

PDX-1

Demonstration of Oncogenic Properties in Pancreatic Cancer

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BACKGROUND: Pancreatic-duodenal homeobox 1 (PDX-1) is a transcription factor that regulates embryologic pancreas development and insulin expression in the adult islet; however, it is overexpressed in many types of cancer, including pancreatic cancer. The purpose of this study was to investigate the role of PDX-1 in tumorigenesis in human cells. **METHODS:** In vitro cell proliferation, invasion, and transformation were performed in human embryonic kidney cell line (HEK 293), pancreatic cancer cell line MIA PaCa2, and human pancreatic ductal epithelial (HPDE) cells transiently or stably expressing PDX-1 or green fluorescent protein (GFP) PDX-1, with or without cotransfection of PDX-1 short hairpin RNA (shRNA). In vivo tumor formation was carried out in severe combined immunodeficiency (SCID) mice with subcutaneous injection of HEK 293 and MIA PaCa2 stably transfected cells. Cell cycle was analyzed by Western blot or immunostaining. Microarray of RNA from pancreatic adenocarcinoma cells with and without PDX-1 shRNA was performed and analyzed. **RESULTS:** Transient and stable expressing PDX-1 significantly increased cell proliferation and invasion in HEK 293, human pancreatic ductal epithelial (HPDE), and MIA PaCa2 cells versus controls ($P < .05$), human PDX-1 shRNA reversed these effects. Expression of PDX-1 significantly increased colony formation in HEK 293, HPDE, and MIA PaCa2 cells versus controls in vitro ($P < .05$). PDX-1 promoted HEK 293 and MIA PaCa2 tumor formation in SCID mice as compared with that of control ($P < .05$). PDX-1 overexpression disrupted cell cycles proteins. PDX-1 expression was confirmed by Western blot and tracked by viewing of GFP-PDX-1 expression. Microarray data support an oncogenic role of PDX-1 in pancreas cancer cells. **CONCLUSIONS:** PDX-1 induced increased cell proliferation, invasion, and colony formation in vitro, and resulted in markedly increased HEK 293 and MIA PaCa2 tumor formation in SCID mice. These data suggest that *PDX-1* is a potential oncogene that regulates tumorigenesis. *Cancer* 2010;000:000–000. © 2010 American Cancer Society.

KEYWORDS: PDX-1, transcription factor, oncogene, pancreatic cancer, proliferation, invasion, transformation, microarray.

Mechanisms involved in oncogenesis include activation of oncogenes and inactivation of tumor suppressor genes,^{1–4} signaling pathways including Wnt, transforming growth factor (TGF) α , TGF β , endothelial growth factor,^{5–9} and re-expression of embryonic genes such as sonic Hedgehog (SHH), Notch, and pancreatic and duodenal homeobox-factor 1 (PDX-1). *Notch* and *SHH* have been well characterized as oncogenes,^{10,11} but the role of PDX-1 in tumorigenesis remains unclear.

PDX-1 has been demonstrated to play a crucial role in the development and differentiation of the pancreas. In adult pancreas, PDX-1 regulates multiple genes such as insulin, islet amyloid polypeptide, somatostatin, glucokinase, and elastase-1 to maintain homeostasis of the endocrine and exocrine pancreas.^{12,13} PDX-1 is required for proliferation and neogenesis of islet cells after chronic hypoglycemic challenge.^{14–16} Re-expression of PDX-1 has been found in adult duct cells in certain conditions such as pancreatectomy and pancreatitis.^{17,18} PDX-1 expression also was observed in several pancreatic cancer mouse models,^{19–21} supporting the hypothesis that PDX-1 could participate in the carcinogenesis of pancreatic cancer in mice. Furthermore, persistent expression of PDX-1 induced acinar-to-ductal cell metaplasia in the transgenic mouse pancreas, representing a potential initiating event to malignancy.²² Most recently, PDX-1–positive cells have been shown to be an origin of pancreatic cancer, supporting their multipotent role as a stem cell of pancreatic

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cancer.²³ Thus PDX-1 not only is required for pancreatogenesis and maintenance of pancreatic homeostasis, but also plays a key role in mediation of cell differentiation and metaplasia.

Interestingly, PDX-1 was overexpressed in most solid cancers, including pancreatic, liver, prostate, ovarian, kidney, gastric, breast, lung, and colon.²⁴⁻³⁰ It has been shown that 50% to 100% of human pancreatic cancers overexpress PDX-1 and that PDX-1 overexpression is associated with advanced clinical pathological stages and poor prognosis.^{25,26,29,31,32} These observations suggest that PDX-1 could mediate tumorigenesis. In the present study, we have generated in vitro and in vivo data in 4 human cell lines using several oncogenesis techniques that strongly support the hypothesis that PDX-1 is a potential oncogene in mediation of tumorigenesis in pancreatic cancer.

MATERIALS AND METHODS

Cell Lines, Vectors, and Antibodies

Human embryonic kidney cell line (HEK 293) and human pancreas cancer cell lines MIA PaCa2 and pancreatic adenocarcinoma (PANC-1) were obtained from the American Type Culture Collection (Bethesda, Md). HPDE cells were maintained in keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen, Carlsbad, Calif). Human *PDX-1* cDNA and green fluorescent protein (*GFP*)–*PDX-1* fusion were cloned into pCMV5 expression vector and pQICXIP (Clontech, Mountain View, Calif) retrovirus vector, respectively. PDX-1 short hairpin RNA (shRNA) was designed and produced as described.²⁸ PDX-1–scrambled shRNA served as control. Mouse goat anticyclin E2, mouse anti-Cdk2, and rabbit anti-p21, -p27, and -p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Rabbit anti-PDX-1 antibody has been previously reported.²⁸ Goat antirabbit antiserum and sheep antimouse antiserum conjugated with horseradish peroxidase were purchased from Amersham Life Science (Arlington Heights, Ill). Rabbit antigoaat immunoglobulin (Ig)G was obtained from Zymed Laboratories (South San Francisco, Calif).

Transient and Stable Transfection of Cell Lines

Transient transfection of cell lines was performed with 24 µg of plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif). Stable transfections were established in HEK 293 or MIA PaCa2 cells with retrovirus carrying

PDX-1 or *GFP*–*PDX-1*, which was produced by pQICXIP expressing PDX-1 or GFP–PDX-1 transfection of the AmphoPack 293 cell line (Clontech, Mountain View, Calif). PDX-1 shRNA or scrambled shRNA was used to cotransfect cells overexpressing PDX-1 or GFP–PDX-1.

Cell Proliferation Assay

Cell proliferation assay was performed on cells with transient or stable PDX-1 or GFP–PDX-1 expression, respectively, and then determined by MTS assay (Promega, Madison, Wis) at 24, 48, and 72 hours after transfection.²⁸

In Vitro Invasion Assay

Invasion assays were performed in a 24-well cell culture insert containing invasion chambers (Chemicon, Temecula, Calif). Invasive cells migrating to the lower surface of the membrane of insert were determined by staining. Invasiveness was quantified by dissolving stained cells in 10% acetic acid and measured in the dye/solution mixture in a Multiskan EX plate reader (Thermo Fisher Scientific Inc. Waltham, Mass) at 560 nm. Experiments were repeated 5×, and representative data are shown.

Anchorage-Independent Cell Growth Assay

Stably transfected cells (2500/well) were suspended in 1.0 mL of Dulbecco modified Eagle medium with 0.35% agarose (UltraPure, Invitrogen), and the suspension was placed on top of 1.0 mL of solidified 0.5% base agar (Difco Agar, Noble, Becton, Dickinson, and Company, Sparks, Md). Triplicate cultures for each cell type were maintained at 37°C in a 5% CO₂ atmosphere, and fresh medium was added after 1 week. Colonies were photographed at 21 days under a phase contrast microscope equipped with fluorescence. The numbers and size of colonies were counted and calculated from each experiment, which was reproduced 5×.

Western Blot Analyses

Western blot analysis of protein levels in transfected cells were performed.²⁸ Antibodies against PDX-1, cyclin E, Cdk2, Cdk4, and p21, p27, and p53 were used. Images were captured using the UVP imaging system, and the band was analyzed using ImageJ software.

Tumorigenicity in Severe Combined Immunodeficiency Mice

Cells (3×10^7) in a 0.1 mL volume of phosphate-buffered saline were inoculated subcutaneously into the right flank

of a 20-g male with 5 mice for each group. Tumor formation was observed 4 weeks later. The formed tumors were dissociated and frozen for further immunostaining studies. Tumors were measured and recorded as the larger (A) and smaller (B), and tumor volume (V ; a rotational ellipsoid) was calculated according to the formula: $V (\text{mm}^3) = A(\text{mm}) \times B^2(\text{mm})/2$. Mice were scored according to the presence or absence of tumor.

Immunohistochemical Staining

Fluorescein isothiocyanate-conjugated antirabbit IgG antibodies were purchased from Sigma (St Louis, Mo). Tissue process, section preparation, hematoxylin and eosin staining, and immunostaining were done as described.²⁸ Anti-PDX-1, P53, and proliferating cell nuclear antigen (PCNA) antibodies were used. Images were recorded using a digital camera (Diagnostic Instruments, Sterling Heights, Mich) on a fluorescent microscope (Olympus IX70; Olympus Optical, Tokyo, Japan). Immunostaining for PDX-1 expression was quantified using ImageJ.

Microarray of PANC-1 Cells Before and After Treatment With Liposome (L)-PDX-1 shRNA

PANC-1 cells, which have high endogenous expression of PDX-1, were transfected with huPDX-1 shRNA and empty vector. Total mRNA was extracted from treated cells using a RNAqueous Kit (Ambion, Austin, Tex). Quality RNA was then subjected to microarray assay using Affymetrix GeneChip arrays (Human Genome U133 Plus 2.0 Array) in the BCM Microarray Core Facility. Each experiment was repeated 3 \times . ComGene expression levels were analyzed, and differentially expressed genes were selected using significance analysis of microarrays.³³ Genes were normalized using log-transformed expression values with GC Robust Multiarray Average.³⁴ Functional gene analysis was carried out using Gene Ontology annotation, followed by computational signal processing analysis (CSPA) using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, Calif), which takes microarray gene expression data and models them into known signaling networks from the primary literature.

Statistical Analysis

The Student t test was used to analyze differences in means of the continuous data. Pearson chi-square and Fisher exact test were used to analyze differences in ratios of the enumeration data. For microarray, P values of specific

nodes and motif networks were defined by the Fisher exact test. All numeric data were expressed as mean \pm standard error of the mean, with $P < .05$ indicating significance.

RESULTS

Generation of Stable Transfections in HEK 293, HPDE, and MIA PaCa2 Cell Lines

All 3 cell populations stably expressed PDX-1 and GFP-PDX-1 after retroviral infection and puromycin selection. Empty vector transfection served as control. Expression was confirmed by Western blot and immunostaining using anti-PDX-1 antibody against PDX-1 as will be described. GFP-PDX-1 fluorescence also was confirmed by microscopy.

Increased Cell Proliferation Related to Transient Expression of PDX-1

Transient overexpression of PDX-1 resulted in a significant increase of cell proliferation by $150.3\% \pm 16.1\%$ in HEK 293, $151.4\% \pm 10.5\%$ in MIA PaCa2, and $130.1\% \pm 5.1\%$ in HPDE cells as compared with empty vector-transfected cells at 48 hours (Fig. 1A). Cotransfection of PDX-1 shRNA to knock down PDX-1 expression blocked cell proliferation ($P = .01$) (Fig. 1B).

Stable Overexpression of PDX-1 Is Associated With Increased Cell Proliferation

Stably transfected cell lines overexpressing PDX-1 were used to determine long-term influence of PDX-1 on cell proliferation. The cells expressing PDX-1 had $133.7\% \pm 11.2\%$ in HEK 293 ($P < .05$) (Fig. 2A), $163.9\% \pm 15.2\%$ ($P < .05$) in MIA PaCa2 (Fig. 2B), and $145.1\% \pm 8.2\%$ ($P < .05$) in HPDE (Fig. 2C) increased cell proliferation as compared with control. PDX-1 shRNA reversed these effects, as shown in Figure 2. We noted that the proliferation in stable transfected HPDE cells was much higher than that in transient transfection of HPDE. To observe the PDX-1 expression, we developed cells expressing GFP-PDX-1 in HEK 293 and MIA PaCa2 cells. The expression of PDX-1 was seen by GFP expression under microscopy (Fig. 2D, E). During the 3 days of observation after PDX-1 shRNA transfection, we found a continuous reduction of signal strength and cell number in both HEK 293 (Fig. 2D) and MIA PaCa2 cells (Fig. 2E).

Overexpression of PDX-1 Induces Cell Invasion Ability

To determine PDX-1's effect on cell invasion, we performed invasion studies using stably transfected HEK

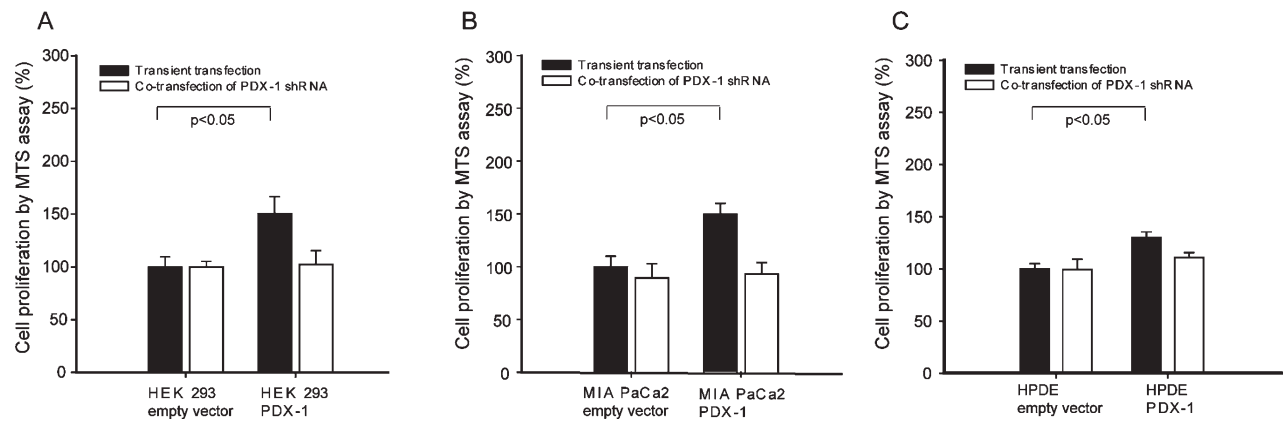


Figure 1. Transient expression of PDX-1 increases cell proliferation. HEK 293, MIA PaCa2, and HPDE cells were transfected with human PDX-1 with and without human PDX-1 short hairpin RNA (shRNA). Transient burst expression of PDX-1 results in increased cell proliferation in HEK 293 (A), MIA PaCa2 (B), and HPDE (C) cells ($P < .05$, respectively). PDX-1 shRNA cotransfection blocks the effect of cell proliferation (white bars).

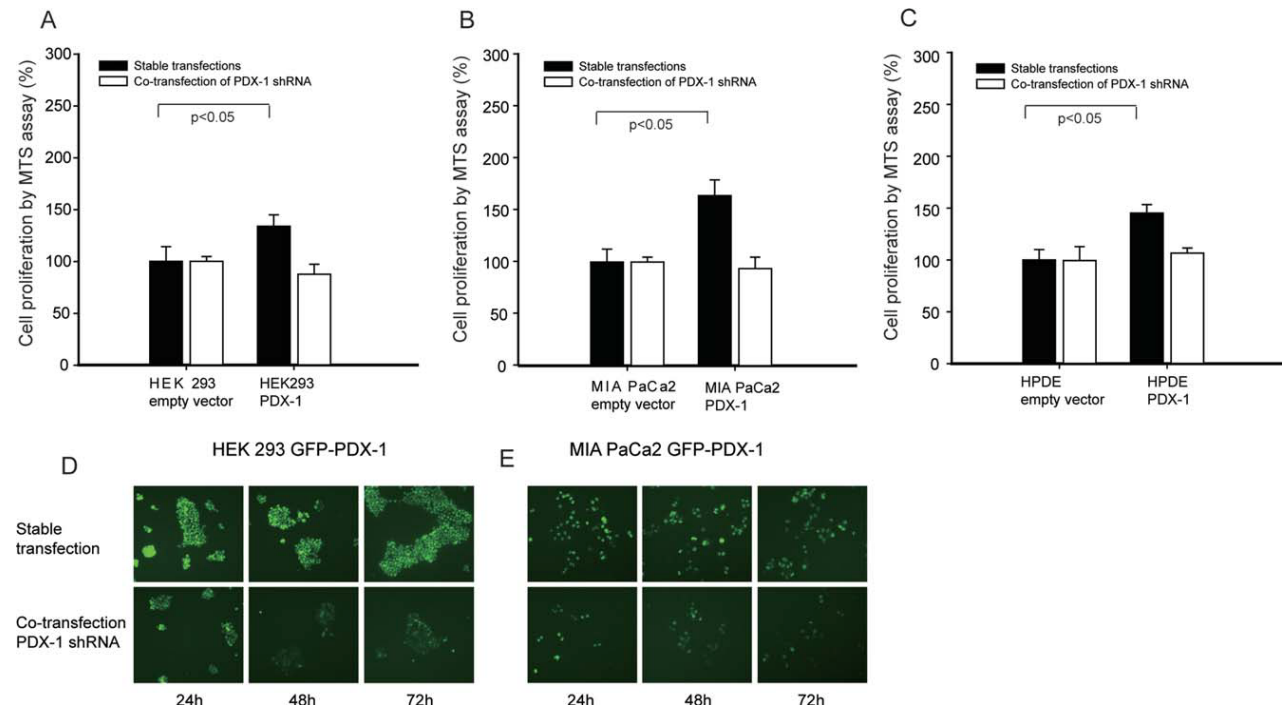


Figure 2. HEK 293, MIA PaCa2, and HPDE cells stably expressing PDX-1 with and without human PDX-1 short hairpin RNA (shRNA) had increased cell proliferation. (A-C) PDX-1 overexpression caused significant increase of cell proliferation in all cell lines. PDX-1 shRNA cotransfection was able to reverse the effect induced by PDX-1.

293, MIA PaCa2, and HPDE cell lines with or without cotransfection PDX-1 shRNA. Invasive cells were seen by staining of migrated cells (Fig. 3A, representing HEK 293 cells), and invasiveness was quantified by colorimetric assay as shown in Figure 3B to D. PDX-1 overexpression induced an increase of cell invasion by $278.6\% \pm 16.5\%$

in HEK 293, $127.6\% \pm 5.1\%$ in MIA PaCa2, and $162.4\% \pm 9.6\%$ in HPDE cells compared with controls ($P < .05$). Cotransfection of PDX-1 shRNA reversed the effect induced by PDX-1 ($P = .01$). These data indicate that PDX-1 overexpression increases cell invasion in both benign cells and pancreatic cancer cells, although the

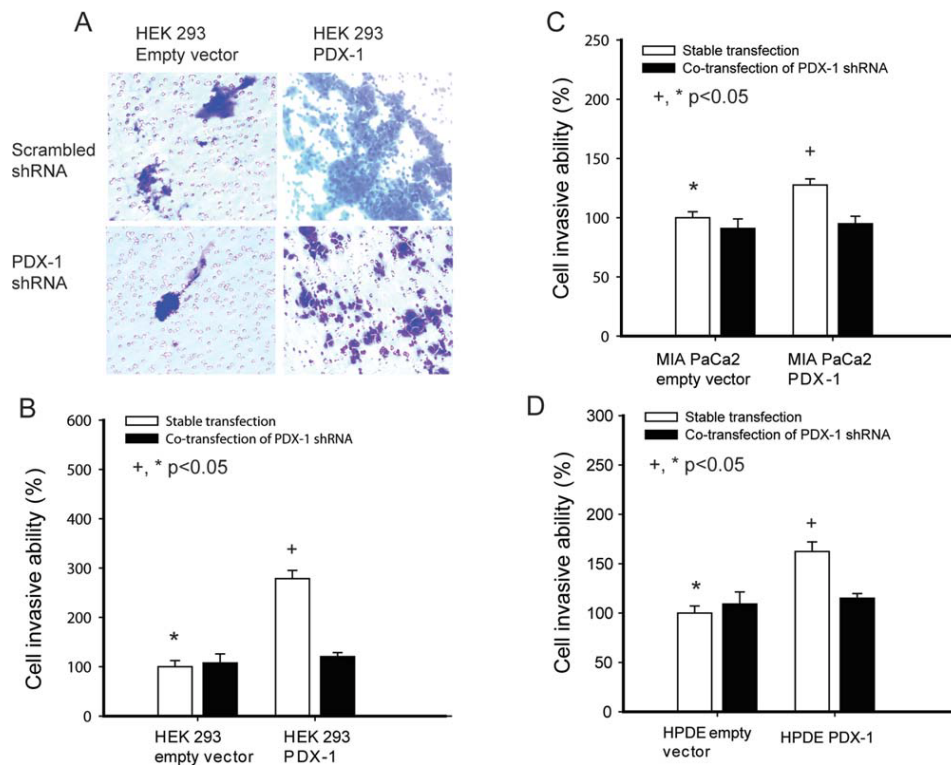


Figure 3. (A) HEK 293, MIA PaCa2, and HPDE cells stably overexpressing PDX-1 were cultured with and without human PDX-1 short hairpin RNA (shRNA), then transferred into invasion inserts for 48 hours. (B-D) Increased quantitative invasiveness is shown.

increase in MIA PaCa2 cells was lower than in the other cell lines presumably because of the presence of endogenous PDX-1.

Overexpression of PDX-1 Enhances the Ability of Colonial Formation

To test whether PDX-1 induces and promotes cell transformation in human cell lines, we performed anchorage-independent growth assay in soft agar plates. HEK 293 and MIA PaCa2 cells expressing PDX-1, GFP-PDX-1, or empty vectors were seeded in the soft agar plates to grow colonies. During culture, colony formation was studied by observing the number and size of colonies (Fig. 4A, D), and PDX-1 expression was tracked by viewing GFP signal under microscopy in GFP-PDX-1 expressing colony formation (Fig. 4A3, D3). Colony numbers and size are shown Figure 4B, C, E, and F. A significant increase in colony formation was found in all cells expressing PDX-1 as compared with controls. PDX-1 overexpression resulted in significantly increased colony numbers (71.3 ± 2.6) in HEK 293 cells (Fig. 4B), 42.7 ± 3.8 in MIA PaCa2 cells (Fig. 4E), and 121.4 ± 17.7 (not shown) in

HPDE cells compared with empty vector controls, 24.1 ± 4.7 ($P = .005$) in HEK, 28.3 ± 3.3 in MIA PaCa2 ($P = .01$), and 0 in HPDE cells. PDX-1 overexpression also increased colony size by 960.2 ± 75.7 in HEK 293 cells (Fig. 4E) and 1983.1 ± 235.2 in MIA PaCa2 cells (Fig. 4F) as compared with empty vector controls.

Overexpression of PDX-1 Promotes Tumor Growth in Severe Combined Immunodeficiency Mice

To study the role of PDX-1 overexpression in vivo, we implanted HEK 293 or MIA PaCa2 cells, expressing PDX-1, GFP-PDX-1, or empty vector, in severe combined immunodeficiency (SCID) mice by subcutaneous injection at a dose of 3×10^7 cells/mouse. The mice were sacrificed at Day 30 after cell inoculation, and tumor information was collected. Five of 5 mice developed tumors from PDX-1-overexpressing HEK 293 cells, whereas only 1 of 5 mice developed a very small tumor from HEK 293 empty vector cells. The volume of PDX-1-overexpressing HEK tumor (Fig. 5A, B) was $239.9 \pm 84.4 \text{ mm}^3$ versus 5.8 mm^3 for the empty vector cells ($P <$

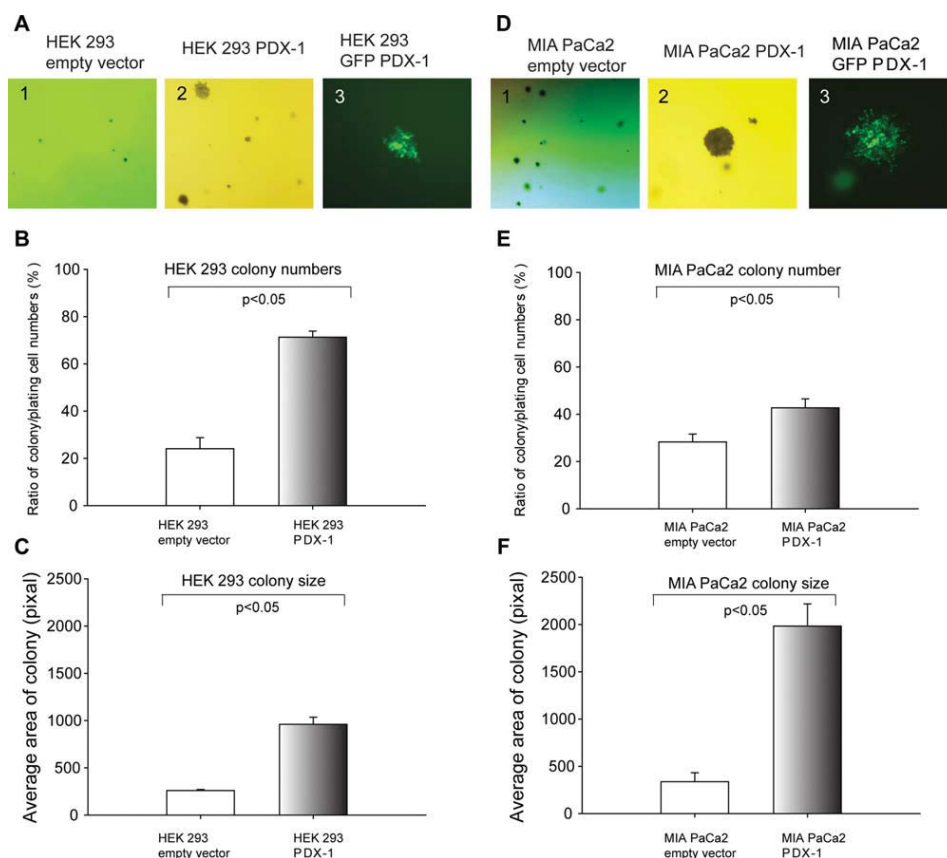


Figure 4. PDX-1 overexpression in HEK 293 (A-C) and MIA PaCa2 (D-E) cells causes cell transformation in anchorage-independent growth assay. Colonies were photographed at 21 days under a phase contrast fluorescent microscope. The number and size of colonies were counted and calculated from each experiment in B, C, E, and F, and the experiment was reproduced 5 \times . GFP indicates green fluorescent protein.

.05 between all groups). As expected, both PDX-1–overexpressing and empty vector MIA PaCa2 cells generated tumors in mice; however, the volume of PDX-1–overexpressing MIA PaCa2 tumor was $545.3 \pm 35.2 \text{ mm}^3$ in PDX-1 and $325.8 \pm 19.3 \text{ mm}^3$ in empty vector control cells ($P < .05$) (Fig. 5C, D). Tumor volume in HEK 293 empty vector control cells was markedly smaller than that of the MIA PaCa2 control cells, reflecting that HEK 293 cells can serve as benign cells. PDX-1 expression in tumors was confirmed by immunostaining, as shown in Figure 6A (top panel). Changes of P53 and PCNA were detected in HEK 293 tumor samples in the presence or absence of PDX-1. As shown in Figure 6A (middle and bottom panel), PDX-1 overexpression down-regulated P53 ($34.5\% \pm 6.3\%$) expression and up-regulated PCNA expression ($45.6\% \pm 9.4\%$) as compared with controls at $86.4\% \pm 10.2\%$ ($P = .005$) and $22.4\% \pm 5.3\%$ ($P = .01$), respectively.

Stable Expression of PDX-1 Leads to the Disruption of Cell Cycle

To study potential mechanisms by which PDX-1 regulates cell proliferation, expression of a series of cell cycle-related proteins was evaluated by Western blot analysis in HEK 293 cells stably expressing PDX-1 with and without PDX-1 shRNA. As shown in Figure 7A, stable overexpression of PDX-1 in HEK 293 cells resulted in down-regulation of expression of P21, P27, and P53 and up-regulation of expression of cyclin E and Cdk2 as compared with control cells ($P < .05$, excluding P27). Protein levels in PDX-1 stably transfected cells versus control vector-treated cells were quantified as p21, 23.4 ± 2.1 vs 30.2 ± 2.3 ; P27, 9.1 ± 1.2 vs 21.1 ± 1.2 ; P53, 61.9 ± 6.0 vs 100.1 ± 9.2 ; cyclin E, 55.0 ± 4.6 vs 35.7 ± 6.7 ; and Cdk2, 39.7 ± 3.7 vs 28.1 ± 2.8 , as shown in Figure 7B. Cotransfection with hPDX-1 shRNA reversed the effect induced by PDX-1 overexpression.

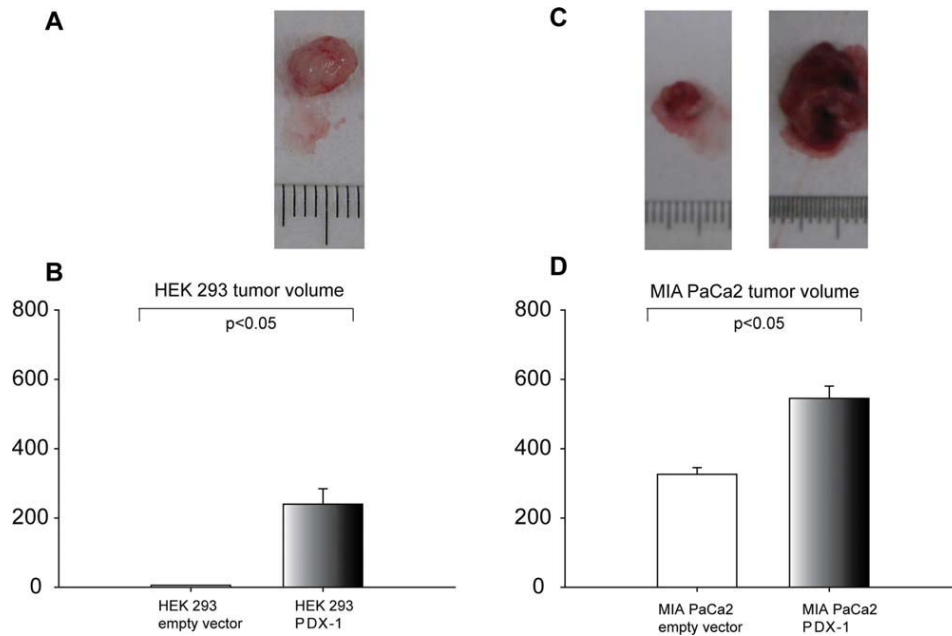


Figure 5. PDX-1 overexpression promotes tumor formation and growth in severe combined immunodeficiency (SCID) mice. HEK 293 or MIA PaCa2 cells overexpressing PDX-1 versus empty vector were implanted in SCID mice. (A) Gross tumor volume at 30 days was compared using a chi-square test, and (B) tumor size was measured and compared using Student *t* test. Tumor size was significantly induced by PDX-1 overexpression in HEK 293 cells (A, B) and MIA PaCa2 cells (C, D).

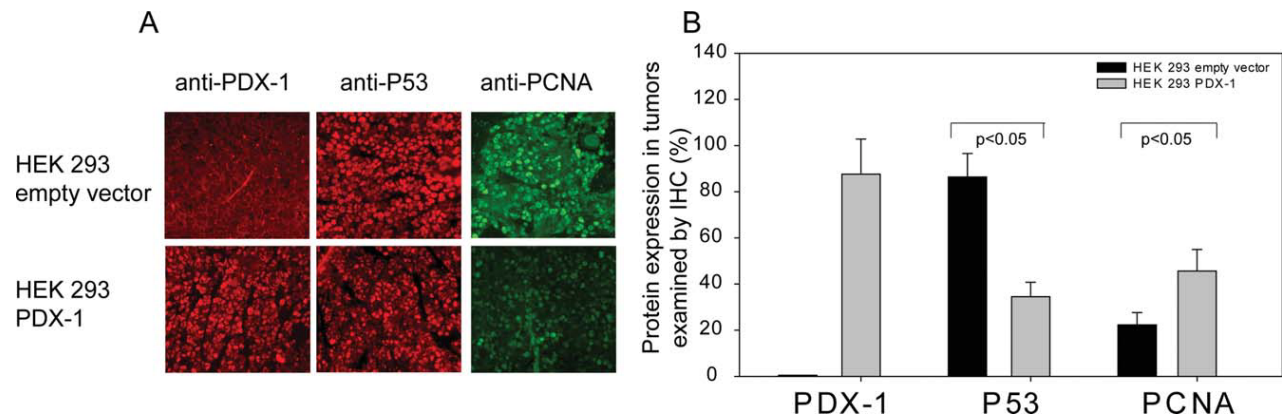


Figure 6. PDX-1 regulates cell cycle proteins. Immunostaining study was performed on tumor sections using antibodies against PDX-1, P53, and proliferating cell nuclear antigen (PCNA). (A) Red staining in the upper panel and middle panels indicates PDX-1 and P53 expression, respectively, and green staining at bottom A represents PCNA expression. (B) Quantity was defined as positive cells in total cells as analyzed by ImageJ software. Original magnification, $\times 200$. IHC indicates immunohistochemistry.

Knockdown PDX-1 Expression in PANC-1 Cells Is Associated With Alterations of Signaling Pathways of Cell Proliferation, Invasion, and Apoptosis

PANC-1 cells were used for this study because they have high endogenous PDX-1 expression. Microarray analysis showed that the highest scoring biological networks were

decreased cancer ($P = 1 \times 10^{-23}$ -.0002), decreased cell cycle ($P = 2.02 \times 10^{-28}$ -.0003), and increased cell death ($P = 2.02 \times 10^{-37}$ -.0002). These networks contained at least 190 molecules from the gene list. For these 3 networks, expression of sp-1, HDAC, p53, CDKN1A, CDKN1B, CDKN2A, v-rel, and E2Fs were critical to direct and indirect pathways for significant genes.

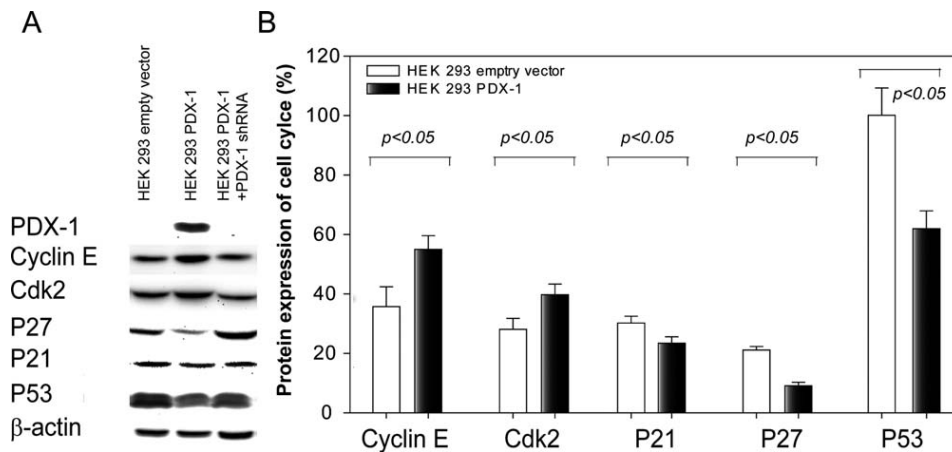


Figure 7. PDX-1 overexpression results in cell cycle disruption. Western blot was performed on PDX-1 stably transfected HEK 293 cells with or without PDX-1 short hairpin RNA (shRNA). Protein levels of PDX-1, cyclin E, cdk2 P21, p27, and P53 were determined by band profiles (A) and quantified by relative density of aimed band versus internal control (B).

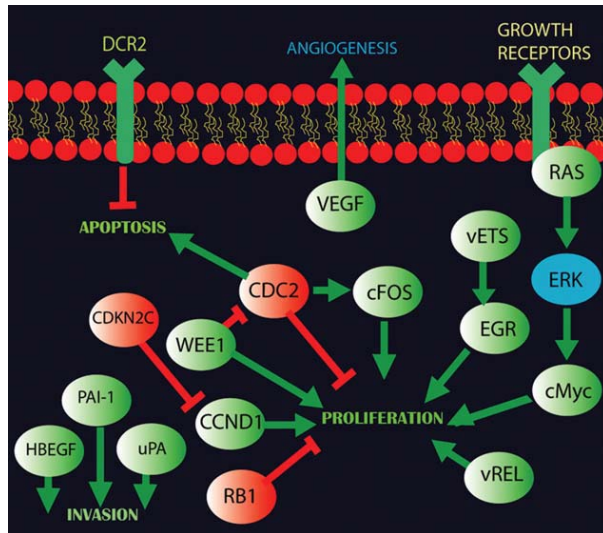


Figure 8. PDX-1 knockdown of pancreatic adenocarcinoma cells with high endogenous PDX-1 expression affected gene expression in signaling pathways of cell proliferation, invasion, apoptosis, and angiogenesis using Affymetrix GeneChip arrays (Human Genome U133 Plus 2.0 Array). Molecules in red represent gene overexpression, whereas molecules in green represent down-regulation. Direct interactions are indicated as (green) promoting or (red) inhibiting interacting arrows. PDX-1 knockdown stimulates apoptosis and cell cycle arrest by down-regulation of cyclins. Decreased gene expression related to angiogenesis (vascular endothelial growth factor [VEGF]) and invasion (heparin binding epidermal growth factor [HBEGF], plasminogen activator inhibitor type 1 [PAI-1], urokinase plasminogen activator [uPA]) was also seen in the analysis.

Figure 8 represents over-represented motifs and proliferative pathways identified by Ingenuity Systems and Database for Annotation, Visualization, and Integrated Discovery/Expression Analysis Systematic Explorer. Red molecules represent overexpression, whereas green mole-

cules represent down-regulation. Direct interactions are indicated as (green) promoting or (red) inhibiting interacting arrows. The data suggest that knockdown of PDX-1 in PANC-1 cells stimulates apoptosis through blockade of NFkB signaling, as well as arrest of cell cycle by down-regulation of cyclins. Interestingly, decreased gene expression related to angiogenesis (vascular endothelial growth factor) and invasion were also demonstrated (heparin binding epidermal growth factor [HBEGF], plasminogen activator inhibitor type 1 [PAI-1], urokinase plasminogen activator [uPA]), along with overexpression of DNA methylating proteins/histone de-acetylases. These data support the hypothesis that PDX-1 stimulates cell proliferation, invasion, and angiogenesis and inhibits cell apoptosis.

DISCUSSION

It is well known that PDX-1 is required for embryologic development of the pancreas and for maintenance of hormone expression in the adult islet.³⁵⁻⁴⁰ PDX-1 has also been found to regulate proliferation of islet cells in mice¹⁵ as well as proliferation and invasion of pancreatic cancer cells in vitro.¹⁴⁻¹⁶ Re-expression of PDX-1 in adult duct cells, under certain conditions such as pancreatectomy and pancreatitis, suggests that PDX-1 may be associated with the regenerative responses that accompany these conditions. It has been demonstrated to be a key regulator of the induction of cell differentiation from nonislet cells to insulin-secreting cells,⁴¹ especially acinar to ductal cell metaplasia, a common cellular change that may progress to malignancy.²² These studies, together with the finding

that PDX-1 is overexpressed in most solid cancers, which is associated with advanced clinical pathological stages and poor prognosis of patients with pancreas cancer, suggests that PDX-1 could play a role in oncogenesis.^{25,26,29,31,32} To test the hypothesis that *PDX-1* is an oncogene, we used techniques similar to those performed on *Kras*, which is a well-known pancreas cancer oncogene that induces malignant cellular transformation when overexpressed as an activating point mutation at codon 12 (*Kras*^{G12D}).^{1,2,42-44} Our cumulative data show that overexpression of PDX-1, in addition to affecting cell proliferation and invasion in both benign and malignant human cell lines, induced significant cell transformation by colony formation and promoted tumor growth in vivo, strongly supporting the role of PDX-1 as a potential oncogene. An opposite view has been mentioned in a study in which PDX-1 was considered as a tumor suppressor gene in human gastric cancer.⁴⁵ Further studies are needed to clarify this issue, because most studies showed a high level of PDX-1 in gastric cancer specimens,⁴⁶⁻⁵⁰ as opposed to this study, which showed low expression of PDX-1 in a single human gastric cancer cell line.

To strengthen the evidence of PDX-1's role in tumorigenesis, we also used visualized PDX-1 expression and PDX-1 siRNA knockdown techniques for in vitro studies in multiple human cell lines. HEK 293 cells are routinely used as a normal utility cell to test the function of oncogenic or tumor suppressor genes.⁵¹ HPDE cells, which originate from human pancreatic ductal epithelial, have been widely used as tool to investigate the oncogenic property in pancreatic cancer studies.^{52,53} MIA PaCa2 cells and PANC-1 cells are human pancreatic cancer cell lines with low and high endogenous expression of PDX-1, respectively. Consistent results were obtained from different human cell lines, demonstrating reliable approaches used in the study, emphasizing the crucial role of PDX-1 in mediating tumorigenesis. Second, observed PDX-1 expression offered kinetic observation of cellular responses to PDX-1 expression. It was particularly useful in monitoring PDX-1 expression in colony formation of PDX-1-transformed cells. Lastly, RNA interference technique was used to validate the role of PDX-1 in regulating cell proliferation and invasion. By knockdown of PDX-1 expression, the effectiveness of PDX-1 induction was accordingly reversed, providing confirmative evidence of PDX-1's role in cell proliferation and invasion. By combining 3 approaches, the study provides reliable evidence to demonstrate that PDX-1 mediates tumorigenesis and

to further support our previous view that PDX-1 is a therapeutic target for pancreatic cancer.²⁸ By using a xenograft human cell-SCID mouse model, this study demonstrates in vivo evidence that PDX-1 overexpression induces tumor formation and growth. HEK 293 cells harboring PDX-1 expression grew large tumors in all implanted mice, whereas HEK 293 control cells developed only a very small tumor in 1 of 5 mice. The results are consistent with other studies using HEK 293 cells to study the function of other oncogenes.^{51,54} PDX-1 also promoted MIA PaCa2 cell tumor growth in SCID mice, indicating that PDX-1 also has a cumulative effect on tumorigenesis, as MIA PaCa2 control cells have low endogenous PDX-1 expression. These data further support the hypothesis that *PDX-1* is an oncogene.

In terms of the mechanism by which PDX-1 is involved in pancreas cancer tumorigenesis, we have shown that overexpression mouse PDX-1 in human pancreas cancer cell lines, as well as PDX-1 shRNA knock down of PDX-1, results in disruption of cell cycle proteins.²⁸ Previous PDX-1 studies have shown dependence on several signal transduction pathways such as those involving Stat3,²² MAPK,⁵⁵ and phosphatidylinositol 3-kinase/Akt/mTOR signaling pathways.⁵⁶ There is great deal of overlap between these transduction cascades and those described in SHH regulation of proliferation in pancreatic cancer.⁵⁷ The data in this study demonstrate that PDX-1 up-regulates expression of Cdk2 and cyclin E, and down-regulates p27, p21, and P53 expression in human PDX-1-overexpressed HEK 293 cells both in vivo and in vitro; these data are consistent with our previous observations in other cell lines, addressing the role of PDX-1 in G1 to S transition in the cell cycle, emphasizing the critical role of PDX-1 in the mediation of cell proliferation.

Further mechanistic evidence was obtained from microarray analysis of genes involved in signaling pathways after PDX-1 knockdown in human PANC-1 cells, which have high endogenous expression of PDX-1. The computerized analysis of the microarray data also helped to identify additional potential molecular targets involved in the PDX-1 pathway, which provides potential insight into the molecular mechanism of PDX-1 in tumorigenesis. These data are consistent with our findings that PDX-1 regulates proliferation and invasion of PC cells and that suppression of PDX-1 expression via PDX-1 shRNA activates apoptotic pathways, as well as suppression of proliferation and invasion pathways.²⁸ However, further studies are needed to determine precisely how PDX-1 regulates transformation.

In conclusion, the data in the present study demonstrate that PDX-1 overexpression resulted in: 1) increased cell proliferation and invasion, as well as transformation of nonmalignant human cells; 2) promotion of tumor formation and growth of human cells implanted in SCID mice; and 3) disruption of the cell cycle in nonmalignant cells. These data, along with the demonstration that PDX-1 is overexpressed in >80 pancreatic cancer specimens, support the hypothesis that *PDX-1* is an oncogene mediating tumorigenesis in pancreatic cancer.

CONFLICT OF INTEREST DISCLOSURES

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