

# Growth factor-induced signaling of the pancreatic epithelium

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## Abstract

Activated signaling proteins regulate diverse processes, including the differentiation of the pancreatic islet cells during ontogeny. Here we uncover the *in vivo* phosphorylation status of major growth factor-activated signaling proteins in normal adult mice and during pancreatic islet regeneration. We report elevated phospho-mitogen-activated protein kinase (phospho-MAPK), phospho-c-Jun-NH<sub>2</sub>-terminal kinase (phospho-JNK), and phospho-p38 MAPK expression during pancreatic regeneration. Immunoblotting experiments demonstrated elevated phosphorylation of p52 Src-homology/collagen (SHC) in the ductal network as well, substantiating the activation of this pathway. Furthermore, protein kinase B (PKB/Akt), a key signaling protein in the anti-apoptotic pathway, was

phosphorylated to a greater extent in the ductal network from regenerating pancreas. We observed fibroblast growth factor (FGF)10 and platelet-derived growth factor (PDGF)AA expression in embryonic as well as regenerating adult pancreas. Epidermal growth factor (EGF) and PDGFAA stimulated MAPK and Akt phosphorylation, while FGF10 stimulated MAPK but not Akt phosphorylation in a time-dependent manner in freshly isolated cells from the adult ductal network. These data suggest that a heightened level of expression and stimulation of key signaling proteins underlie the expansion and differentiation processes that support pancreatic ontogeny and regeneration.

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## Introduction

Transduction of cellular signals is critical for development of mature cells and tissues. The regulation of mitogenesis by growth factors such as epidermal growth factor (EGF), erbB ligands, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) is primarily enacted via two pathways: the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3 kinase) pathways. Once growth factors phosphorylate their receptors, the receptor can bind the adaptor proteins Grb2 or Src-homology/collagen (SHC). Upon phosphorylation, SHC can also associate with Grb2, which binds the exchange factor Son-of-sevenless (SOS); SOS translocates to the plasma membrane where Ras and Raf are activated, after which they can activate MAPK (Bonfina *et al.* 1996, Schlessinger 2000). Another major signaling pathway activated by growth factors, the PI3 kinase pathway phosphorylates phosphatidylinositols, yielding lipid products that bind the downstream signaling intermediate Akt, targeting it to the cell membrane, where it is activated by serine and threonine phosphorylation. Akt has been studied extensively for its role as a survival signal (Datta *et al.* 1997, Coffey *et al.* 1998). The phosphorylation state of the aforementioned signaling molecules at any point in

time reflects the convergence of signals from different growth factors, cytokines, chemokines, integrins and other agents.

The activity of transcription factors such as fos, jun, atf2 and MEF2C is regulated by phosphorylation by the MAPK pathways (Widmann *et al.* 1999). During pancreatic ontogeny, transcription factors such as Pdx-1/Ipf1, Isl-1 and PAX6 regulate the differentiation of pancreatic cell types (reviewed in Edlund (2002)). There is interesting emerging evidence that the activity of transcription factors such as Pdx1 and the members of the PAX family may also be modulated by phosphorylation by MAPK, c-Jun-NH<sub>2</sub>-terminal kinase (JNK) or p38 MAPK (Cai *et al.* 2002). Glucose stimulation of Pdx-1 activation involves phosphorylation by p38 MAPK (Macfarlane *et al.* 1999). Interestingly, overexpression of p38 MAPK mimics stimulation by glucose. MAPK and p38 MAPK have been reported to phosphorylate PAX6 *in vitro* and cotransfection of PAX6 plasmids with p38 MAPK or MKK6 (the kinase upstream of p38 MAPK) was found to enhance the transactivation potential of PAX6 (Mikkola *et al.* 1999).

Several growth factors have been demonstrated to be critical for pancreatic development. Epidermal growth factor receptor (EGFR) and several related members of the EGFR family are expressed in the developing

pancreas (Kritzik *et al.* 2000a, Miettinen *et al.* 2000, Huotari *et al.* 2002). Mice with targeted disruption of the EGFR gene show disturbed migration and delayed differentiation of beta cells in the developing islets in the embryonic pancreas (Miettinen *et al.* 2000, Cras-Meneur *et al.* 2001). FGFs are a large family with over 22 members involved primarily in proliferation, migration and differentiation in the embryo (Ornitz & Itoh 2001). In the adult, FGFs are associated with tissue repair and response to injury (Floss *et al.* 1997). The expression of dominant negative forms of either FGFR1 or FGFR2 do not affect growth or morphogenesis of the pancreas, implying redundant pathways growth control by these FGF receptors (Hart *et al.* 2000). One particular ligand, FGF10, is important in pancreas development. The FGF10 knockout mouse, besides lacking limb and lung development, displays a marked attenuation of insulin-expressing cells, suggesting a requirement of FGF10 for adequate islet development (Ohuchi *et al.* 2000, Bhushan *et al.* 2001, Cras-Meneur & Scharfmann 2002, Elghazi *et al.* 2002). A very recent study elegantly demonstrated that transgenic expression of FGF10 under the control of the Pdx-1 promoter results in an increased proliferation of pancreatic progenitor cells, coupled with an absence of endocrine cells (Norgaard *et al.* 2003). Finally, PDGFs have been extensively studied in development by utilizing loss of function mutations for the PDGFs and PDGFRs in mice (Hoch & Soriano 2003). What is particularly interesting in relation to the study of a regeneration model is that the PDGFs appear to act on specific populations of progenitor cells that generate distinct cell types in a number of developmental processes (Betsholtz *et al.* 2001). In a classical study of the expression of tyrosine kinase receptors in embryonic pancreas development, striking expression of PDGF receptors was reported in the embryonic day 13 (E13) pancreas (LeBras *et al.* 1998).

We have undertaken the present study to investigate the signaling proteins involved in growth responses in the pancreatic epithelium. We have examined epithelial responses since pancreatic cells arise from this tissue during embryonic development and pancreatic regeneration (Pictet *et al.* 1972, Slack 1995, Apelqvist *et al.* 1999, Edlund 2001). For these studies we have utilized regenerating pancreatic tissue, which we have previously demonstrated to express a number of growth factors including EGF (Arnush *et al.* 1996) and other members of the EGF family of ligands and receptors (Kritzik *et al.* 2000a). This spontaneous model recapitulates pancreatic ontogeny (Gu & Sarvetnick 1993, 1994, Kritzik *et al.* 1999, 2000b) and provides access to pools of duct epithelial progenitor cells for detailed analysis *in vitro*. We have tested the hypothesis that key signaling intermediates demonstrate a heightened state of activation in these regenerating pancreatic ducts. We also defined which signaling pathways are stimulated in the pancreatic epithelium following exposure to growth factors potentially critical for pancreatic

differentiation. Our work reveals potential ligands that can be utilized for the *in vitro* expansion and differentiation of islets.

## Materials and Methods

### Experimental animals

The transgenic mice expressing interferon (IFN) $\gamma$  in the pancreatic beta cells have been previously described (Sarvetnick *et al.* 1988, 1990, Gu & Sarvetnick 1993, 1994). The IFN $\gamma$  transgenic mice used in the present study were maintained on the non-obese-diabetic (NOD) background. The NOD background mice were used as controls. The presence of the transgene was confirmed by PCR. Eight- to ten-week-old non-transgenic NOD and littermate transgenic mice, which display significant expansion and regeneration in the pancreas, were utilized for the immunohistochemical evaluation of the subcellular localization of signaling proteins and the pancreatic duct cell purification procedure. Embryos were obtained from timed pregnancies of NOD mice on embryonic day 16. All animal experiments were performed in accordance with the ethical guidelines specified by the Scripps Research Institute.

### Pancreatic ductal cell purification

Cells of the pancreatic ductal network were purified from 8-week-old and IFN $\gamma$ NOD mice for assessment of *in vivo* and *in vitro* phosphorylation of signaling proteins. The pancreata were digested with collagenase (1 mg/ml) (Roche) for approximately 45 min. The digest was filtered through a 200  $\mu$ m mesh. Subsequently, the ductal network above the mesh was treated with 0.05% trypsin, 0.53 mM EDTA. The cell suspension was filtered through a 70  $\mu$ m cell strainer, and the cells in the filtrate were resuspended in RPMI medium. Approximately 1 million duct cells were derived from each pancreas preparation.

### Assessment of the expression of signaling proteins in NOD and IFN $\gamma$ NOD pancreas by immunoblotting

Freshly isolated cells from the ductal network of NOD and IFN $\gamma$ NOD mouse were lysed in ice-cold radioimmuno-precipitation assay (RIPA) buffer containing 20 mmol/l Tris, pH 7.5, 1 mmol/l EDTA, 140 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride and 10  $\mu$ g/ml aprotinin. Cell lysates were solubilized in Laemmli's buffer and boiled for 5 min and the proteins were resolved on a 12% SDS-PAGE gel.

The proteins were subsequently transferred to nitrocellulose membranes and immunoblotted with antibodies to dually phosphorylated phospho-MAPK, phospho-JNK

and p38 phospho-MAPK as well as to phospho-Akt and phospho-SHC. The polyclonal phospho-MAPKinase antibodies were generated against synthetic phospho-threonine phospho-tyrosine peptides corresponding to residues around Thr202/Tyr204 of human p44 MAPK (extracellular regulated kinase (ERK)1), Thr180/Tyr182 of human p38 MAPK and Thr183/Tyr185 of human JNK/stress-activated protein kinase (SAPK), which have all been demonstrated to recognize mouse tissue (Cell Signaling Technology, Beverly, MA, USA). The polyclonal phospho-Akt antibodies were generated against a synthetic phospho-Ser473 peptide corresponding to residues around mouse Akt (Cell Signaling Technology). The polyclonal phospho-SHC antibodies were generated against a synthetic phospho-Tyr317 peptide corresponding to the residues surrounding human SHC, and were demonstrated to recognize mouse tissue (Cell Signaling Technology). The expression of the non-phosphorylated form of the signaling molecules was assessed using polyclonal antibodies to ERK1 and Akt1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), SAPK/JNK and p38 MAP Kinase (Cell Signaling Technology), and SHC (BD Biosciences, San Diego, CA, USA). Mouse monoclonal antibodies to tubulin- $\alpha$  (Neomarkers, Fremont, CA, USA) were used to examine the relative expression of tubulin in ductal cell lysates from IFN $\gamma$ NOD transgenic and NOD pancreas. Autoradiograms similar to those presented in Fig. 1 were scanned and analysed by densitometry using National Institutes of Health (NIH) image 1.63 for quantitation.

#### *Immunohistochemical assessment of in vivo expression of signaling proteins in NOD and IFN $\gamma$ NOD pancreas*

Pancreata from IFN $\gamma$ NOD transgenic mice, NOD mice and E16 embryos were fixed in 10% neutral buffered formalin or Bouin's fixative and processed for paraffin embedding. Embedded tissue was cut into 4  $\mu$ m thick sections and stained with the primary antibodies to PDGFAA and FGF10 (Santa Cruz Biotechnology, Inc.) and the antibodies indicated in the preceding section and counterstained with hematoxylin. For phospho-p38 MAPK staining the pancreata from IFN $\gamma$ NOD and NOD mice were flash frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Finetechnical Co. Ltd., Tokyo, Japan), and 4  $\mu$ m sections were cut for staining.

#### *Assessment of EGF-, FGF10- and PDGFAA-stimulated MAPK and Akt phosphorylation*

Freshly isolated cells from the pancreatic ductal network from IFN $\gamma$ NOD transgenic mice were serum-starved overnight and stimulated with 10 ng/ml recombinant human EGF (Research Diagnostics, Inc., Flanders, NJ, USA), 50 ng/ml human recombinant FGF-10 (Research Diagnostics, Inc.) or 10 ng/ml human recombinant PDG-

FAA (Research Diagnostics, Inc.) for 0, 2, 5, 10, 30, 60 min at 37 °C. Cells were then lysed with RIPA buffer described in the preceding section, and solubilized on ice, and the proteins were resolved on a 12% SDS-PAGE gel. The proteins were subsequently transferred to nitrocellulose membranes and immunoblotted with antibodies to dually phosphorylated phospho-MAPK and phospho-Akt. The antibodies utilized are described in the previous section. Membranes were stripped and reblotted with mouse mAb to actin (ICN Biomedicals, Aurora, OH, USA) to confirm equal protein loading except in the case of PDGFAA stimulation of MAPK, where the membrane was reblotted with a rabbit polyclonal antibody to ERK (Santa Cruz Biotechnology, Inc.).

#### *Statistical analysis*

A *t*-test was performed to determine whether the fold-increase in the expression of phosphorylated and non-phosphorylated signaling proteins was different from a hypothetical mean of 1.

## Results

#### *Stimulation of the MAPK signaling pathway*

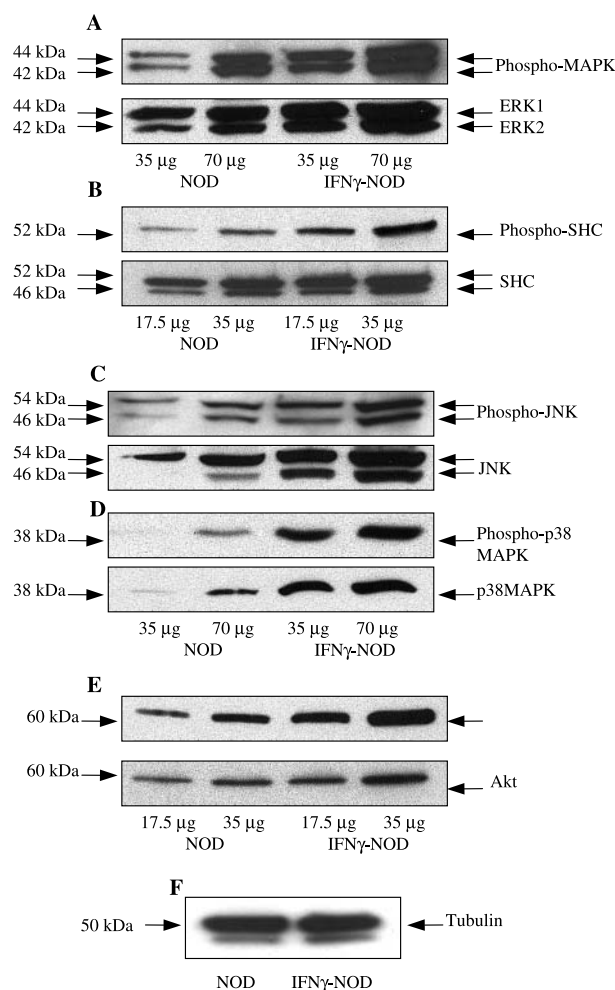
Peptide growth factors constitute an important component in the regulation of pancreatic development and regeneration, and are the major external signals that activate the MAPK pathway (Denhardt 1996, Widmann *et al.* 1999). We determined the endogenous *in vivo* phosphorylation state of ERK1 (p44 MAPK) and ERK2 (p42 MAPK) protein in the ductal networks of the IFN $\gamma$ NOD transgenic mouse pancreas and NOD controls. Although we could isolate fewer pancreatic duct cells from NOD mice compared with the IFN $\gamma$  transgenics, we found equivalent proportions of CD49f+ (epithelial) cells and cytokeratin+ (ductal) cells in these two populations (FACS analysis; data not shown). We observed an increasing trend in ERK1 (1.3-fold,  $n=4$ , N.S.) and ERK2 (1.6,  $n=4$ , N.S.) and a statistically significant enhancement in phospho-MAPK expression in the ductal network of IFN $\gamma$ NOD compared with NOD mice by immunoblotting (Fig. 1A). We found a 2.2-fold increase in ERK2 (p42) ( $n=5$ ,  $P=0.04$ ) and a 2.7-fold increase in ERK1 (p44) ( $n=5$ ,  $P=0.02$ ) phosphorylation in the IFN $\gamma$ NOD ductal network compared with NOD mice. Since an increase in actin expression was observed in the regenerating rat liver (Goldsworthy *et al.* 1993), rendering this marker unsuitable for quantitation, Amido Black 10B (BioRad) staining was utilized to confirm equal protein loading. Furthermore, we have found that tubulin- $\alpha$  expression remains constant in the IFN $\gamma$ NOD pancreas duct cells compared with the NOD duct population (Fig. 1F).

One of the ways that peptide growth factors activate the MAPK pathways is through the interaction of their

transmembrane receptors with the adaptor protein SHC (Bonfini *et al.* 1996, Ravichandran 2001). We therefore examined the expression of SHC and phospho-SHC in the ductal networks of NOD and IFN $\gamma$ NOD mice. The dominant isoform of SHC expressed in the duct cells of both the NODs and the transgenic mice was p52 SHC, and its phosphorylation was significantly enhanced in the regenerating pancreas (Fig. 1B,  $P=0.02$ ). It is important to note that there is no significant increase in p52 SHC protein expression ( $n=2$ ) despite an average 3-fold increase in phospho-SHC expression ( $n=3$ ).

#### Stimulation of the stress-activated MAPK pathway

Cytokines constitute a major category of molecules that activate the stress MAP kinases (Davis 2000, Kyriakis & Avruch 2001). We hypothesized that IFN $\gamma$  and the chemokines that it induces *in vivo* (Kayali *et al.* 2003) may result in the stimulation of the stress MAP kinases, JNK and p38 MAPK. We therefore examined the expression and phosphorylation state of JNK and p38 MAPK *in vivo*.



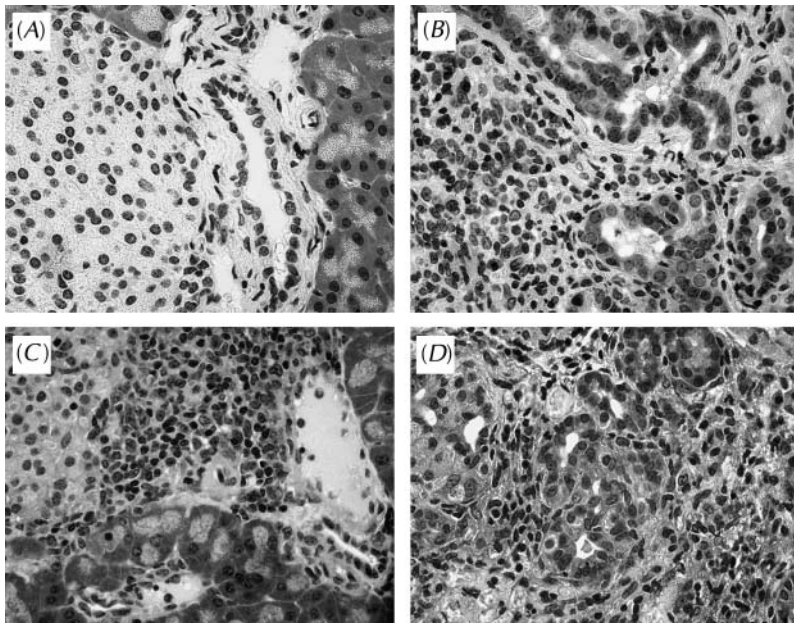
In whole-cell lysates of the ductal network both the p46 and p54 JNK were phosphorylated to a greater extent in the regenerating ductal epithelium (Fig. 1C) (2- to 3-fold,  $n=4$ ). The expression of the unphosphorylated JNK proteins exhibited the same pattern of enhancement (approximately 2-fold,  $n=4$ ,  $P=0.06$ ). Phospho-p38 expression was elevated significantly in the expanding pancreas (Fig. 1D; 4.2-fold,  $n=6$ ,  $P=0.05$ ). The augmentation in p38 protein was comparable to the rise in the phosphorylated species (4.5-fold,  $n=6$ ,  $P=0.03$ ).

#### *In vivo* serine 473 phosphorylation of Akt in duct epithelial cells

Activation of phospho-Akt has been described as a key event of signal transduction pathways regulating cellular proliferation, glucose metabolism and protection from apoptosis (Coffer *et al.* 1998, Vanhaesebroeck & Alessi 2000, Brazil & Hemmings 2001). Growth factors such as insulin-like growth factor (IGF)-I, FGF-2, EGF, PDGF, nerve growth factor (NGF), leukemia inhibitory factor (LIF), stem cell factor (SCF) and vascular endothelial growth factor (VEGF) have been demonstrated to regulate the Akt kinase activity through the stimulation of PI3 kinase (Coffer *et al.* 1998). To assess the *in vivo* phosphorylation state of Akt, whole-cell lysates were solubilized and the proteins were immunoblotted with antibodies to phospho (Ser473)-Akt or Akt 1/2. We found that serine 473 phosphorylation of Akt was elevated 4.7-fold in the transgenic pancreatic ductal cells compared with the cells isolated from the NOD pancreas (Fig. 1E;  $n=5$ ,  $P=0.02$ ). By immunoblotting, Akt protein expression displayed a small but significant increase (1.4-fold,  $n=5$ ,  $P=0.04$ ).

In summary, the expression of: (i) total Akt and p38 MAPK, (ii) dually phosphorylated ERK1, ERK2, p54 JNK and p38 MAPK; and (iii) phosphorylated SHC

**Figure 1** The *in vivo* expression of phosphorylated and non-phosphorylated signaling proteins in the ductal network cells from the IFN $\gamma$ NOD and NOD pancreas. (A) Expression of ERK1, ERK2 and dually phosphorylated MAPK are increased in the ductal network cells from IFN $\gamma$ NOD transgenic mouse pancreas compared with the NOD mouse pancreas. (B) Elevated *in vivo* phosphorylation of p52 SHC in the IFN $\gamma$ NOD pancreatic ducts. Note that both the transgenic and non-transgenic mice express the 46 and 52 kDa isoforms of SHC. Of these two isoforms, the p52 seems to be preferentially phosphorylated, its phosphorylation being significantly enhanced in the IFN $\gamma$  transgenic. (C) Expression of JNK and expression of the dually phosphorylated phospho-JNK are elevated in the duct cells from IFN $\gamma$ NOD transgenic mouse pancreas compared with the NOD mouse pancreas. (D) The expression of p38 MAPK protein and the extent of p38 MAPK phosphorylation are both potently elevated in the duct cells from IFN $\gamma$ NOD transgenic mouse pancreas compared with the NOD mouse pancreas. (E) Expression of Akt and phospho-Akt are elevated in the duct cells from the IFN $\gamma$ NOD transgenic mouse pancreas compared with the NOD mouse pancreas. (F) Tubulin expression in duct cells from NOD pancreas and the IFN $\gamma$ NOD pancreas (40 μg protein loaded in each case) does not vary.



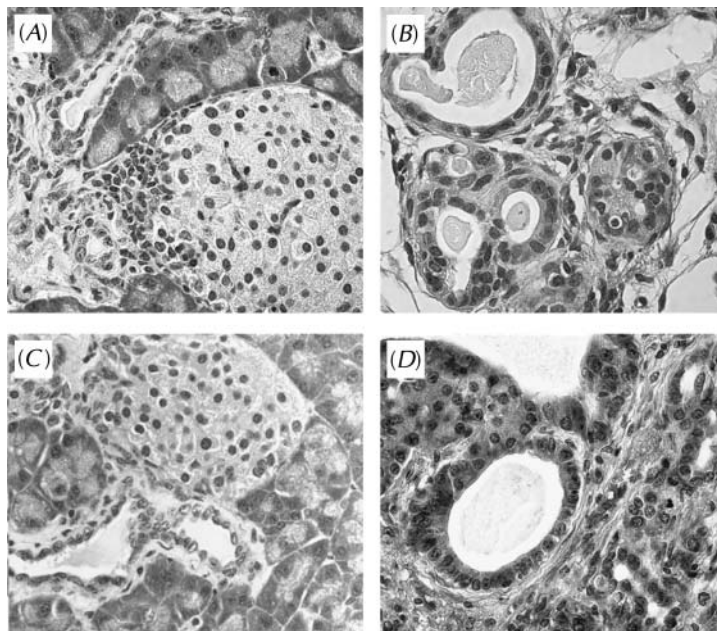
**Figure 2** Phospho-MAPK and ERK1/2 staining in the IFN $\gamma$  transgenic pancreas. Pancreatic sections from NOD (A and C) and IFN $\gamma$ NOD (B and D). (A and B) Sections stained with an antibody to the dually phosphorylated MAPK (Thr202/Tyr204). Note the nuclear expression of phospho-MAPK in the ducts. MAPK translocates to the nucleus upon activation. (C and D) Sections stained with anti-ERK1/2 antibody. ERK1/2 expression is evident in the ducts, the infiltrating lymphocytes and the islets in the transgenic pancreas. Original magnification  $\times 50$ .

(Tyr316) and phosphorylated Akt (Ser473) were significantly enhanced in the IFN $\gamma$  pancreatic ductal network compared with its NOD counterpart. These findings suggest that the regulation of the signaling protein activation occurs at the level of protein expression as well as at the level of phosphorylation.

#### *Immunolocalization of the signaling proteins in the regenerating pancreas*

To determine the localization of these events in the pancreas we proceeded to do histological studies using antibodies to phosphorylated MAPK, JNK, p38 and Akt, and antibodies to the corresponding non-phosphorylated proteins. We determined that the ductal cells of the IFN $\gamma$ NOD pancreas express phospho-MAPK (Fig. 2B) whereas ductal staining was absent in the NOD (Fig. 2A). ERK1/2 expression was diffuse but extensive in the expanding ductal regions of the IFN $\gamma$  pancreas (Fig. 2D), whereas ERK1/2 staining was faint and appeared to be localized to both the islet cells and surrounding infiltrating cells in the NOD pancreas (Fig. 2C). We next examined the localization of phosphorylated JNK and p38. Phosphorylated JNK was expressed in the expanding ducts, both within the ductal cells and in the infiltrating cells surrounding the ducts (Fig. 3B). In the NOD mouse, a

small amount of phospho-JNK was expressed in the infiltrate around the islets (Fig. 3A). JNK protein was also expressed in the ducts in the IFN $\gamma$  transgenic pancreas as well as in the immune cells surrounding the ducts (Fig. 3D). In the NOD mice, the expression of JNK protein was faint and appeared in a few of the duct cells and islet cells (Fig. 3C). In the adult regenerating pancreas, the expression of phosphorylated p38 MAPK was observed in a number of duct cells and in the infiltrate surrounding the ducts (Fig. 4B), in contrast to the NOD pancreas where there was no evidence of p38 phosphorylation (Fig. 4A). In the regenerating pancreas the expression of p38 protein was enhanced primarily in the duct cells and in neighboring cells of immune origin (Fig. 4D), while in the NOD mouse pancreas a modest level of p38 protein expression was seen in the pancreatic ducts and in the infiltrated areas adjacent to islets (Fig. 4C). Immunolocalization experiments demonstrated elevated phospho-Akt expression in the ductal and periductal cells of the regenerating pancreas in the transgenic mice (Fig. 5B). In the NOD pancreas phosphorylated Akt was expressed in a subset of cells in the periphery of the islets (Fig. 5A). The ducts and some of the infiltrating cells in their vicinity displayed extensive staining for Akt protein (Fig. 5D) while very few of the ductal cells and a number of islet cells in the NOD pancreas exhibited subtle Akt staining (Fig. 5C).



**Figure 3** Phospho-JNK and JNK staining in the IFN $\gamma$  transgenic pancreas. Pancreatic sections from NOD (A and C) and IFN $\gamma$ NOD (B and D). (A and B) Sections stained with an antibody to the dually phosphorylated phospho-JNK (Thr183/Tyr185). The proliferating ducts and the surrounding infiltrating immune cells exhibit augmented phospho-JNK expression compared with the NOD pancreas. There is limited phospho-JNK expression, however, in the regions of islets, which display lymphocytic infiltration in the NOD pancreas (A). (C and D) Sections stained with anti-JNK antibody. Original magnification  $\times 50$ .

#### *Expression of FGF10 and PDGF in the developing and regenerating pancreas*

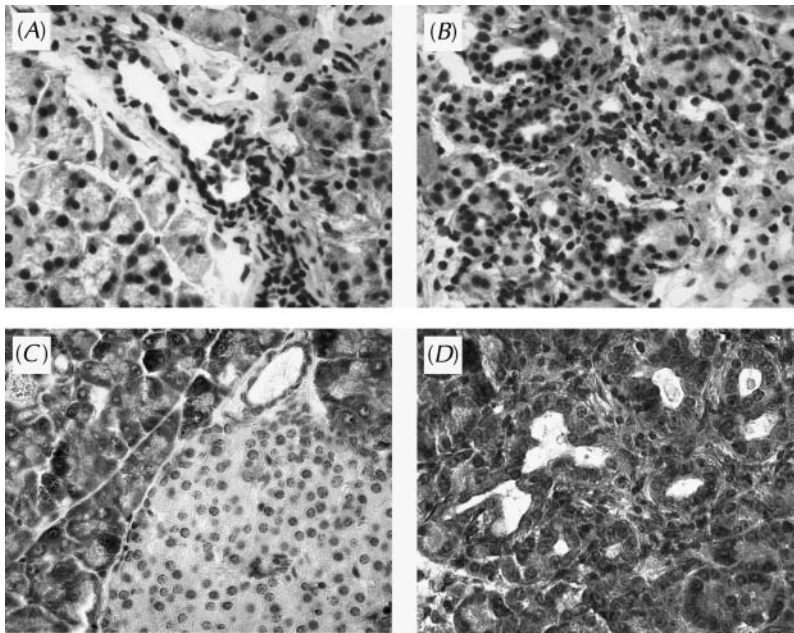
The forgoing results demonstrate enhanced stimulation of critical signaling pathways during pancreatic regeneration. We next asked which growth factors might induce this hyper-activated state. As mentioned in the Introduction, there is extensive literature on the expression and involvement of EGF in developing pancreas. As other potential activators of islet development, we assessed the expression of FGF10 and PDGFAA in both embryonic pancreas in the NOD mice and the regenerating adult pancreas in the IFN $\gamma$  mice. Immunostaining of the pancreas from E16 embryos revealed expression of FGF10 (Fig. 6A) in the acini, emerging ducts and islets. Cells within or contiguous to proliferating ducts of the adult IFN $\gamma$ NOD pancreas also displayed FGF10 staining, suggesting FGF10 involvement in islet neogenesis in this adult regeneration model where emerging islets bud out of the ducts (Fig. 6B). The FGF10 antibody utilized has been reported to cross-react with mouse tissue by immunohistochemistry (Santa Cruz Biotechnology, Inc). PDGFAA immunoreactivity was observed in emerging ducts and acini in E16 embryos (Fig. 6C). Remarkably, the ductal cells and surrounding infiltrate in adult IFN $\gamma$ NOD pancreas also stained positive for PDGFAA (Fig. 6D), identifying this growth factor as a

potential regulator of pancreatic regeneration. Published reports have demonstrated that EGFR and several related members of the EGFR family and their ligands are expressed in the developing and regenerating pancreas (Miettinen *et al.* 1995, 2000, Arnush *et al.* 1996, Krakowski *et al.* 1999b, Kritzik *et al.* 2000a).

#### *Responsiveness of the ductal epithelial cells to EGF, FGF10 and PDGFAA*

The growth factors EGF and FGF10 have previously been demonstrated to be involved in the development of embryonic pancreas. As mentioned in the Introduction we have observed enhanced PDGFAA expression in the IFN $\gamma$  transgenic pancreas compared with NOD control results from our laboratory (M Kritzik & N Sarvetnick, unpublished observations). Moreover our forgoing results have demonstrated that FGF10 and PDGFAA are expressed in the ductal network of the IFN $\gamma$  transgenic mouse. Therefore, we isolated the ductal cell population and stimulated this population *in vitro* with EGF, FGF10 and PDGFAA. Responsiveness was determined by the ability of these growth factors to stimulate the phosphorylation of MAPK and Akt.

EGF stimulation of MAPK and Akt phosphorylation was assessed in whole-cell lysates of freshly isolated ductal



**Figure 4** Phospho-p38 and p38 staining in the IFN $\gamma$  transgenic pancreas. Pancreatic sections from NOD (A and C) and IFN $\gamma$ NOD (B and D). (A and B) Frozen sections stained with an antibody to the dually phosphorylated phospho-p38 MAPK (Thr180/Tyr182). Note the extensive staining in the area surrounding the ducts in the transgenic mouse pancreas (B). Ducts or islets from the NOD mouse pancreas did not display phospho-p38 MAPK staining. (C and D) Sections stained with an antibody to p38 MAPK. Original magnification  $\times 50$ .

cells, serum-starved overnight and then stimulated with EGF. Figure 7A shows the time course of phosphorylation of ERK1 and ERK2. EGF stimulation of ERK1 and ERK2 was maximal by 5 min and started to decrease by 10 min. The stimulation of Akt phosphorylation by EGF followed a similar time course to that of the ERKs (Fig. 7B). Taken together, these results indicate that EGF can stimulate both the MAPK and Akt signaling pathways in the ductal cells.

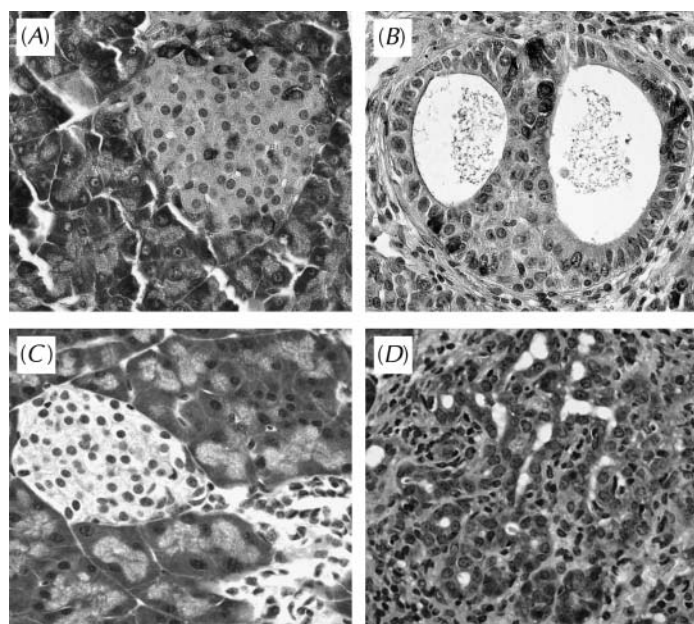
During development, the mesenchyme affects cell fate determination of epithelial cells. Elegant *in vitro* studies utilizing physical separation of mesenchymal and epithelial layers have suggested, however, that the default pathway of epithelial differentiation in the pancreas is the formation of endocrine cells (Gittes *et al.* 1996). Interesting genetic depletion studies with FGF10, one of the mesenchymal growth factors involved in early organogenesis, have demonstrated arrested epithelial development and branching in the pancreas (Bhushan *et al.* 2001). Based on the premise that the ductal network includes progenitors that give rise to new islets, and our observations of FGF10 expression in embryonic and regenerating pancreas (Fig. 6A and B), we assessed the responsiveness of the freshly isolated ductal population to FGF10 stimulation. As shown in Fig. 7C, ERK1 and ERK2 phosphorylation peaked at 5 min and started declining by 10 min in response to FGF10. In

contrast to EGF, FGF10 failed to stimulate Akt phosphorylation (Fig. 7D). This suggests that the ductal network contains a population of cells that may be proliferating in response to FGF10. The absence of FGF10-induced Akt phosphorylation indicates that repression of apoptosis does not appear to be mediated by FGF10.

In the embryonic pancreas the PDGF receptors are one of the most highly expressed tyrosine kinase receptors (LeBras *et al.* 1998). Based on gene chip analyses showing elevated PDGFAA expression in the IFN $\gamma$ NOD pancreas compared with NOD pancreas (M Kritzik & N Sarvetnick, unpublished observations) and our immunohistochemical and immunoblotting results described in the preceding section, we explored the potential responsiveness of ductal epithelial cells to PDGFAA, a ligand for the PDGF $\alpha$  receptor. Figure 7E shows that PDGFAA stimulates ERK1 and ERK2 phosphorylation. The phosphorylation of Akt was also very rapid in onset, demonstrating (Fig. 7F) that the ductal cells are responsive to the PDGFAA ligand. Clearly, PDGFAA is both a potential activator of proliferation and repressor of apoptosis.

## Discussion

In this study we unveiled the stimulation of the p42 and p44 MAPKs ERK1 and ERK2 during pancreatic islet



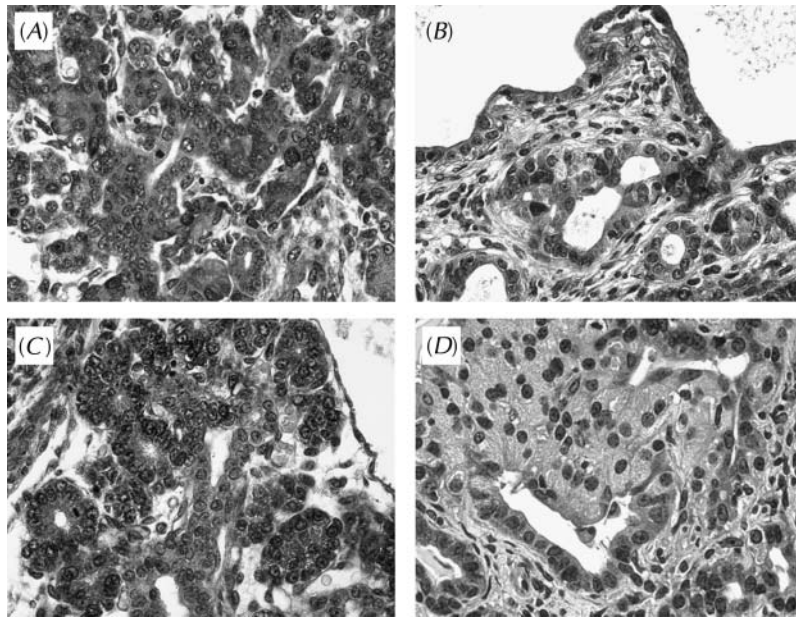
**Figure 5** Phospho-Akt and Akt staining in the IFN $\gamma$  transgenic pancreas. Pancreatic sections from NOD (A and C) and IFN $\gamma$ NOD (B and D). (A and B) Sections stained with an antibody to phospho-Akt (Ser473). A significant number of cells in the proliferating ducts and cells contiguous to the duct cells exhibit phospho-Akt expression in the transgenic mouse pancreas. Interestingly, phospho-Akt is also expressed in the periphery of the islets in the NOD pancreas in a pattern similar to glucagon staining. (C and D) Sections stained with an antibody to Akt. Original magnification  $\times 50$ .

regeneration in a spontaneous model that recapitulates pancreatic ontogeny. We report here a predominant endogenous expression of phosphorylated MAPK in the ductal epithelial cells of the expanding ducts in the transgenic pancreas, as well as an enhanced expression of the ERK proteins supporting the hypothesis that growth factors expressed locally in the proliferating cells of the pancreas would activate the MAPK pathway (Arnush *et al.* 1996, Krakowski *et al.* 1999a,b, Kritzik *et al.* 2000a). The duration of ERK activation is associated with different outcomes (Marshall 1995). In fibroblasts, sustained activation of MAPK results in S phase entry (reviewed in Murphy *et al.* (2002)). In PC12 cells, a rat pheochromocytoma cell line that can be induced to differentiate into neurons *in vitro* in response to NGF, sustained ERK activation accompanies differentiation into neurons (Heasley & Johnson 1992). During pancreatic development and regeneration, adjacent cells in the ducts are at different stages of differentiation. We have observed repeatedly that some cells in the regenerating ducts express only epithelial markers such as cytokeratins while others express PAX6, Pdx1, insulin or glucagon, markers of endocrine differentiation (Kritzik *et al.* 1999, 2000b, Liu *et al.* 2001, Zhang & Sarvetnick 2003). Therefore our present findings of enhanced MAPK phosphorylation in

the ductal epithelial cells may reflect an involvement of MAPK either due to EGF-induced proliferation (Cras-Meneur *et al.* 2001) or perhaps differentiation of endocrine cells in response to a yet undetermined factor. An extensive list of potential stimulators of endocrine cell proliferation and differentiation is presented in a recent review by Nielsen *et al.* (2001). Our *in vitro* data demonstrating an increase in phosphorylation of ERK1 and ERK2 in response to the growth factors EGF, PDGF and FGF10 show that freshly isolated ductal cells from regenerating adult pancreas display MAPK stimulation.

The expression of phosphorylated JNK in the ductal epithelial cells is noteworthy. Activation of JNK can lead to several possible outcomes. Mice lacking both the JNK1 and JNK2 isoforms demonstrate embryonic lethality with severe dysregulation of apoptosis in the brain, resulting in the absence of neural tube closure (Kuan *et al.* 1999). These studies suggest region-specific stimulation or inhibition of apoptosis by JNK (Ip & Davis 1998). The significance of JNK in embryonic morphogenesis is also corroborated by studies in *Drosophila* where mutations in DJNK (the drosophila homolog of JNK) result in incomplete dorsal closure (Sluss & Davis 1997). Finally, there is evidence for the involvement of JNK in epithelial regeneration in *Drosophila* wound healing





**Figure 6** FGF10 and PDGFAA expression in the embryo and IFN $\gamma$  transgenic pancreas. E16 NOD pancreas (A) and pancreatic sections from IFN $\gamma$ NOD pancreas (B) were stained with a rabbit polyclonal antibody to FGF10, which has been reported to cross-react with mouse tissue both by immunoblotting and immunohistochemistry. The E16 NOD pancreas displays FGF10 expression in emerging ducts, islets and acini. Note the expression of FGF10 in the duct cells in the IFN $\gamma$ NOD pancreas (B). E16 NOD (C) pancreas and pancreatic sections from IFN $\gamma$ NOD pancreas (D) were stained with a rabbit polyclonal antibody to PDGFAA also reported to cross-react with mouse tissue by immunoblotting and immunohistochemistry (Santa Cruz Biotechnology, Inc). Original magnification  $\times 50$ .

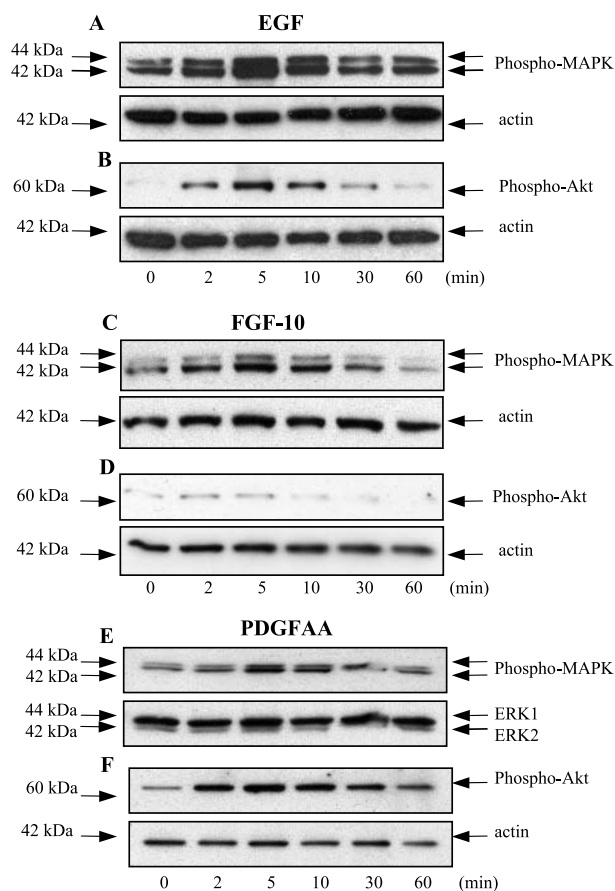
(Ramet *et al.* 2002). Our results suggest an involvement of JNK in the remodeling of the transgenic pancreas.

In this study we report enhanced expression of both p38 MAPK protein and phosphorylated p38 MAPK in the ductal epithelial cells of the regenerating adult pancreas. The enhanced expression and activation of p38 MAPK may be a reflection of diverse processes occurring in these cells. p38 MAPK promotes cell death as well as stimulating cell growth and survival (Dent *et al.* 2003). An obligatory role for p38 MAPK has been shown in chondrocyte differentiation (Stanton *et al.* 2003). Chondrogenesis is a process involving several FGF receptors, which are relevant factors in the pancreatic development and regeneration programs (Edlund 2002; S Dabernat & N Sarvetnick, unpublished observations). In the present work we also demonstrate FGF10 expression in both embryonic and regenerating adult pancreas.

The regulation of apoptosis is an important component of tissue remodeling. One of the key signaling proteins regulating survival is the serine threonine kinase Akt (Datta *et al.* 1999). The major targets of Akt signaling are the Bcl-2 family member BAD, caspase 9 and forkhead family members. Akt regulates the subcellular localization of the forkhead family members by phosphorylating them

and preventing their translocation into the nucleus. In the present study both immunolocalization and immunoblotting studies have shown enhanced Akt phosphorylation in the regenerating pancreas. Our results are corroborated by Jetton *et al.* (2001) who observed phospho-Akt expression in ductal cells in the rat 60% pancreatectomy model. We propose that Akt activation promotes regeneration by protecting ductal progenitor cells from programmed cell death. As mentioned in the results, Akt can also regulate metabolism and cellular proliferation. It could therefore also have a role in maintaining and inducing the proliferation of subgroups of ductal cells.

The responsiveness of the ductal epithelial cells to PDGFAA is intriguing. PDGFAA knockout mice either die before E10 or survive either to a couple of days or up to 6 weeks after birth. The surviving mice develop a number of defects including oligodendrocyte deficiency, lack of alveolar smooth muscle cells, lack of testicular leydig cells and a disturbance of intestinal villus formation (Betsholtz *et al.* 2001). In an oligodendroglial cell line the treatment of cells with PDGFAA resulted in the increased expression and polarization of the notch-1 receptor (Bongarzone *et al.* 2000), which is important in maintaining cells in an undifferentiated state (Lardelli *et al.* 1995, Simpson 1998).



**Figure 7** Time course of EGF-, FGF10- and PDGF-AA-stimulated phosphorylation of MAPK (ERK1 and ERK2) and Akt in freshly isolated ductal cells from the IFN $\gamma$ NOD mouse pancreas. Following isolation, cells were serum-starved overnight and stimulated with 10 ng/ml EGF, 50 ng/ml FGF10 or 10 ng/ml PDGF-AA for 0, 2, 5, 10, 30 and 60 min at 37 °C. Whole-cell lysates were immunoblotted with antibodies to phospho-MAPK or phospho-Akt. The membranes were stripped and reblotted with anti-actin or anti-ERK antibody. Autoradiograms representative of at least three experiments for each ligand are presented.

Therefore, it is likely that cross-talk between PDGF-AA and the notch signaling pathways maintain the pancreatic progenitor cells in an undifferentiated state, allowing regeneration in the adult.

In conclusion, we have demonstrated the enhanced phosphorylation of SHC, three families of MAPKs, and Akt in the expanding pancreatic epithelium. These stimulated signaling molecules reflect signal transduction by growth factors, cytokines and integrins. EGF, FGF10 and PDGF-AA were able to stimulate MAPK and Akt *in vitro*. We propose that these three factors are morphogens during regeneration and will be valuable in the *in vitro* expansion of ductal cells for therapies involving beta cell replacement.

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