a matrix specific protein, is associated with proliferation and invasion of pancreatic cancer

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Abstract. Overexpression of periostin is present in various malignant tumors and correlates with disease progression. However, its clinicopathological significance in pancreatic cancer is currently not known. Expression of periostin was analyzed by RT-PCR and Western blotting in pancreatic cancers and cell lines. Using immunohistochemistry, expression of periostin in pancreatic cancers was evaluated according to factors influencing overall survival with Kaplan-Meier analysis. Ectopic expression of periostin was used to examine the effects of periostin on proliferation and invasiveness of pancreatic cancer cells *in vitro*. There was no detectable periostin mRNA and protein expression in the 4 pancreatic cell lines. Expression of periostin was found to be up-regulated in pancreatic cancer compared to the adjacent tumor free (TF) tissues by Western blotting. The positive ratio of periostin expression in the neoplastic stroma was significantly correlated with the depth of invasion (p=0.007) and lymph node metastasis (p=0.027). Survival analysis showed that stromal or epithelium expression of periostin was associated with poor survival (p=0.035, p=0.022, log-rank test, respectively). *In vitro* studies showed that periostin was able to promote proliferation and invasiveness of pancreatic cancer cells. These results suggest that periostin may be involved in the progression and invasion of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most malignant gastrointestinal tumors, accounting for over 30,000 deaths yearly in the United States alone (1). In China, pancreatic cancer is the sixth leading cause of malignancy-related death, with an overall cumulative 5-year survival rate of 1-3% (2). At the time of diagnosis, only 10-15% of these cancers are typically found to be resectable (1), due to the presence of locally advanced disease or distant metastases. Effective systemic therapy for advanced or recurrent disease is currently unavailable (3). Therefore, research efforts have been increasingly focused on elucidation of molecular targets and on development of novel therapeutic approaches for this disease.

Periostin (PN), originally designated osteoblast-specific factor 2, has a typical signal peptide sequence at its N-terminus and four repeated domains. Each of the latter shares a structural homology to insect fasciclin I (4,5). The human periostin is found on chromosome 13q (6), which encodes a protein of 811 amino acids with a molecular weight of 90.2 kDa (4). Periostin is known to play a role in bone and tooth formation and maintenance of structure in these tissues (5). Periostin-null mice exhibit incisor enamel defects, dwarfism (7) and impaired scar formation in the infarct zone after acute myocardial infarction (8).

The expression of periostin is reported to be associated with both tumor suppression and tumor progression in a number of human cancers, and its role in tumorigenesis remains controversial. In human tumors, including head and neck cancer (9), breast cancer (10,11), pancreatic cancer (12,13), non-small cell lung cancer (NSCLC) (14) and colon cancer (15), periostin expression has been shown to be up-regulated; however, this is not true for bladder cancer (16). Re-expression of periostin in colon cancer cells or in head and neck cancer cells promoted tumorigenicity in nude mice (9,15). In addition, Erkan *et al* demonstrated that periostin was produced solely by pancreatic stellate cells (PSCs), which created a tumor-supportive microenvironment (12). Specifically, periostin is suggested to promote the invasiveness or growth rate and confer resistance to hypoxia in pancreatic cancer cells via the α6ß4 integrin complex (17). Accordingly, periostin is reported to be a ligand for αvß3 and αvß5 integrins inducing integrin-dependent cell adhesion and motility (18).

In this study, we relied on semi-quantitative immunohistochemistry to investigate periostin expression in pancreatic cancer and to correlate it with clinicopathological features of this tumor. Next, we also examined the role of periostin in pancreatic cancer growth and progression *in vitro*. Based on these studies, we verified that periostin has a critical role in pancreatic cancer progression.

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Materials and methods

Study population, follow-up and TMA construction. The study was approved by the ethics committee of the chamber of physicians of Ruijin Hospital, Shanghai, China. The cohort of study population and tissue microarray (TMA) construction were as described previously (19). Briefly, 94 patients who underwent potentially curative surgery for pancreatic cancer were recruited for this study between January 1, 2002 and December 31, 2007. All cases were staged according to the guidelines of the International Union Against Cancer (2002) (20). Patient follow-up was obtained through reviewing the hospital records, contacting with family members of the patients, or the Cancer Registry of Shanghai until November 1, 2008, with a median observation time of 20 months (range from 3 to 45 months). Original paraffin-embedded specimens were used to construct a TMA. Duplicates of 1-mm diameter cylinders from tumor center for each case and 1 1.0-mm tissue core from 'normal' adjacent pancreas for 22 patients were punched using an automated tissue arrayer (Beecher Instruments, Sun Prarie, Wisconsin). Thus, a tissue microarray block containing 210 cylinders was constructed.

Immunohistochemistry and scoring. The TMA slides were analyzed by immunostaining using the Dako Envision system (Dako, Carpinteria, CA) as described previously. Following quenching of endogenous peroxidase activity and blocking of non-specific binding, the slides were incubated with polyclonal antibody to periostin (Abcam, Cambridge, UK) in 1:200. Assessment for the abundance of periostin expressing cells was performed by two experienced pathologists (J.X. and Y.F.). Neoplastic epithelial cell staining was considered positive if the chromogen was detected in at least 10% of the cells within a microscopic field. Staining intensity included 4 scores: no staining (score 0), weak staining (score 1), moderately positive (score 2), and strongly positive (score 3). Scores 0 and 1 were regarded as negative and scores 2 and 3 as positive. Focal, multifocal, or diffused stainings were also scored. For neoplastic stroma, the immunohistochemical staining of anti-periostin were evaluated and classified by the area of positive-staining stroma into negative (<25%) and positive (>25%).

Cell lines and tissue sample. The four established pancreatic cancer cell lines (AsPC-1, SW-1990, BxPC-3 and Panc-1) in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RMPI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 units/ml of penicillin-streptomycin (Invitrogen) under the following conditions: 5% CO_2 in air at 37˚C.

RT-PCR. RT-PCR were performed as previously described (21). Briefly, after cDNA template was synthesized according to the manufacturer's protocol, PCR was performed on 2 μl of RT products in a 25-μl reaction mixture. An internal control gene, ß-actin, was included to control for the amount of RNA. The amplification products were then electrophoresed on 1.5% agarose gels.

Western blotting. Protein was isolated from exponentially growing cells and fresh-frozen pancreatic tissue samples. After protein concentrations measured, the proteins were transferred to polyvinylidene fluoride membranes and blotted with human monoclonal anti-periostin (R&D Systems, Minneapolis, MN). Immunoreactive bands were detected by using chemiluminescence (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Construction of recombinant adenoviruses and cell transfection. Periostin full-length cDNA was cloned into a pDC316 carrier plasmid (Benyuanzhengyang, Beijing, China) to generate pDC316-PN. The plasmid pDC316-PN and the skeleton plasmid pBHG-fiber5/35 (Benyuanzhengyang) were co-transfected into HEK293 cells, and which yielded the recombinant Ad5/F35-PN plasmid. Successful recombination was confirmed by observation of cytotoxicity as well as by PCR. The control vector Ad5/F35-Null was obtained by the same methods. Cell infections were done by using 0-100 multiplicities of infection (MOI) per target cell. The recombinant adenovirus Ad5-PN was used after purification, characterization, and titration of the viral infectivity by plaque assay.

Proliferation assay. Proliferation of cells infected with Ad5-PN or Ad5-null at MOI of 100 pfu/cell was determined by MTT assay at 12, 24, 48, and 72 h. The mock reaction contained no virus. Absorption was measured at 490 nm. The experiment was repeated three times.

Soft agar colony formation assay. Assays of colony formation in soft agar were done in 6-well plates with a base layer containing 0.6% agar. Cells ($1x10^4$) were plated onto the previously prepared underlayers (1.2% agar). The plates were incubated at 37˚C in a humidified 5% CO_2 atmosphere for 10 days, and afterwards, colonies >50 μm were counted 15 days after plating.

Cell migration and invasion assay. Cell migration and invasion assays were conducted by using 8-μm pore size transwell chambers (Chemicon). After cells ($1.5x10^6$ cells/ml) were placed in the upper compartment, the lower chambers of the transwell were filled with 500 μl of medium with 10% FBS as a chemoattractant. The plates were incubated at 37˚C for 24 and 48 h. Non-invasive cells and matrigel were removed by scraping with sterile cotton swabs. After stained with hematoxylin, the migrated or invaded cells were counted under a microscope at x400 magnification. Assays were done thrice, and 10 fields were randomly selected and counted for each assay.

Statistical methods. All statistical analyses were performed with SPSS13.0 software (SPSS, Inc., Chicago, IL). To compare the mean difference in various clinicopathological factors between groups, the χ^2 test and Fisher's exact test were used. Survival curves were calculated according to the method of Kaplan and Meier and p-values were evaluated by the log-rank test. The difference between means was performed with Student's t-test. For all analyses, a two-sided p-value of <0.05 was defined as statistically significant.

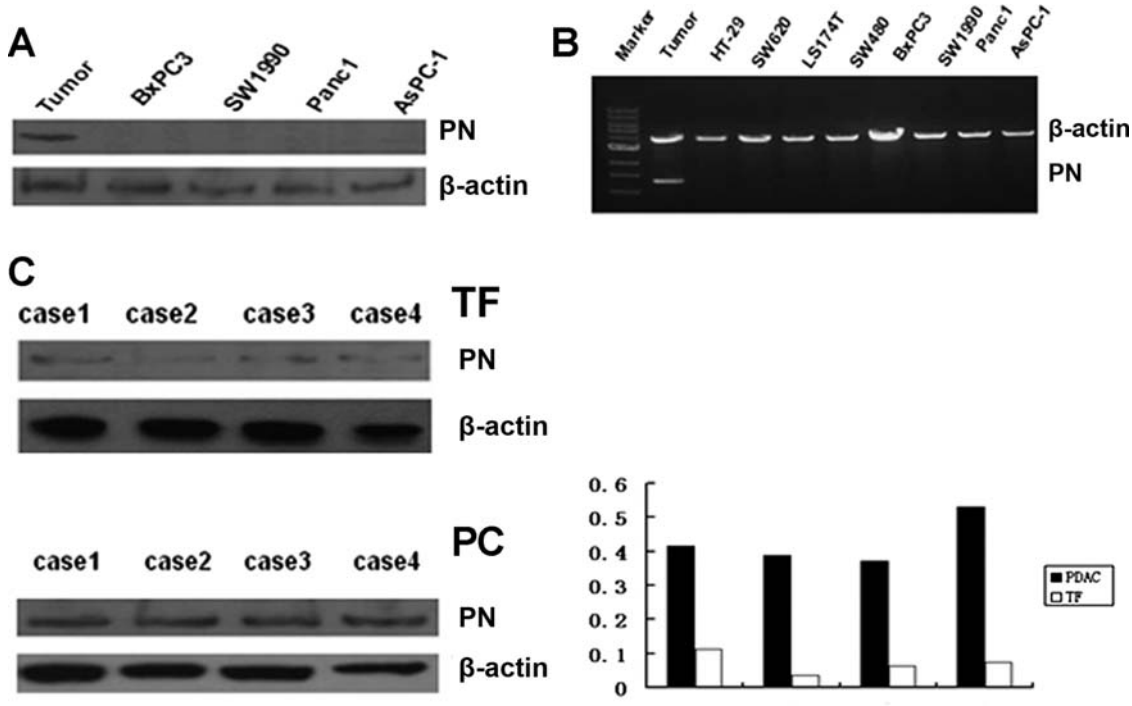

Figure 1. Periostin expression in a panel of pancreatic cancer cell lines and tissues. (A) Protein expression of periostin in cell lines assayed by Western blot analysis with a monoclonal anti-periostin antibody. Human periostin protein was not present in pancreatic cancer cell lines. (B) RT-PCR was used to assay periostin mRNA in cell lines. Human periostin mRNA was not present in pancreatic cancer cell lines and colon cancer cell lines (previously reported). (C) Periostin was differentially expressed in pancreatic cancer samples compared with adjacent tumor free (TF) pancreas from the same patient. The expression of ß-actin was used as an internal control. Bands were quantified by Quantity One software (Bio-Rad). M, Marker; T, human colorectal cancer tissue; PN, periostin; TF, tumor-free tissues; PDAC, pancreatic ductal adenocarcinoma.

Results

Expression of periostin in human pancreatic cancer cell lines and tissues. As shown in Fig. 1A and B, periostin mRNA and protein were not present in the four cell lines (AsPC-1, SW-1990, BxPC-3, Panc-1) after RT-PCR and Western blot analysis. In order to determine the expression of periostin protein in human pancreatic cancer tissues, we performed Western blot analysis on four tumors and matched tumor-free tissues. The average periostin expression level (periostin/ß-actin signal intensity ratio) was significantly higher in tumors than in matched TF tissues ($p<0.05$, Fig. 1C).

Expression of periostin on TMA. To determine the distribution and clinical significance of periostin in pancreatic cancer, we analyzed the expression pattern of periostin on TMA by using immunohistochemistry. In adjacent normal pancreas, periostin was slightly expressed in capillary endothelial cells, fibroblasts; no or faint expression was found in ductal cells (Fig. 2A). In pancreatic cancer, strong periostin expression was observed close to the neoplastic cells in the neoplastic stroma (Fig. 2B and C). Interestingly, most of the neoplastic epithelium, only moderate levels of periostin expression could be observed (Fig. 2D and E). Collectively, up to 80% of tumors were strongly positive for periostin staining in the neoplastic stroma, whereas about 30% showed strong staining in neoplastic epithelium.

We then examined whether periostin expression is correlated with the development of pancreatic cancer. Periostin expression was significantly up-regulated in pancreatic cancer than that of matched 'normal' tissues ($p<0.001$, data not shown). In addition, the positive expression of periostin in the neoplastic stroma was significantly correlated with depth of invasion ($p=0.007$) and lymph node metastasis ($p=0.027$) (Table I). Survival analyses showed that the median survival time in periostin negative group (19.4 months) was significantly longer than that in periostin positive group (8.7 months, $p=0.035$, Fig. 3A). Interestingly, elevated expression of periostin in neoplastic epithelium was also associated with poor prognosis in pancreatic cancer ($p=0.022$, log-rank test), although which was not correlated with the advanced clinicopathological factors in this disease (Table I and Fig. 3B).

Periostin overexpression promotes anchorage-independent growth. To determine the effect of periostin on pancreatic cancer development, we constructed periostin recombinant adenovirus plasmid Ad5-PN. After the recombinant adenovirus vectors were constructed successfully, the virus was generated at 1-5x10^9 pfu/ml in titer. Human pancreatic cancer cells BxPC-3 or Panc-1, which had no expression of periostin, were infected with Ad5-PN adenovirus vectors. Our data showed that infection of BxPC-3 cells with Ad5-null at a MOI of 100 pfu/cell conferred transgene expression in nearly 97% of cells by detecing green fluorescent protein in microscope (Fig. 4A).

As shown in Fig. 4B, after virus infection, periostin did not significantly promote cell growth compared with parental cells or mock infection. However, the effect of periostin

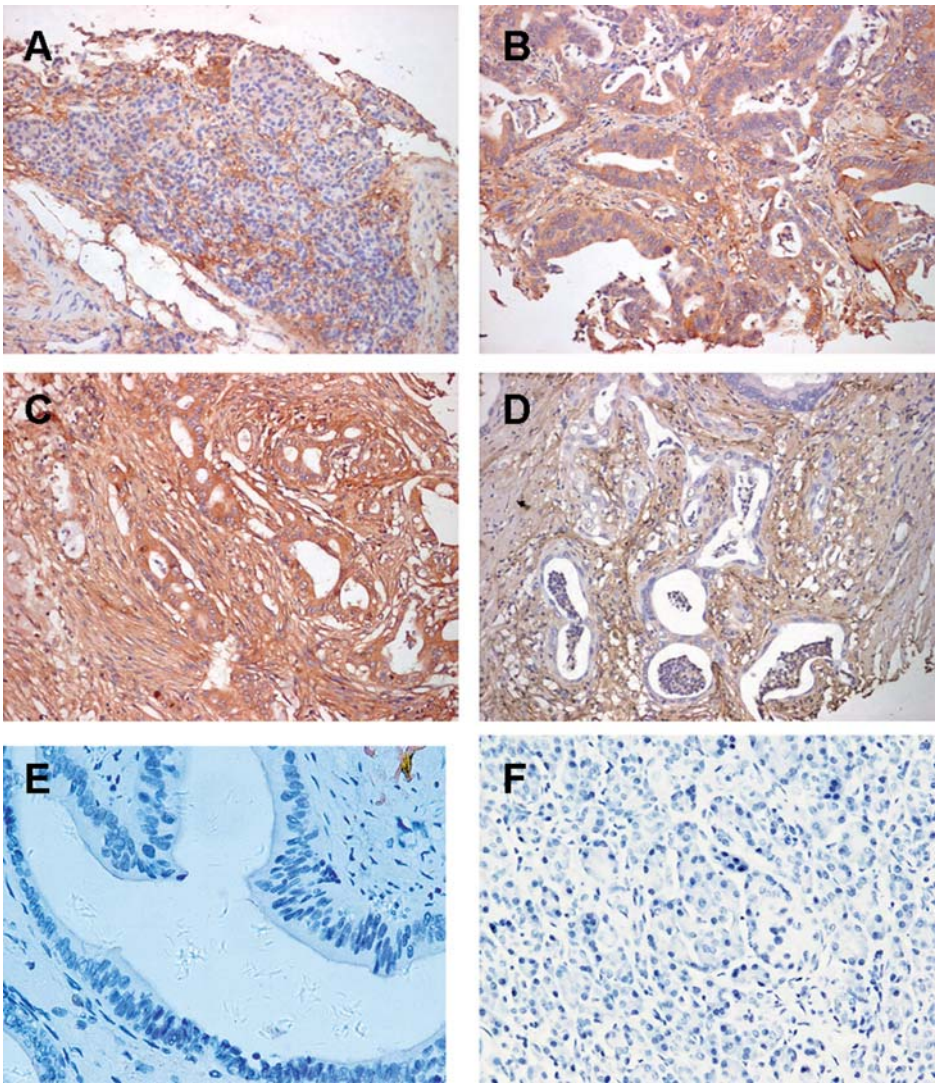

Figure 2. Immunohistochemistrical staining of periostin in human pancreatic tissues. The tissue cores were immunostained with a polyclonal periostin antibody. (A) Faint stromal immunostaining in adjacent 'normal'tissues. (B) Moderate staining in neoplastic stroma and strong staining in epithelium. (C) Strong periostin staining in neoplastic stromal and epithelial cells. (D) Strong staining in neoplastic stroma and no staining in neoplastic epithelium. (E) No staining in neoplastic epithelium. (F) Negative controls. (A-D, original magnification x200; E and F, magnification x400).

was more pronounced on promoting anchorage-independent growth, measured by the number of colony formation in soft agar assays. This finding was also found for another cell line, Panc-1, which showed more numerous and larger colonies with a soft agar assay (Fig. 4C).

Periostin promoted cell motility and invasion in vitro. To examine whether periostin had a role in cancer cell metastatic processes, we performed cell migration and invasion assays using transwell chambers, respectively. Surprisingly, periostin infected cells migrated faster at 24 and 48 h than did controls (Fig. 5A and B). With the invasion assay, the number of invaded periostin-overexpression cells was significantly more than the number of invaded parental cells or GFP infected cells (Fig. 5C and D).

Discussion

To develop adequate therapeutic modalities against pancreatic cancer, an increased number of molecular markers have been identified to correlate with tumor development and progression in this disease (22-24). The present study indicated an overall higher expression of periostin in pancreatic cancer, especially in the tumor-associated stroma. We also found a correlation between periostin expression in neoplastic stroma and epithelium and overall survival of patients with pancreatic cancer after potentially curative surgery. In addition, endogenous periostin might efficiently promote invasion and anchorage-independent growth of pancreatic cancer cells, which highlights its potential role as a therapeutic target.

The source of periostin in tumor is still under debate. Recent researches indicated that periostin protein is a matrix-specific protein, with high expression in the stromal cells surrounding the carcinoma epithelium (12,25-28), whereas other experiments suggested that periostin was detected in cancer cells (17,29). Interestingly, our previous study on colorectal cancers found that serum periostin levels after curative surgery showed a significant decrease below pre-operative levels (21). In the present study, we found that periostin mRNA and protein were not present in pancreatic

Table I. Clinicopathological variables with the epithelial or stromal expression of periostin in pancreatic cancers.

Parameters	n	Periostin epithelial expression			Periostin stromal expression		
		Negative	Positive	P-value[a]	Negative (<25%)	Positive (>25%)	P-value[a]
Age (years)				0.382			0.419
≤60	54	36	18		8	46	
>60	40	30	10		9	31	
Gender				0.527			0.173
Male	55	40	15		7	48	
Female	39	26	13		10	29	
Tumor classification				0.135			0.007
T1	8	5	3		4	4	
T2	25	14	11		7	18	
T3	61	47	14		6	55	
Node involvement				0.134			0.027
No	61	46	15		15	46	
Yes	33	20	13		2	31	
Histological grading				0.460			0.133
1/2	69	47	22		8	61	
3/4	25	19	6		9	16	
Tumor size (cm)				0.590			0.287
<4	43	29	14		10	33	
≥4	51	37	14		7	44	
Vascular invasion				0.544			0.538
Yes	56	38	18		9	47	
No	38	28	10		8	30	

[a]Tested by the χ^2 test and Fisher's exact test.

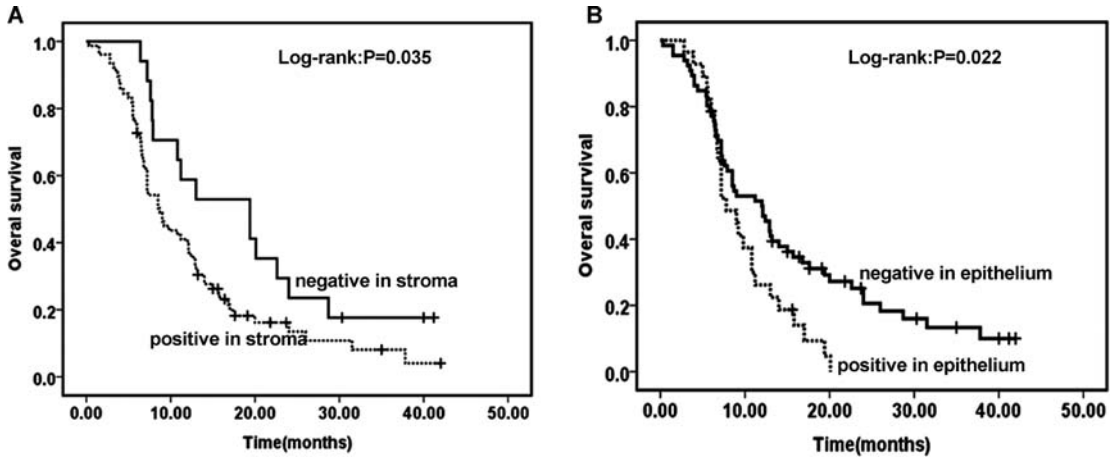

Figure 3. Kaplan-Meier analysis of overall survival of patients with pancreatic cancer according to periostin expression. The median survival time in the stromal (A) or epithelial (B) expression-negative group were significantly longer than those in the stromal or epithelial expression-positive group (P=0.035, P=0.022, log-rank test, respectively).

cancer cell lines, and that no or faint periostin protein was present in adjacent human non-cancerous pancreas, while expression of periostin was up-regulated in pancreatic cancer tissue samples. In addition, we also found that up to 80% of

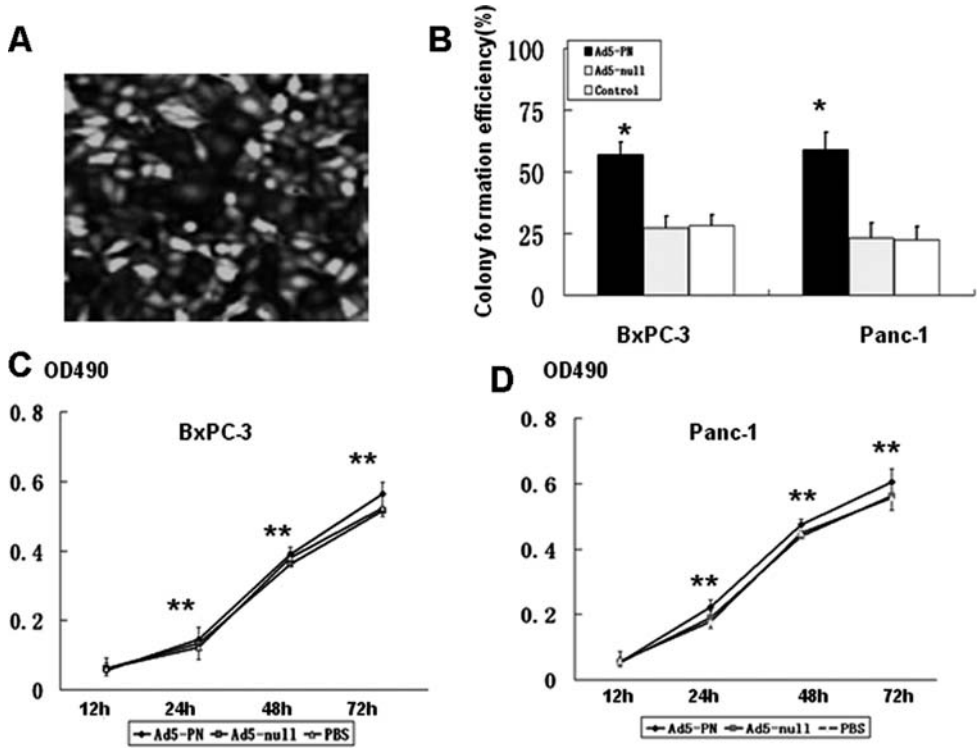

Figure 4. Cell infection of recombinant adenovirus and effects of periostin on cell proliferation and colony formation *in vitro*. (A) BxPC-3 cells infected with Ad5-null at a multiplicity of infection (MOI) of 100 pfu/cell conferred transgene expression in nearly 97% of cells by detecting fluorescent protein in microscope (x200). (B) BxPC-3 or Panc-1 cells were infected with Ad5-PN or Ad5-null at MOI of 100. Cells were also treated by phosphate-buffered saline (PBS) as a mock infection. The viability of cells was determined by MTT (3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at 12, 24, 48, and 72 h. Cell viability was expressed as absorption at 490 nm. The results shown are the mean of three independent experiments. Proliferation of BxPC-3 or Panc-1 cells infected with adenovirus vectors was slightly increased but there were no significant differences. $p>0.05$, bars, Standard error. (C) Colony formation assay was performed on BxPC-3 or Panc-1 cells infected with adenovirus for 48 h. The number of colonies per well was counted 2 weeks after plating $1x10^3$ cells. The cells without any infection were used as control. The data are the average of 3 independent triplicate experiments. PN, periostin.

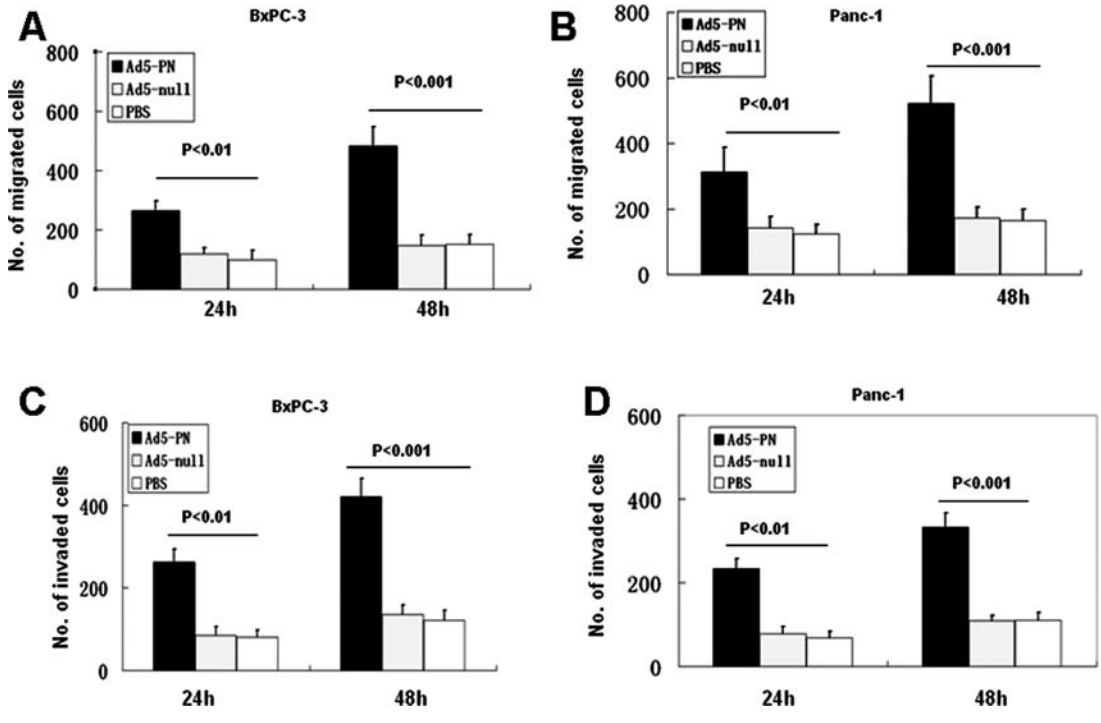

Figure 5. Effect of periostin on cell migration and invasion. Migration and invasion assays of BxPC-3 (A and C) or Panc-1 (B and D) cells infected with Ad5-PN. Statistically significant reductions were calculated by Student's t-test. (Top) *In vitro* motility potential of cells across transwell chambers was measured for BxPC-3 (A) or Panc-1 (B) cells. (Bottom) Cell invasion assays were done for BxPC-3 (C) or Panc-1 (D) cells by using matrigel coated transwell chambers. Invaded cells were counted in 10 random fields. Bovine serum albumin was used as a control. PN, periostin.

tumors were strongly positive for periostin staining in the stroma, whereas about 30% showing strong periostin staining in neoplastic epithelium. These findings were in accordance with previous reports by Erkan *et al* (12), who identified periostin as a protein exclusively secreted by PSCs in pancreatic carcinoma. Building on these results and previous studies, we speculate that pancreatic cancer cell-mediated humoral factor(s) may stimulate periostin secreted from PSCs.

Overexpression of periostin has been reported to correlate with the progression of a number of human tumors, such as papillary thyroid carcinoma (30), NSCLC (28), breast cancer (11,31) and melanoma (32). In the present study, we found that the positive expression of periostin in the neoplastic stroma was clearly and significantly correlated with the degree of malignancy and poor prognosis. This observation is consistent with previous studies, which reported that elevated expression of periostin was associated with the invasion and progression of NSCLC, and periostin status acted as an prognostic factor in NSCLC (28,33).

Accumulating evidence suggests that tumor microenvironment may produce many of the signals driving the proliferation and invasion of cancer cells (34,35). The interactions between cancer and stromal cells, as well as between cancer cells and the extracellular matrix (ECM), are required in invasion and metastatic process. One critical step in tumor metastasis is termed epithelial-mesenchymal transition (EMT), which enables epithelial cancer cells to acquire invasive and metastatic potential (36,37). Periostin has been shown to facilitate the migration and differentiation of cells that have undergone EMT, both during embryogenesis and in pathological conditions (38). Stable expression of periostin induces 293T cells to undergo EMT and promotes cell migration, invasion, and adhesion (39). This is also true for NSCLC (28). Therefore, we hypothesized that a cancer microenvironment in which periostin is present may have a tumor-supportive phenotype.

The roles of periostin in tumorigenesis and development have been proposed previously. First, it is recognized that periostin has adhesive activity because its receptors, integrins, all mediate cell adhesion (15,17,18) and the periostin protein shares structural and sequence homology with fasciclin I, which is an insect adhesion molecule (4,18,40). Second, periostin promotes the migration, inhibits apoptosis and stimulates survival and growth of diverse cells (9,15,18). Third, several studies have suggested that periostin increases tumor invasiveness by promoting tumor angiogenesis through complex signaling pathways, such as PI3kiase/Akt or focal adhesion kinase (FAK)-mediated signaling pathway (15,29,41). In the present study, we established periostin ectopic expression pancreatic cancer cell lines and found that ectopic expression of periostin did not promote proliferation, but instead promoted anchorage-independent growth of pancreatic cancer cell lines. In addition, periostin significantly increased the migration and invasion of pancreatic cancer cells. Therefore, this study suggests that overexpression of periostin may confer on pancreatic cancer cells the ability to survive in the absence of anchorage and invade aggressively. However, the underlying mechanisms of invasion and anchorage-independent growth by periostin are still required.

Taken together, our studies have revealed a critical role of periostin in invasion and anchorage-independent growth in the metastatic process of pancreatic cancer. Importantly, periostin not only is associated with advanced pancreatic cancer but also is a prognostic marker for this disease. The findings that effect on metastatic potential of periostin raise the possibility that it could be used as a molecular target in the anti-metastatic therapy of pancreatic cancer.

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