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Activation of phosphatidylinositol-3 kinase regulates pancreatic duodenal homeobox-1 in duct cells during pancreatic regeneration

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Abstract

OBJECTIVE—The purpose of our study was to determine whether the phosphatidylinositol 3-kinase (PI3K)/Akt pathway contributes to expression of pancreatic duodenal homeobox-1 (PDX-1) in duct cells and the cell differentiation during pancreatic regeneration.

METHODS—The role of PI3K in PDX-1 expression and duct cell differentiation with pancreatic regeneration in mice after partial pancreatectomy (Px) was examined using either wortmannin, a pharmacologic PI3K inhibitor, or siRNA directed to the p85 α regulatory subunit of PI3K. Akt phosphorylation, a marker of PI3K activation, and PDX-1 expression were assessed by Western blot analysis and immunohistochemistry.

RESULTS—Both PDX-1 levels and Akt phosphorylation were concomitantly increased in pancreatic ducts following partial Px, and, conversely, blocked by treatment with wortmannin or p85 α siRNA. Pancreatic duct cell differentiation, as assessed by appearance of insulin-positive cells 3 days after partial Px, was effectively reduced by wortmannin.

CONCLUSIONS—PI3K/Akt activation plays a critical role for both PDX-1 expression and pancreatic duct cell differentiation into insulin-producing cells during pancreatic regeneration.

Keywords

Insulin; PI3K; PDX-1; Pancreatectomy; Pancreatic regeneration

INTRODUCTION

Phosphatidylinositol 3-kinase (PI3K), a ubiquitous lipid kinase involved in receptor signal transduction, is composed of a regulatory subunit, p85, and a catalytic subunit, p110.^{1,2} PI3K catalyzes the production of phosphatidylinositol-3, 4, 5-triphosphate, which recruits a subset of signal proteins with pleckstrin homology domains to the membrane, where they are phosphorylated.^{1,2} These proteins include the protein serine-threonine kinase Akt and phosphoinositide-dependent kinase 1. Activation of Akt results in phosphorylation of downstream target proteins that affect cell growth, cell cycle distribution, apoptosis and survival.^{1,2} The PI3K pathway plays various important roles in pancreatic function, such as

insulin signaling, insulin-stimulated glucose transport and glycogen synthesis.³⁻⁷ In addition, we have previously reported that the PI3K pathway is critical for the proliferation of pancreatic acinar cells and plays a major role in pancreatic regeneration after partial pancreatectomy (Px).⁸

Pancreatic regeneration has been extensively studied in various animal models.⁹⁻¹¹ Increases in islet mass and islet neogenesis are noted in the regenerating pancreas after partial Px in the animal models.^{9, 12} Islet morphogenesis during embryogenesis is thought to occur predominately through the budding of islet cells from pancreatic ducts, followed by their migration away from the duct to form clusters.^{13, 14} In the normal adult pancreas, islet β -cell mass is replenished by two major mechanisms, β -cell cell replication and neogenesis from progenitor cells.¹⁵⁻¹⁸ The origin of β -cell progenitor cells is not clearly understood at present; pancreatic duct cells represent likely candidates for these progenitor cells since insulin within the pancreatic duct epithelium has been well documented in the regenerating pancreas.^{19, 20}

Pancreatic duodenal homeobox-1 (PDX-1), which is also known as IDX-1, IPF-1 and STF-1, is a key regulator of β -cell differentiation and function.²¹ It is expressed in the primitive gut during the early embryonic stage, diminished after birth,¹¹ and predominantly expressed in β -cells of adult pancreatic islets.²² PDX-1 plays critical roles in endocrine pancreatic function through its regulatory action on the expression of functional pancreatic genes including insulin.^{23, 24} PDX-1 is also essential for β -cell neogenesis as demonstrated by several *in vitro* models of cell differentiation to insulin-producing cells.²⁵⁻²⁹ Expression of PDX-1 increases in the duct during β -cell neogenesis in an animal model of pancreatic regeneration after partial Px.¹² These findings strongly suggest that PDX-1 is an important mediator and marker of ductal cell differentiation into β -cells. However, the molecular mechanisms contributing to PDX-1 expression during β -cell neogenesis are not fully understood. In the present study, we examined whether the PI3K/Akt pathway contributes to PDX-1 expression and β -cell differentiation in the pancreatic ducts.

MATERIALS AND METHODS

Materials

Anti-pAkt (Ser473) (#9271) and anti-Akt (#9272) antibodies for Western blot analysis were purchased from Cell Signaling (Beverly, MA). Anti-pAkt (Ser473) (#sc-7985-R) antibodies for immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-PI3K p85 α antibody was purchased from NeoMarker (Fremont, CA). Rabbit anti-PDX-1 antibody (#AB3243) was purchased from Chemicon International (Temecula, CA). EnVision+ system and anti-insulin (A0564) antibodies were purchased from DAKO Cytomation (Carpinteria, CA). Secondary antibodies (goat anti-rabbit or mouse IgG) for immunoblotting were obtained from Upstate (Waltham, MA). Wortmannin, type IV collagenase, monoclonal anti- β -actin antibody (#A5441; clone AC-15) and other molecular biology grade reagents were purchased from Sigma (St. Louis, MO). The siSTABLE SMARTpool siRNA directed to PI3K p85 α regulatory subunit and non-specific control siRNA duplexes were synthesized by Dharmacon (Lafayette, CO). To prevent recognition and cleavage of unintended mRNA targets (off-target effect),³⁰ this siRNA was modified by an ON-TARGET technique from Dharmacon. *Trans IT In Vivo* Gene Delivery System and *Trans* TKO transfection reagent was purchased from Mirus (Madison, WI).

Animals and Partial Px model

Male C57BL/6 and Swiss-Webster mice (7-8 wks old) were obtained from Charles Rivers Laboratories (Wilmington, MA) or Harlan (Indianapolis, IN). Before experiments, mice were acclimated for at least 7 days in an environment with controlled temperature (21-23°C), and

lighting (12 h light/12 h dark) with free access to tap water and regular chow diet. A detailed procedure of 75% partial Px in mice has been described previously.⁸ All procedures were approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee (IACUC).

Immunohistochemical analysis

Immunohistochemical staining was performed by the dextran polymer method using Dako EnVision+ system as we have described previously.⁸

Protein extraction and Western blot analysis

Protein extraction and Western blot analysis were performed as previously described⁸. Dilution factor for primary antibodies was 1:1000 for antibodies against phosphorylation of Akt (pAkt), Akt, and p85 α , 1:5000 for anti-PDX-1 antibody, and 1:2000 for anti- β -actin antibody, respectively.

siRNA *in vivo* delivery

Non-targeting control or p85 α siRNA (20 μ g/mouse) was delivered to Swiss-Webster mice by hydrodynamic tail vein injection^{31, 32} using *Trans IT In Vivo* Gene Delivery System as described previously⁸. siSTABLE SMARTpool *in vivo* siRNA from Dharmacon was injected into the mice, and 2 days later, the mice underwent either partial Px or sham operation. We have previously shown that administration of siRNA targeting the PI3K p85 α subunit effectively blocked PI3K/Akt activation and pancreatic regeneration after partial Px.⁸

Statistical analysis

The results of insulin-positive duct cell index *in vivo* were analyzed using a two-group t-test. The effect was assessed at the 0.05 level of significance as the experiment-wise error rates. Data analysis was conducted using Statview software for Windows version 5.0 from SAS (Cary, NC).

RESULTS

Akt phosphorylation and PDX-1 expression are concomitantly increased in the ducts of regenerating pancreas

To determine whether PI3K/Akt activation may play a role in PDX-1 expression, we first assessed the remnant pancreas from mice after partial 75% Px for expression of both pAkt and PDX-1 by Western blot and immunohistochemistry. Western blot analysis on total protein samples from the remnant pancreas detected an increased expression of pAkt and PDX-1 after partial Px (Fig. 1A) confirming previous observations.^{8,12} Immunohistochemical analysis found that PDX-1 and pAkt were increased in pancreatic ducts on day 3 compared to day 0 after partial Px; both PDX-1 and pAkt were still detected in several ducts on day 7 after partial Px (Fig. 1B). Increased pAkt was also detected on day 3 in acinar cells as we have previously reported⁸ (data not shown). Analysis of serial sections of the remnant pancreas revealed that the majority of PDX-1 positive cells after partial Px were also positive for pAkt (Fig. 1C). These results demonstrate concomitant PI3K activation and PDX-1 induction in the ducts of regenerating pancreas.

PI3K inhibition effectively blocks PDX-1 expression in regenerating pancreas

To examine whether inhibition of PI3K can block PDX-1 expression in pancreatic ducts during pancreatic regeneration, mice received either control siRNA or p85 α siRNA and underwent either partial 75% Px or sham operation (n=3 per group). Both PDX-1 and pAkt were increased in the remnant pancreas of mice treated with control siRNA 3 days after partial Px; these

increases were blocked by p85 α siRNA (Fig. 2A). To confirm that treatment with p85 α siRNA effectively suppressed pancreatic protein levels of p85 α and pAkt, Western blot analysis was performed on total protein extracts. Treatment with p85 α siRNA effectively reduced pancreatic p85 α protein levels by approximately 80% (Fig. 2B). Moreover, p85 α siRNA effectively blocked induction of pAkt and PDX-1 levels in the remnant pancreas of mice after partial Px (Fig. 2C). Taken together, these results demonstrate that the PI3K p85 α regulatory subunit is important for PDX-1 expression in the remnant pancreas during pancreatic regeneration.

Using wortmannin, we confirmed the effect of PI3K inhibition on PDX-1 expression in the pancreatic ducts during pancreatic regeneration. Mice were treated with either wortmannin or vehicle, underwent partial Px or sham operation, and sacrificed 3 days later; pAkt and PDX-1 expression were determined in the remnant pancreas by immunohistochemistry (n=3 per group). In the vehicle-injected group, pAkt and PDX-1 were abundantly localized to duct and islet cells of the remnant pancreas 3 days after Px (Fig. 3). However, treatment with wortmannin effectively blocked both pAkt and induction of PDX-1 in the ducts of the remnant pancreas (Fig. 3); pAkt and PDX-1 expression in islet cells was similar to the control (i.e., vehicle injection) group (data not shown).

Taken together, these complementary techniques utilizing the chemical inhibitor wortmannin and p85 α siRNA demonstrate that PI3K activation plays an important role in induction of PDX-1 expression in the pancreatic ducts.

Wortmannin blocks appearance of insulin-positive duct cells in remnant pancreas during pancreatic regeneration

Next, we examined the effect of PI3K inhibition on the differentiation of pancreatic duct cells to insulin-positive cells. Young adult mice underwent either partial 75% Px or sham operation (n=12 per group); each group was further subdivided to receive either vehicle (n=5) or wortmannin (n=7), an irreversible PI3K inhibitor. Mice were sacrificed 3 days after the surgery, and insulin-positive duct cells were immunohistochemically analyzed in the remnant pancreas from the Px groups and the remnant equivalent tissues from the sham-operated groups (Fig. 4). As previously reported,²⁰ insulin-positive duct cells are observed in the remnant pancreas of mice after partial Px and saline injections; however, wortmannin significantly reduced the number of insulin-positive duct cells in the remnant pancreas (p<0.05). In total, 4761 and 9209 duct cells were counted in the control (ie, saline injection) and wortmannin groups with 35 (0.73%) and 25 (0.27%) duct cells positive for insulin staining, respectively. There were no insulin-positive duct cells in the pancreas of sham-operated mice. These findings suggest that the PI3K signaling pathway plays a role in the differentiation of pancreatic duct cells to insulin-positive cells during pancreatic regeneration.

DISCUSSION

The molecular mechanisms of pancreatic duct cell differentiation into islet cells in adult animals have not been clearly defined. Although PI3K is a critical step for proliferation of various cell types and insulin signaling, its role in duct differentiation into endocrine cells is not known. pAkt, an indicator of PI3K activation, is significantly increased in the remnant pancreas during pancreatic regeneration after partial Px.⁸ Expression of PDX-1 is increased in remnant pancreas a few days after partial Px.¹² The PI3K pathway, but not the p38 MAPK pathway, has an important role in glucose-stimulated preproinsulin gene transcription and nuclear trans-location of PDX-1 in islet β -cells.³³ Since duct-specific pAkt and induction of PDX-1 in remnant pancreas has not been studied, we examined whether pAkt and induction of PDX-1 occurs in duct cells during pancreatic regeneration. We found that PI3K/Akt activation (as assessed by Akt phosphorylation) and PDX-1 induction occurred in the ducts

concomitantly during pancreatic regeneration, and that inhibition of PI3K suppressed expression PDX-1 in duct cells during pancreatic regeneration.

Partial Px induces pancreatic regeneration and islet neogenesis,³⁴ insulin-positive cells are detected in the duct of the remnant pancreas during pancreatic regeneration.²⁰ Given the role of PI3K/Akt in acinar cell regeneration noted in our previous study,⁸ we next hypothesized that PI3K activation is important for duct-derived islet cell neogenesis during partial Px-induced pancreatic regeneration. Our result demonstrated that inhibition of PI3K effectively blocked duct cell differentiation into insulin-producing cells in the regenerating pancreas. These results clearly show that activation of the PI3K pathway plays a critical role in PDX-1 induction in pancreatic ducts during pancreatic regeneration. PDX-1 is an important factor for the pancreatic duct cell differentiation; pancreatic ductal-derived cancer cells, Panc-1, differentiated to insulin-producing cells upon forceful expression of exogenous PDX-1.²⁵ Thus, our results also suggest a possible involvement of the PI3K pathway in duct cell differentiation into insulin-producing cells.

There may be two distinct roles for PI3K activation in the ducts during pancreatic regeneration. One role is to promote differentiation of pancreatic duct cells into insulin-producing cells. This function requires induction of PDX-1 in the duct cells and is clearly supported by our results with wortmannin and p85 α -siRNA. The second role for PI3K activation in the ducts may be to promote proliferation of duct cells for tissue regeneration. As we have previously reported, activation of the PI3K pathway is critical for acinar cell proliferation during pancreatic regeneration.⁸ Thus, it is reasonable to speculate that PI3K has a similar function for duct cells. In our previous study, we also found increased phosphorylation of ERK in the ducts during pancreatic regeneration raising a possibility that MAPK/ERK plays a role in the ducts during pancreatic regeneration. The involvement of PI3K and/or MAPK pathway in the duct cell proliferation during pancreatic regeneration is currently being evaluated.

PI3K is divided into three classes (ie, Class I, II and III). Class IA PI3K, which is composed of a p85 regulatory and p110 catalytic subunit in mammalian cells, regulates cell proliferation, cell survival and apoptosis in various cell types.¹ In this study, we clearly demonstrate that Class IA PI3K plays a central role in PDX-1 expression in pancreatic duct cell during pancreatic regeneration. At present, it is not known whether other classes of PI3K also mediate PDX-1 expression in duct cells. Blocking specific PI3K subunits by RNA interference techniques should be able to answer this question.

The physiological significance of the differentiation of pancreatic duct cells into insulin-producing cells is not currently clear. While islet β -cell mass is replenished mostly by β -cell replication, a certain role for the neogenesis of β -cells from stem cells cannot be excluded.¹⁵⁻¹⁸ The occasional detection of insulin in the pancreatic duct epithelium by a number of investigators has led to the hypothesis that the duct cells possess a stem cell-like function to differentiate into endocrine cells under certain conditions (eg, regeneration).^{19, 20} Detection of insulin-positive duct cells only after partial Px noted in our study supports this hypothesis.

Our current study strongly suggests that activation of PI3K, especially Class IA PI3K, plays a critical role in PDX-1 expression and differentiation of pancreatic duct cells into insulin-secreting cells. These findings provide a further understanding of the molecular mechanisms of pancreatic duct cell differentiation in the adult pancreas following physiologic stimulation. Consistent with our previous findings⁸, it is possible that PI3K/Akt activation is critical for not only pancreatic acinar cell regeneration but also duct cell proliferation following partial pancreatectomy. These findings further support the important role of PI3K in the regulation of physiologically stimulated growth of various organs including the pancreas, intestinal mucosa and heart.^{8, 35-38}

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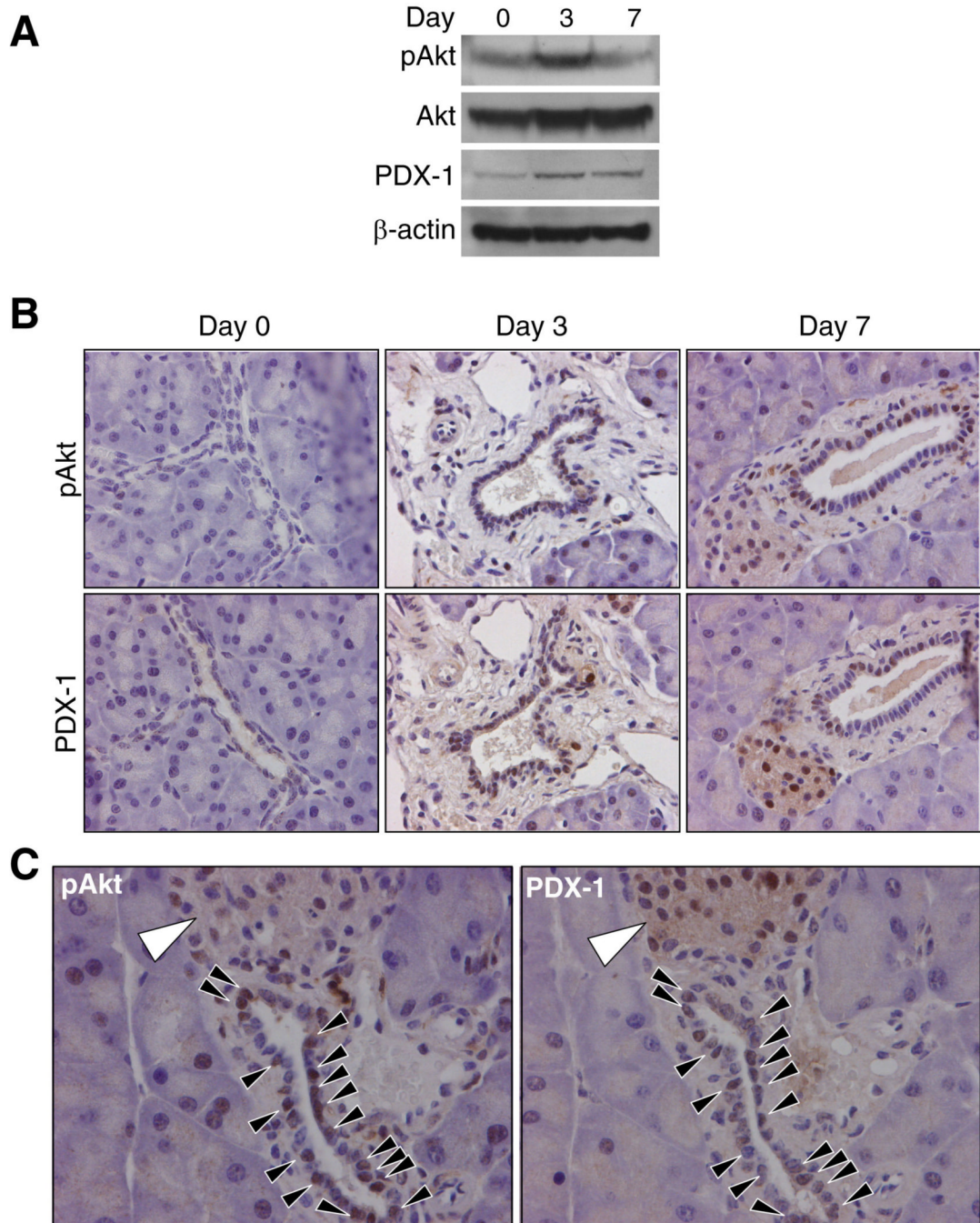


Figure 1. Concomitant PDX-1 expression with Akt phosphorylation in remnant pancreas during pancreatic regeneration

(A) Western blot analysis assessing expression of pAkt and PDX-1 in remnant pancreas at Day 0 (before surgery), and Day 3 and 7 after 75% partial Px. This figure shows one experiment testing 3 mice per group; results were similar in a repeated experiment. (B) The expression of pAkt and PDX-1 in remnant pancreas was immunohistochemically determined on Day 0, 3 and 7 after partial Px (magnification $\times 400$). (C) Simultaneous expression of pAkt and PDX-1 in pancreatic duct cells was immunohistochemically determined on serial sections of remnant pancreatic tissues after partial Px. Closed arrow heads indicate pAkt or PDX-1 positive duct

cells. Islets, which contain pAkt or PDX-1 positive cells, are indicated by large open arrow heads. Slides were counterstained with hematoxylin.

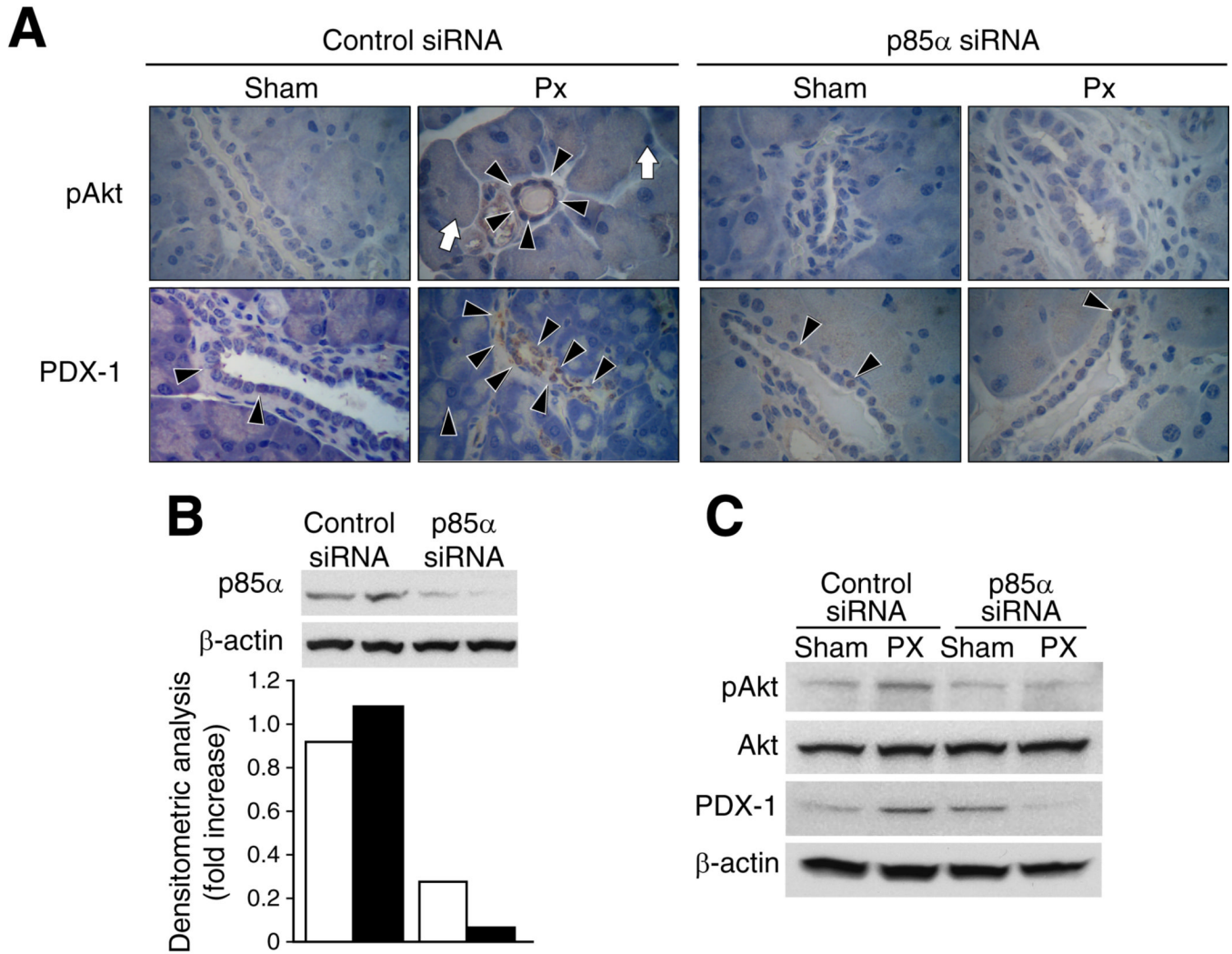


Figure 2. PI3K p85α regulatory subunit siRNA suppresses PDX-1 expression in remnant pancreas during pancreatic regeneration

(A) Non-targeting control or p85α siRNA (20 μg/mouse) was delivered to mice by hydrodynamic tail vein injection 2 days prior to either partial Px or sham operation. All mice were sacrificed 3 days after the operation, and the remnant pancreas of Px-mice or the remnant equivalent tissue segments of sham-operated mice were collected, and Akt phosphorylation (pAkt) and PDX-1 expression assessed by immunohistochemistry (n=3 per group). Typical positive areas are indicated as follows: arrow head = pAkt or PDX-1 positive duct cell (magnification × 400), white arrow=pAkt positive acinar cells. Slides were counterstained with hematoxylin. (B) To confirm whether p85α siRNA effectively reduces target protein, p85α protein levels were assessed by Western blot; the blot was stripped and reprobbed with β-actin. Densitometric analysis of the blot is shown in the bottom panel. Mice were sacrificed 2 days after siRNA delivery. (C) Western blot analysis of pAkt, Akt and PDX-1 expression in remnant pancreas was assessed 3 days after partial Px. Each lane represents pooled samples from 2 mice at the indicated times.

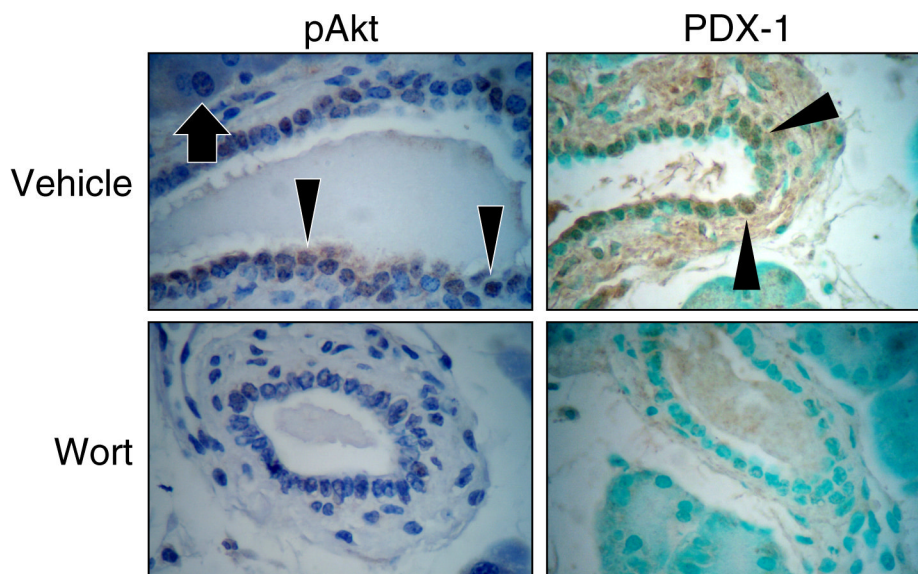


Figure 3. PI3K inhibitor wortmannin suppresses PDX-1 expression in remnant pancreas during pancreatic regeneration

The effect of PI3K inhibition by wortmannin (Wort) on pAkt and PDX-1 expression in duct cells of remnant pancreas was assessed by immunohistochemistry as described in Fig. 1. Mice underwent partial Px and sacrificed 3 days after operation (n=3 per group). Arrow heads indicate typical pAkt or PDX-1 positive duct cell (magnification $\times 400$). Arrows indicates pAkt-positive acinar cell. Slides were counterstained with hematoxylin.

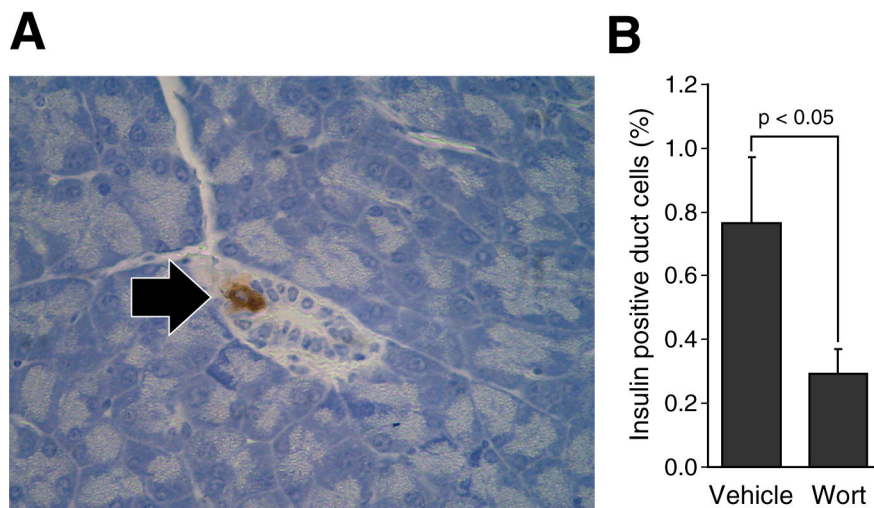


Figure 4. Wortmannin, a pharmacological PI3K inhibitor, significantly reduced the induction of insulin-positive duct cells in remnant pancreas after partial Px
 (A) An example of immunohistochemistry detecting an insulin-positive cell (arrow) in the duct of regenerating remnant pancreas 3 days after partial Px. (B) Insulin-positive duct cell index was calculated in pancreatic sections from mice after partial Px mice that were injected either with wortmannin (wort: 0.75mg/kg, i.p.) (n = 7) or vehicle (physiological saline with 5% ethanol) (n = 5) 2 h before the operation and then every 12 h thereafter. Mice were sacrificed 3 days after the surgery, and three independent sections were prepared from each mouse. All insulin-positive duct cells were counted in one section and insulin-positive duct cell index was calculated by division of all pancreatic duct cells in the same section (Values are mean \pm SEM; * = $p < 0.05$).