

Phosphatidylinositol 3-Kinase Is a Negative Regulator of Cellular Differentiation

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Abstract. Phosphatidylinositol 3-kinase (PI3K) has been shown to be an important mediator of intracellular signal transduction in mammalian cells. We show here, for the first time, that the blockade of PI3K activity in human fetal undifferentiated cells induced morphological and functional endocrine differentiation. This was associated with an increase in mRNA levels of insulin, glucagon, and somatostatin, as well as an increase in the insulin protein content and secretion in response to secretagogues. Blockade of PI3K also increased the proportion of pluripotent precursor cells coexpressing multiple hormones and the total number of terminally differentiated cells originating from these

precursor cells. We examined whether any of the recently described modulators of endocrine differentiation could participate in regulating PI3K activity in fetal islet cells. The activity of PI3K was inversely correlated with the hepatocyte growth factor/scatter factor-induced downregulation or nicotinamide-induced upregulation of islet-specific gene expression, giving support to the role of PI3K, as a negative regulator of endocrine differentiation. In conclusion, our results provide a mechanism for the regulation of hormone-specific gene expression during human fetal neogenesis. They also suggest a novel function for PI3K, as a negative regulator of cellular differentiation.

THE identification of mechanisms involved in the formation and function of the mammalian endocrine system is one of the most important issues in developmental biology. The differentiation and growth of endocrine organs can be regulated at several levels. One level is the regulation of reactions controlled by intracellular signal messengers. This type of regulation provides a different gene expression response to various external stimuli, which is critical for rates of hormone biosynthesis and release.

Phosphatidylinositol 3-kinase (PI3K)¹ is a ubiquitous enzyme that has been shown to be an important mediator of intracellular signaling in mammalian cells. To date, the expanding family of mammalian PI3K consists of three members, each containing a different p110 catalytic subunit (Hiles et al., 1992; Hu et al., 1993; Stoyanov et al., 1995).

Upon activation, PI3K phosphorylates inositides at the D-3 position of the inositol ring to generate such lipid messengers as: PtdIns(3)P, PtdIns(3,4)P₂, and PtdIns(3,4,5)P₃ (see abbreviations footnote for explanation of nomenclature). The exact role and downstream molecular targets of these lipid products are unknown. However, it is known that overall increases in the levels of these messengers correlates with mitogenic signaling by growth factors (Cantley et al., 1991), secretion, and vesicle trafficking (Brown et al., 1995), as well as chemotaxis, cell shape changes, and membrane ruffling (Traynor-Kaplan et al., 1988; Eberle et al., 1990; Wennstrom et al., 1994). PI3K was reported to be important for the regulation of insulin receptor-induced intracellular pathways, including glucose transport (Backer et al., 1993). Similarly, members of the seven transmembrane-spanning receptor family, hormone and sensory receptor system in mammalian cells, were recently shown to use PI3K to transduce signals to the interior of the cell and to assemble the ras activation complex (Ptasznik et al., 1995, 1996; Touhara et al., 1995). Several studies have shown that the PI3K lipid products are signaling intermediates in the induction of cellular differentiation of PC12 pheochromocytoma cells (Hempstead et al., 1992; Kimura et al., 1994) as well as of adipocytic 3T3-F442A cells (Saad et al., 1994), suggesting that this enzyme may function as a positive regulator of cellular differentiation in these cell lines.

While the process of endocrine cell differentiation has

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1. *Abbreviations used in this paper:* HGF/SF, hepatocyte growth factor/scatter factor; ICC, islet-like cell cluster; NIC, nicotinamide; PI3K, phosphatidylinositol 3-kinase; PI, phosphatidylinositol; PIP, phosphatidylinositol 4 phosphate; PIP₂, phosphatidylinositol 4,5 bisphosphate; PIP₃ or PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5 trisphosphate; PKC, protein kinase C; PtdIns(3)P, phosphatidylinositol 3 phosphate; PtdIns(3,4)P₂, phosphatidylinositol 3,4 bisphosphate.

been extensively studied, no specific intracellular signaling pathway directly involved in regulating expression of endocrine-specific genes has been identified. Because of the role of PI3K in mitogenesis, differentiation, and stimulus-secretion pathways, we have investigated the possibility that this enzyme regulates endocrine differentiation in mammalian cells. Until recently, most of the studies addressing the role of PI3K in cellular proliferation and differentiation were undertaken using a variety of cell lines and transfection methodologies. Such transformed cells are capable of indefinite replication in culture and express only some of the differentiated properties of their cell of origin. Thus, these approaches provide only limited information about the potential link between PI3K activity and development. With the identification of the drugs wortmannin (Powis et al., 1994) and Ly294002 (Vlahos et al., 1994) as potent PI3K inhibitors, it became possible to directly inhibit the endogenous PI3K activity in cultured primary cells. In the present experiments, we have used, as a model for endocrine differentiation, human fetal-derived pancreatic cells, growing in vitro as islet-like cell clusters (ICCs) (Sandler et al., 1989). The cellular composition of ICCs consists mostly of undifferentiated epithelial cells (~80%) containing putative precursors of the hormone-producing cells (Sandler et al., 1989; Otonkoski et al., 1993; Beattie et al., 1994). Endocrine cells developing in vitro within ICCs originate from undifferentiated, pluripotent epithelial cells and contain insulin-producing β cells and the three other cell types, α , δ , and pp, secreting glucagon, somatostatin, and pancreatic polypeptide, respectively. An advantage of this model system is the ability to mimic steps of the differentiation process in cell culture, as evidenced by the fact that after being transplanted into athymic nude mice, ICCs develop into morphologically and functionally mature endocrine tissue (Sandler et al., 1985; Beattie et al., 1994).

We now report that wortmannin or Ly294002 blockade of PI3K activity significantly increased the number of hormone-producing cells growing in ICCs. These unexpected results indicate that PI3K plays a role as a negative regulator of cellular differentiation during fetal neogenesis of endocrine system.

Materials and Methods

Tissue Culture

The use of human fetal tissue for these studies was reviewed and approved by the Institutional Review Board at our university. Human fetal pancreases at 18–24 gestational wk were obtained with appropriate permissions and patient consent through nonprofit organ procurement programs (Advanced Bioscience Resources, Oakland, CA; Anatomic Gift Foundation, Laurel, MD). Experiments were started by the enzymatic digestion with collagenase P (Boehringer Mannheim Corp., Indianapolis, IN) of the human fetal pancreases followed by tissue culture for 5 d, which led to the formation in vitro of ICCs, as previously described in detail (Otonkoski et al., 1993). The cells, cultured for 5 d, were treated continuously with 10 μ M Ly294002 (Calbiochem Corp., La Jolla, CA), 100 nM wortmannin (Sigma Chemical Co., St. Louis, MO), 10 mM nicotinamide (NIC) (Sigma Chemical Co.), or 25 ng/ml hepatocyte growth factor/scatter factor (HGF/SF) (a kind gift of J.S. Rubin, National Cancer Institute, Bethesda, MD). These concentrations of wortmannin and Ly294002 were found to be effective in inhibiting PI3K activity in our preliminary, dose-response experiments. The effective doses for NIC and HGF/SF as modulators of endocrine differentiation in fetal islet cells were established in our laboratory previ-

ously (Otonkoski et al., 1993; Beattie et al., 1996a). PI3K inhibitors, NIC, or HGF/SF was added to the culture medium from the very beginning. Medium plus factor was changed every day for 5 d. For direct comparison, portions of the same pancreases were grown, treated with factors, and used for transcriptional analyses, insulin content and secretion, PI3K activities, and DNA synthesis.

Isolation of Fetal Pancreatic Islets

Effects of PI3K inhibitors on endocrine function were tested not only in ICCs, but also in purified islets. Undifferentiated epithelial cells account for ~75–80% of the total cell mass in ICCs. By contrast, purified fetal islets contain about 10-fold fewer undifferentiated epithelial cells (Beattie et al., 1996b). Purification of human fetal pancreatic islets was performed as recently described (Beattie et al., 1996b). Fetal islets, identified as homogeneous differentiated clusters of dithizone-positive cells, were incubated with factors as above.

RNA Isolation and Analysis

Transcriptional analyses on total RNA (0.5 μ g) were performed using a multiprobe ribonuclease protection assay, as previously described (Otonkoski et al., 1993). The housekeeping gene cyclophilin was used as an internal control, and yeast tRNA (10 μ g) was included as a negative control. Probes used were of human origin and were described previously (Otonkoski et al., 1993). Target RNAs were quantitated in autoradiographs by scanning densitometry (LKB Ultrascan XL Laser) and integrated using Gel Scan XL software (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The probe-specific mRNA signals were normalized to the cyclophilin signal in each sample to account for differences in sample loading between lanes.

Insulin Content, Insulin Secretion, and DNA Synthesis

After incubation with various factors in culture, the ICCs were harvested, and measurements of insulin content, insulin release in response to glucose plus theophylline, and [3 H]thymidine incorporation into DNA were performed as described previously (Otonkoski et al., 1993).

PI3K Assay

Aliquots of cell lysates normalized for protein content were incubated for 3 h with anti-PI3K antibodies directed against the 85-kD regulatory subunit or with antiphosphotyrosine antibodies (Upstate Biotechnology, Inc., Lake Placid, NY). The immune complexes were absorbed onto protein A-Sepharose and washed as described (Ptasznik et al., 1995). PI3K assays were performed directly on beads. Briefly, the reaction was carried out for 10 min in a buffer containing 40 mM Hepes, pH 7.2, 6 mM MgCl₂, 1 mM EDTA, 10 μ g of PI (Avanti Polar Lipids, Alabaster, AL), 10 μ M ATP, and 10 μ Ci [γ - 32 P]ATP (6,000 Ci/mmol; DuPont/NEN, Wilmington, DE). Adenosine (0.2 mM) was added to the reaction mixture to inhibit residual PI⁴-kinase activity. After the incubation, the reaction was stopped with methanol plus 2.4 N HCl (1:1, vol/vol), and lipids were extracted, analyzed by thin-layer chromatography, and quantified as described previously (Ptasznik et al., 1995). In some experiments, the direct binding of PI3K to p190Met after HGF/SF stimulation of islet cells was determined, as previously described in detail (Graziani et al., 1991).

Determination of Total Cellular PIP₃

The ICCs, which were pretreated for 5 d with PI3K inhibitors, NIC, HGF/SF, or control buffer, were subsequently harvested and suspended at a concentration of 2×10^5 cells/ml in buffer A (30 mM Hepes, pH 7.2, 110 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 10 mM glucose), and 1 mCi/ml [32 P]orthophosphate (HCl-free; DuPont/NEN) was added. The cells were incubated at 37°C for 2 h and then washed 3 \times with buffer A. Depending on the type of experiment, the labeled islet cells were either directly lysed by addition of 3 ml chloroform/methanol (1:2, vol/vol), followed by 4 ml chloroform/2.4 M HCl (1:1, vol/vol) (to measure basal levels of PIP₃), or labeled cells were first stimulated with 25 ng/ml HGF/SF for the indicated times, and subsequently the reaction was stopped as above (to measure the inducible levels of PIP₃). Phospholipids were extracted and analyzed by thin-layer chromatography, and the total cellular PIP₃ was quantified, as we described previously in detail (Ptasznik et al., 1996).

Triple Immunofluorescence and Confocal Microscopy

ICCs cultured for 5 d in either control medium or medium containing 10 μM Ly294002 were paraffin embedded, and 5- μm sections were stained for hormone immunoreactivity. To simultaneously identify cells producing insulin, glucagon, somatostatin, and pancreatic polypeptide (pp), we followed a modification of our previous protocol for multiple labeling (Otonkoski et al., 1996). Briefly, sections were incubated for 1 h at room temperature with a mixture of primary antibodies: IgG fraction of a sheep anti-human insulin antiserum (The Binding Site, Birmingham, England) (5 $\mu\text{g}/\text{ml}$), mouse monoclonal anti-human glucagon (Sigma Chemical Co.) (1 $\mu\text{g}/\text{ml}$), rabbit anti-human somatostatin antiserum (Dako Corporation, Carpinteria, CA) (used at 1:100 dilution), and rabbit anti-human pp antiserum (Chemicon International, Inc., Temecula, CA) (used at 1:100 dilution). In separate sections, a mixture of normal sheep, rabbit, and mouse IgGs was used as control reference for specificity of primary antibodies. After washings in PBS-DS (5 mM glycine, 0.2% donkey serum, 0.1% BSA), sections were incubated for 1 h at room temperature with a cocktail of F(ab')₂ fractions from secondary antibodies: lissamine-rhodamine-conjugated affinity-purified donkey anti-sheep IgGs (5 $\mu\text{g}/\text{ml}$); FITC-conjugated affinity-purified donkey anti-rabbit IgGs (5 $\mu\text{g}/\text{ml}$); and indocarbocyanine-conjugated affinity-purified donkey anti-mouse IgGs (5 $\mu\text{g}/\text{ml}$). All F(ab')₂ were preadsorbed on appropriate multiple species to eliminate the possibility of cross-reactivity in multiple labeling protocols (Jackson ImmunoResearch Labs, Inc., West Grove, PA). The sections were processed as previously described (Otonkoski et al., 1996) and viewed on a laser scanning confocal microscope (model MRC-1024; BioRad Laboratories, Hercules, CA).

Morphometric Analysis and Statistics

Sections were prepared from control and Ly294002-treated ICCs from three independent experiments. After immunostaining, confocal images were acquired from 57 control and 61 Ly294002-treated ICC sections. All images collected (one image per section) were then analyzed for total surface area and insulin-, glucagon-, somatostatin-, and pp-positive cell surface area by using measurement tools in the software NIH Image 1.60 (National Institutes of Health, Bethesda, MD). Data were analyzed in Stat View 4.01 (Abacus Concepts, Inc., Berkeley, CA) for calculation of mean, standard deviation, and parametric statistic (*t* test).

Results

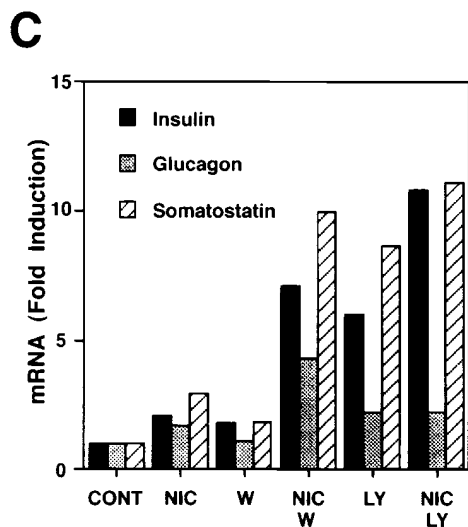
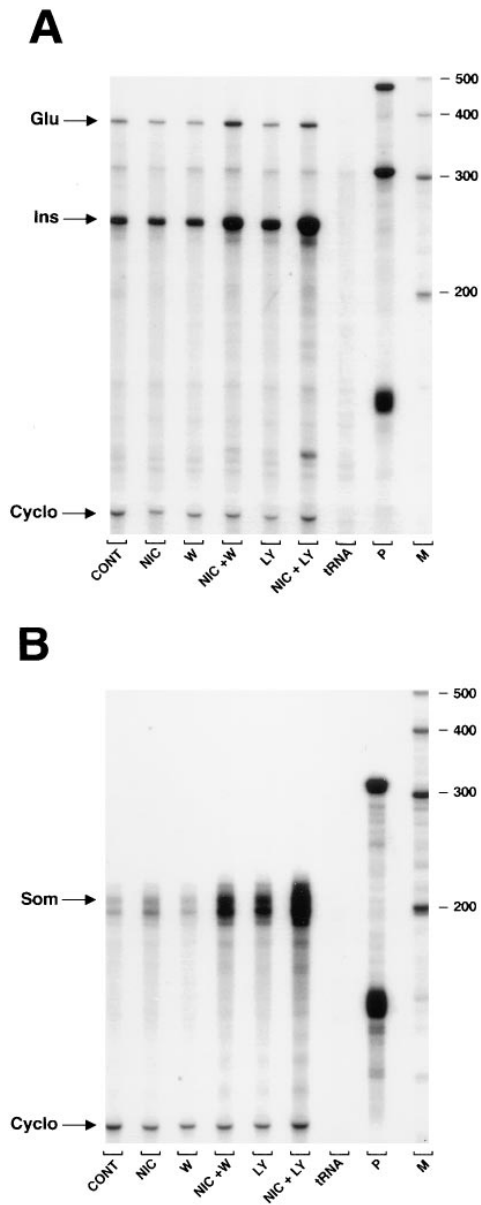
Inhibition of PI3K Increases Islet-specific Hormone Biosynthesis and Hormone Secretion in Developing Fetal Pancreatic Cells

To investigate whether PI3K activation is important for endocrine differentiation of human fetal pancreatic cells, we continuously treated ICCs for 5 d with 100 nM wortmannin or 10 μM Ly294002, concentrations that block over 90% of total PI3K activity in intact fetal islet cells (data not shown; see Fig. 7). We established that these concentrations of wortmannin and Ly294002 almost completely inhibited the rise in PIP₃ formation stimulated by growth factors in intact [³²P]orthophosphate-labeled islet cells. By contrast, these concentrations of inhibitors did not affect significantly the ratio of [³²P]PIP₂ to [³²P]PIP and [³²P]PIP to [³²P]PI in phospholipid labeling experiments where PIP₃ levels were measured, implying that other kinases (PI5K and PI4K) were not inhibited under these conditions. Wortmannin, a fungal metabolite, functions as a covalent inhibitor of the catalytic p110 subunits of PI3Ks at nanomolar concentrations, whereas Ly294002, a structurally and mechanistically distinct compound, functions as a noncovalent, competitive inhibitor of PI3Ks at 100-fold higher concentrations than wortmannin (Okada et al., 1994; Powis et al., 1994; Vlahos et al., 1994; Wymann et al., 1996). At nanomolar concentrations, wortmannin is

thought to be selective for PI3K. Ly294002, even at micromolar concentrations, is quite specific for PI3K and does not affect PI4K or a number of intracellular Ser/Thr and Tyr kinases (Vlahos et al., 1994). Finally, we have also shown that continuous treatment for 5 d with 100 nM wortmannin or 10 μM Ly294002 does not cause notable cytotoxicity nor induce apoptosis in fetal pancreatic cells growing as islet-like cell clusters (data not shown).

We measured the transcriptional expression of islet-specific hormone genes in ICCs growing for 5 d in the presence of PI3K inhibitors. As shown in Fig. 1, wortmannin and Ly294002 increased the transcriptional levels of insulin, glucagon, and somatostatin in cells within the ICCs. The pattern of alterations of mRNA levels was strikingly similar to that of insulin protein (see below). Therefore, these data indicate that two structurally distinct compounds have similar effects on hormone transcription as a consequence of their shared ability to function as specific inhibitors of PI3K. The inhibitors had no effects on cyclophilin mRNA, which was used as an internal control. The quantitative analysis of islet hormone transcription levels, after normalization to cyclophilin expression, is shown in Fig. 1 C. To better understand the effect of these PI3K inhibitors on islet hormone gene expression, we have compared the effect of PI3K inhibitors alone to the NIC-induced increase in expression of islet-specific hormone genes (Otonkoski et al., 1993). NIC is an inhibitor of the enzyme poly(ADP-ribose) synthetase and can potently induce human fetal islet cell differentiation by influencing the transcription of DNA (Yonemura et al., 1984; Sandler et al., 1989). Treatment with the combination of a PI3K inhibitor and NIC resulted in a synergistic increase in mRNA levels of islet-specific hormones (maximum increase is about 10-fold for insulin and somatostatin, and fourfold for glucagon). Taken together, these data suggest that PI3K is a negative regulator of islet-specific gene expression in developing pancreatic cells.

We also measured the insulin content and insulin secretion per cellular DNA in fetal ICCs cultured for 5 d in the presence of the PI3K inhibitors. As shown in Fig. 2, A and B, we found that both insulin content and secretion were significantly increased in Ly294002-treated cells, as compared to untreated control cells. Wortmannin also induced increases in these parameters, but to a lesser extent. (Wortmannin appears to be a much less stable agent than Ly294002 in culture medium [Kimura et al. 1994] [data not shown].) The pattern of alterations of insulin secretion was almost identical to that of insulin content, indicating a close functional association between these two parameters. This would suggest that a continuous blockade of PI3K activity could secondarily increase insulin secretion through potent stimulation of insulin biosynthesis in developing islet cells. Consistent with this, we observed no direct effect of PI3K inhibitors on insulin secretion when islet cells were treated with these inhibitors for a short time (0.5 and 2 h; data not shown). No significant differences were observed in insulin content under these conditions. The observation that short-term inhibition of PI3K does not significantly affect insulin secretion was also recently shown in rat islets and insulin-secreting β -TC3 cells and has already been exhaustively discussed by other investigators (Gao et al., 1996). Taken together, these data suggest that the continu-



ous blockade of PI3K activity triggered changes in mRNA levels and that these changes were followed by a significant increase in hormone biosynthesis and a subsequent increase in hormone secretion.

To determine whether PI3K inhibitors increase the proportion of islet cells expressing hormones, confocal immunofluorescent detection of all islet-specific hormones, followed by morphometric analysis of the ICCs, was carried out. Fig. 3 shows representative fields of a microscopic analysis performed on sections from human fetal ICCs, cultured with or without Ly294002 for 5 d. Only a few hormone-positive cells were visible in ICCs cultured in control medium. By contrast, hormone-positive cells were more common in ICCs cultured with the PI3K inhibitor (4.4-fold increase in the total percentage of endocrine-positive cells in Ly294002-treated ICCs, as compared to control ICCs). Interestingly, in Ly294002-treated ICCs, we detected more cells positive for more than one protein, indicating that the PI3K inhibitor can trigger activation of multiple hormone-specific genes in a single cell. It was previously shown that coexpression of multiple hormones represents an early step in the endocrine differentiation program of islet cell progenitors (Alpert et al., 1988; Herrera et al., 1991). The present results thus suggest that PI3K inhibitors induce a process of endocrine differentiation in fetal islet precursor cells.

Inhibition of PI3K Decreases DNA Synthesis in Fetal Pancreatic Cells

We measured DNA synthesis in fetal ICCs cultured in the presence of the PI3K inhibitors. As shown in Fig. 4 A, wortmannin and Ly294002 significantly decreased the [³H]thymidine incorporation into DNA in cells within the ICCs. Treatment with the combination of the PI3K inhibitor and NIC resulted in a synergistic decrease in DNA synthesis. To make certain that the observed increase in islet-specific hormone expression in PI3K inhibitor-treated cells is not secondary to nonspecific blockade of the cell cycle, we measured insulin protein expression in mitomycin C-treated or serum-starved cells. As shown in Fig. 4, B and C, neither mitomycin C-induced blockade of DNA synthesis nor

Figure 1. Stimulatory effect of PI3K inhibitors on islet hormone gene expression. Total RNA from ICCs cultured for 5 d was hybridized simultaneously to radiolabeled antisense riboprobes for (A) glucagon (*Glu*), insulin (*Ins*), and cyclophilin (*Cyclo*), or (B) somatostatin (*Som*) and cyclophilin (*Cyclo*). Protected fragments for glucagon (389 nucleotides), insulin (262 nucleotides), somatostatin (206 nucleotides), and cyclophilin (135 nucleotides) are indicated. Lanes: *CONT*, control ICCs; *NIC*, *W*, and *LY*, ICCs cultured in the presence of nicotinamide, wortmannin, or Ly294002, respectively; *NIC+W* or *NIC+LY*, ICCs cultured in the presence of the combination of nicotinamide and either wortmannin or Ly294002; *tRNA*, yeast tRNA (10 μg) used as a negative control; *P*, mixture of undigested probes (from top: [*A*] glucagon, insulin, cyclophilin; [*B*] somatostatin, cyclophilin); *M*, RNA molecular size markers, in nucleotides (Ambion, Inc., Austin, Texas). (C) Quantitative analysis of hormone transcription levels. Densitometrically determined band intensities of target mRNAs are shown after normalization to the cyclophilin signal from the RNase protection assays shown in A and B. Values are compared to the control samples. Results shown are representative of two independent experiments.

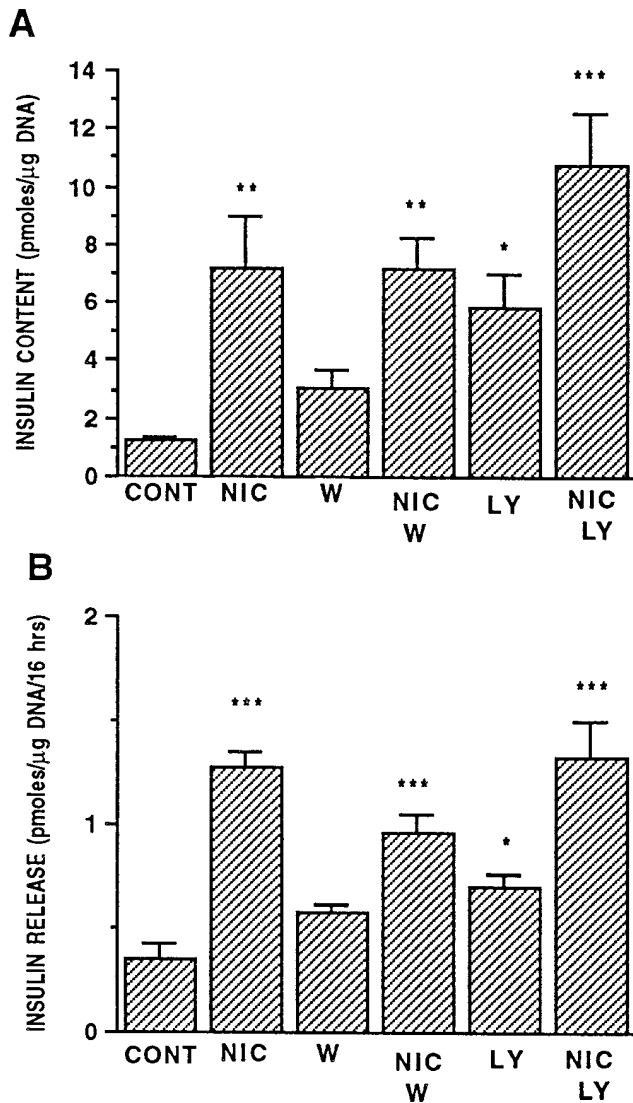


Figure 2. Stimulatory effects of PI3K inhibitors on insulin content and insulin secretion in fetal islet cells. Replicate groups of 50 human fetal ICCs were cultured for 5 d in the presence or absence of the inhibitors wortmannin (W) or Ly294002 (LY). Nicotinamide (NIC), a potent inducer of endocrine cell differentiation, was also included in some cultures. Insulin content (A) and insulin release (B) were measured. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$; $n = 8$.

serum starvation affected insulin protein expression in ICCs. Consistent with this observation, neither mitomycin C, which is known to interact directly with DNA (Tomasz et al., 1987), nor serum starvation significantly affected basal PI3K activity in fetal islet cells (data not shown). Thus, the stimulatory effect of PI3K inhibitors on endocrine differentiation is not secondary to blockade of cell proliferation but is due to the specific blockade of PI3K.

HGF/SF or NIC-dependent Regulation of Endocrine Differentiation Is Inversely Related to PI3K Activity

To clarify the correlation between PI3K activity and endocrine-specific gene expression, we examined whether any

of the recently described modulators of endocrine differentiation could participate in regulating PI3K activity in islet cells. Mesenchyme-derived HGF/SF is a physiological modulator of endocrine differentiation in human fetal islet cells. When fetal ICCs were induced to proliferate by the addition of HGF/SF, a marked downregulation of both insulin and glucagon gene transcription, as well as insulin protein biosynthesis, was observed (Beattie et al., 1996a; Otonkoski et al., 1996). By contrast, addition of NIC under the same conditions resulted in an increase in insulin and glucagon mRNA levels and insulin protein biosynthesis. These data indicate a role for HGF/SF in promoting proliferation and inhibiting the endocrine function of fetal islet cells. The HGF/SF receptor is known to function by activating PI3K in a variety of cellular systems (Graziani et al., 1991). Consistent with this, cellular PI3K is robustly activated in fetal islet cells after acute HGF/SF stimulation, and this activation results from direct binding of PI3K to the HGF/SF receptor (Fig. 5 and data not shown). Similarly, analysis of [32 P]orthophosphate-labeled ICCs, growing continuously in the presence of HGF/SF, showed a threefold increase in levels of the major lipid product of PI3K; PIP₃ (Fig. 6). Thus, the HGF/SF-triggered downregulation of hormone-specific genes is associated with an increase in proliferation of islet cells and with an increase of PI3K activity.

In contrast to HGF/SF, NIC is known to increase both insulin content and insulin release in ICCs (Sandler et al., 1989), as well as to upregulate the expression of islet-specific hormone genes (Otonkoski et al., 1993). Fig. 7 shows the amount of PI3K activity measured in p85 subunit antibody immunoprecipitates from control, NIC-treated, and PI3K inhibitor-treated fetal ICCs. A significantly lower (about fivefold) amount of PI3K activity was present in p85 immunoprecipitates from NIC-treated cells, as compared to untreated cells. We consistently observed that the basal level of PIP₃ was significantly lower in NIC-treated ICCs than in control cells (Fig. 6). Treatment with wortmannin or Ly294002 eliminated >90% of control PI3K activity under these conditions (Fig. 7). Inducibility of cellular PI3K activity by growth factors, as well as the de novo formation of PIP₃, was significantly reduced in NIC-treated cells. There was no change in the actual level of p85 protein during NIC treatment, as determined by Western blotting, nor was there a direct inhibitory effect of various concentrations of NIC on PI3K activity in vitro in p85 immunoprecipitates, indicating that NIC is not a direct inhibitor of PI3K (data not shown). The mechanism by which NIC attenuates cellular PI3K activity in developing fetal islet cells remains to be determined. The cell culture and treatment conditions used in Fig. 7 were identical to those used in Fig. 1 and Fig. 2, indicating that an inverse correlation exists between the amount of PI3K activity and the stage of endocrine maturity in islet cells during fetal neogenesis.

Discussion

The cellular signaling pathways that are required for endocrine differentiation are unknown. The present studies clearly indicate that lipid products of PI3K are an important part of the regulatory network that links differentia-

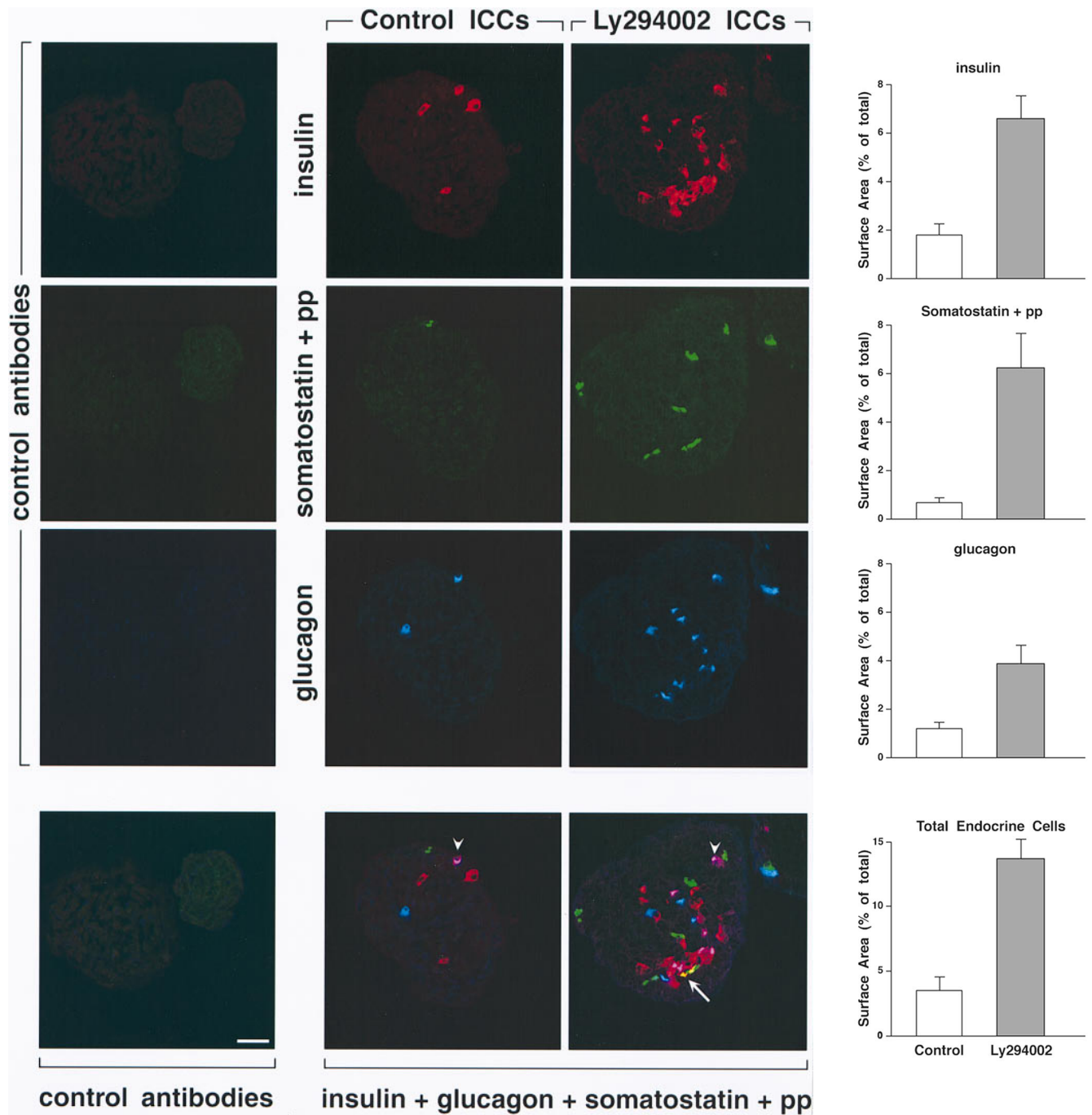


Figure 3. Increased frequency of endocrine-positive cells and endocrine precursors in PI3K inhibitor-treated fetal islet cultures. Representative fields, collected by confocal microscopy, are shown for control and Ly294002-treated ICCs. Immunoreactivity for insulin is shown in red, somatostatin and pp in green, and glucagon in blue. Bar graphs represent the morphometric analysis of endocrine cell frequency for each hormone and show the immunoreactive surface area expressed as percent of total surface area of ICCs. Notably, the endocrine surface area is significantly increased for all hormones in Ly294002-treated ICCs (insulin, $P < 0.01$; somatostatin and pp, $P < 0.03$; glucagon, $P < 0.03$; $n = 3$). When the three fluorescence spectra are merged (*lower panels*) to measure the total endocrine cell surface, hormone colocalization can also be appreciated. Insulin and glucagon coexpression is highlighted by the appearance of a purple color (*arrowheads*) resulting from the overlap of red and blue, whereas colocalization of insulin and somatostatin or pp is shown by the yellow color (*arrow*) resulting from the overlap of red and green fluorescences. Note that the frequency of cells coexpressing multiple hormones is increased in Ly294002-treated ICCs: seven cells coexpressing insulin and glucagon (*purple*) and two cells coexpressing insulin and somatostatin or pp (*yellow*). Morphometric analysis demonstrates that the total endocrine cell surface area is significantly increased in Ly294002-treated ICCs (*lower right bar graph*). Control sections incubated with irrelevant primary antibodies did not show any detectable immunoreactivity (*left panels*). Bar, 12 μm .

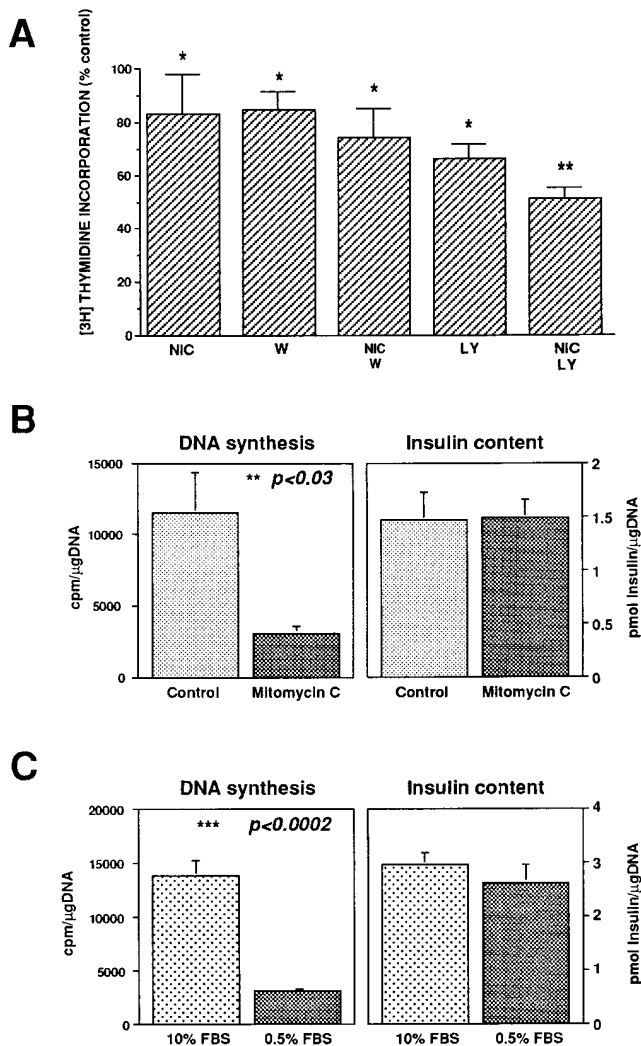


Figure 4. Inhibition of proliferation in fetal islet cells by PI3K inhibitors. (A) Effect of PI3K inhibitors on $[^3\text{H}]$ thymidine incorporation into DNA. ICCs were cultured as described in Fig. 2. $[^3\text{H}]$ thymidine ($1 \mu\text{Ci/ml}$) was added to the cultures 16 h before the assay. Data are combined from three independent experiments and are presented as percentage of control ICCs from each experiment. (Absolute control values were 2086 ± 172 , 4552 ± 583 , and 7625 ± 536 cpm/ μg DNA). * $P < 0.05$ compared to control, ** $P < 0.05$ compared to treatment with nicotinamide or wortmannin. Inhibition of DNA synthesis by mitomycin C or serum starvation does not affect insulin protein expression in fetal ICCs. ICCs were cultured in the presence of $2 \mu\text{g/ml}$ mitomycin C for 5 d (B) or starved in 0.5% FBS for 2 d (C). Subsequently, $[^3\text{H}]$ thymidine incorporation and insulin protein content were measured as described in Materials and Methods.

tion signals at the cell surface of endocrine precursors to transcriptional responses in the nucleus. We demonstrate a previously unrecognized function for PI3K—as a negative regulator of endocrine differentiation in developing mammalian cells. Blockade of PI3K activity in primary cultured fetal pancreatic cells resulted in a robust activation of endocrine differentiation. Treatment of ICCs with PI3K inhibitors increased transcription of islet-specific hormone genes, expression of islet-specific hormone proteins, insu-

lin content, insulin release in response to secretagogues, the total number of endocrine-positive cells developing in islets, and the number of precursor islet cells coexpressing multiple hormones. By contrast, DNA synthesis was significantly decreased in the PI3K inhibitor-treated islet cells, as compared to untreated cells. However, inhibition of DNA synthesis by serum starvation or by treatment with cell cycle-blocking antibiotics had no effect on islet-specific hormone expression. This implies that the observed effects are not secondary to nonspecific blockade of the cell cycle, but they can be attributed directly to specific inhibition of PI3K. As a further indication of the involvement of PI3K in regulating endocrine differentiation, we also observed that the activity of this enzyme was inversely correlated with the HGF/SF-induced downregulation or NIC-induced upregulation of islet-specific hormone gene expression, providing support for the role of PI3K as a negative regulator of endocrine differentiation.

The basis for the interaction of cytoplasmic phospholipid messengers with transcriptional factors in the nucleus is, at present, unknown. Only a few potential biochemical targets of phosphoinositides have been found in mammalian cells (for review see Carpenter and Cantley, 1996). The exact role and immediate downstream molecular targets of $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$ have not been identified. This is accurate for all known cellular functions of PI3K in various systems. Thus, the precise molecular mechanisms that link the inhibition of the PI3K to the induction of endocrine differentiation remain to be elucidated. It has been reported that proteins of the jun family can inhibit islet-specific hormone gene transcription both in vivo (Inagaki et al., 1992) and in vitro (Henderson and Stein, 1994). The jun transcription factors block activity of these genes through the E-box elements, which are common for insulin, glucagon, and somatostatin (Kruse et al., 1993; Cordier-Bussat et al., 1995). The known ability of PI3K to activate ras and subsequently the MAPK-Jun cascade (Pulverer et al., 1991; Thomas et al., 1992; Hu et al., 1995) provides a potentially direct link between PI3K signaling and the inhibition of islet-specific hormone gene expression. This scenario would explain why treatment with PI3K inhibitors can release the blockade of all islet-specific hormone genes, which is described in the present experiments. Alternatively, it is possible that the functional association of PI3K with the islet-specific hormone genes is mediated by protein kinase C (PKC)-dependent pathways. Several recent reports have indicated that PI3K might activate PKC isoforms both in vitro and in vivo (Nakanishi et al., 1993; Toker et al., 1994). PKC-dependent branches of signal transduction pathways are known as upstream regulators of several regulatory genes and transcription factors, including members of the c-rel family (NF- κB), as well as members of the fos/jun family involving AP-1 sites (Leonardo and Baltimore, 1989; De Tata et al., 1993). PKC was previously suggested to be involved in intracellular control of insulin anabolism and secretion (for review see Newgard and McGarry, 1995). Thus, the PI3K could modify islet-specific gene expression in a PKC-dependent manner. Another explanation for our results would have to imply that PI3K can control biosynthesis of transcriptional factors for hormone gene expression during fetal neogenesis. We have recently shown that

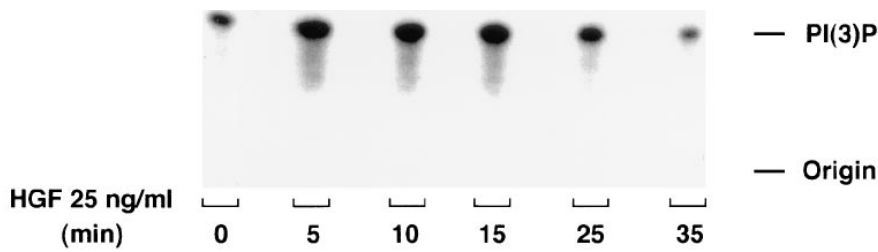


Figure 5. HGF/SF-induced increase of PI3K activity in fetal ICCs. ICCs starved for 24 h were stimulated for the indicated times with HGF/SF (25 ng/ml). Equivalent amounts of cell lysates were immunoprecipitated with antiphosphotyrosine antibody (Upstate Biotechnology, Inc.), and immune complexes were assayed for PI3K activity as described in Materials and Methods. The position of migration of phosphatidylinositol 3 phosphate [*PI(3)P*] is indicated. Results shown are representative of two independent experiments.

phosphoinositides may regulate the expression of islet/duodenum homeobox-1 (IDX-1) transcriptional factor in undifferentiated rat insulinoma cells growing in vitro at low passages (Ptasznik, A., unpublished data). IDX-1 (PDX-1, IPF-1, STF-1) is known to be important for activation of islet-specific genes and development of endocrine pancreas (Josson et al., 1994; Miller et al., 1994; Watada et al., 1996). Experiments to test this alternative in primary growing human fetal cells are in progress.

The data presented here strongly suggest that the functional association between PI3K activity and islet-specific gene expression is part of a more general developmental program that coordinates cell differentiation and cell division. It has previously been suggested that the general function of HGF/SF is to allow various epithelial cells to rearrange during embryogenesis by promoting their proliferation, scattering, and invasiveness (Brinkmann et al., 1995). The ability of PI3K to inhibit islet-specific hormone gene expression, which we show in our present experiments, provides an explanation for the link between the activation of the HGF/SF receptor and the downregula-

tion of islet-specific gene transcription. Since PI3K is activated in islet cells by HGF/SF (Figs. 5 and 6), it could serve in these proliferating cells at the same time to block insulin synthesis and secretion. We have clearly shown that induction of endocrine differentiation by PI3K inhibitors is associated with a decrease in DNA synthesis in ICCs (Figs. 1, 2, 3, 4) and, vice versa, that the transition of fetal ICCs towards proliferation, by the addition of HGF/SF, is associated with downregulation of islet-specific hormone gene expression and a decrease in the hormone protein content (Beattie et al., 1996a; Otonkoski et al., 1996). Thus, according to our present results, it is possible to suggest that PI3K may functionally convert activation of growth factor receptors into downregulation of tissue-specific genes, and in this way accommodate the rates of differentiation versus proliferation in developing tissues. Thus, cell differentiation and cell division would be modulated in a coordinated way, by the common signaling transducer-PI3K. An inverse relationship between proliferation and endocrine differentiation in insulin-producing cells has already been demonstrated previously (Philippe et al., 1987a,b; Oberg et al., 1994). Our observation is also in agreement with the general view that tissue-specific functions inversely correlate with cellular growth during embryogenesis.

It is known that external stimulation of islet cells with insulin inhibits insulin gene expression in these cells (Koranyi et al., 1992). Consistent with our present results, it is possible to suggest that PI3K, which is known to be a downstream target for the insulin receptor (Backer et al., 1993), might functionally link activation of this receptor to downregulation of insulin and other hormone-specific genes in fetal islet cells. This is also consistent with the recent observation that islet β -cells express the insulin receptor mRNA and insulin receptor substrate 1, i.e., the same signal transducers that are known to mediate insulin action in peripheral insulin target tissues (Harbeck et al., 1996). Since PI3K is activated by insulin in target tissues, it could serve in β -cells at the same time to block insulin biosynthesis and secretion. An autocrine feedback loop acting through PI3K would be part of the signaling mechanism maintaining homeostatic control within developing fetal cells.

It has been shown that during development, multipotential epithelial stem cells give rise to all islet cell phenotypes (Teitelman and Lee, 1987; Alpert et al., 1988). The double control mechanism was suggested to be necessary during islet development (Alpert et al., 1988; Herrera et al., 1991;

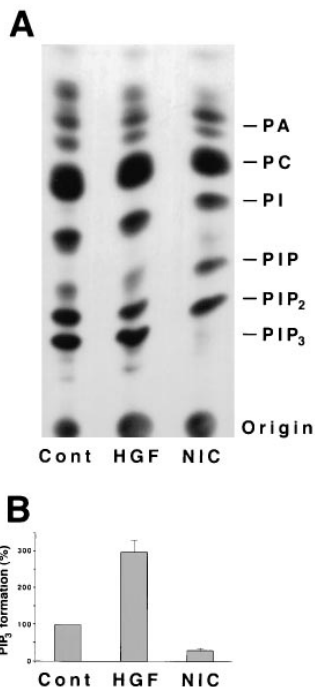


Figure 6. Effect of continuous treatment for 5 d with HGF/SF or NIC on PIP₃ formation in fetal ICCs. (A) Autoradiogram of a thin-layer chromatographic separation of cell phospholipids showing levels of PIP₃ in fetal islet cells treated continuously for 5 d in the absence (*Cont*) or presence of 25 ng/ml HGF/SF (*HGF*) or 10 mM nicotinamide (*NIC*). (B) Radioactivity in PIP₃ spots was quantitated and is expressed relative to control. Results shown are representative of three similar experiments. *PA*, phosphatidic acid; *PC*, phosphatidylcholine.

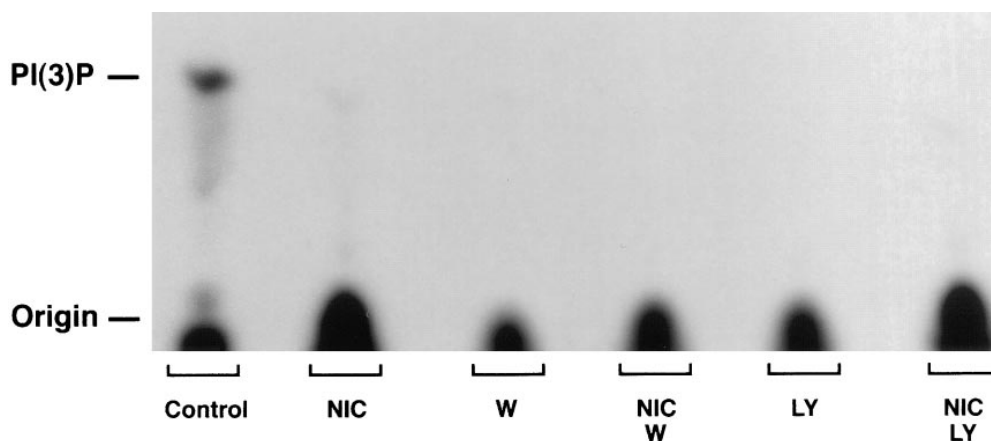


Figure 7. Effect of continuous treatment for 5 d with NIC, wortmannin, or Ly294002 on cellular PI3K activity in fetal ICCs. Fetal islet cells were incubated for 5 d with control medium (*Control*), 10 mM nicotinamide (*NIC*), 100 nM wortmannin (*W*), 10 μ M Ly294002 (*LY*) or combined NIC plus inhibitor. PI3K activity in p85 precipitates from islet cells was analyzed as described in Materials and Methods. Results shown are representative of two independent experiments.

Cordier-Bussat et al., 1995). The first step occurs when all the islet-specific hormone genes are activated, and the cells are able to coexpress multiple hormone genes. The second step occurs when the differentiating cells become restricted to express only one hormone gene. The data presented here strongly point to the early islet progenitor cells as a target, which responded to our treatment with PI3K inhibitors. Thus, we observed that treatment with these inhibitors released the blockade of all hormone genes in undifferentiated pancreatic cells and significantly increased the number of cells coexpressing multiple hormone proteins, which are known to represent precursors of terminally differentiated islet cells. As a further indication of the involvement of progenitor islet cells, we also observed that the PI3K inhibitors combined with NIC caused the synergistic increase of the mRNA levels of islet-specific hormone genes. NIC alone was previously shown to induce these mRNA levels only in precursor cells, without any effect on mature endocrine islet cells (Otonkoski et al., 1993). Finally, we observed no effect of PI3K inhibitors or NIC on endocrine function in purified fetal islets (data not shown). These results would suggest, again, that the effects that we found in primary cultured ICCs, rich in precursor cells, were developmentally dependent. For this reason, these effects cannot be detected in purified islets, which contain mostly terminally differentiated endocrine cells and few precursor cells (see Materials and Methods).

In conclusion, our results describe a role of PI3K in regulating development of the human endocrine system. Interestingly, it was shown just recently that inhibition of the PI3K displays a stimulatory effect on melanogenesis and dendrite outgrowth in B16 murine melanoma cell line (Busca et al., 1996). Thus, the negative regulation of cellular differentiation by PI3K, which we independently discovered in primary growing human cells during fetal neogenesis, may be a general phenomenon. Nevertheless, other authors have previously shown a positive involvement of PI3K in PC12 pheochromocytoma and 3T3-F442A adipocytic cell lines differentiation (Hempstead et al., 1992; Kimura et al., 1994; Saad et al., 1994). Taken together, all available results suggest that the PI3K may play a dual role as both a positive and negative regulator of cellular differentiation in mammalian cells. Future studies di-

rected at the downstream signaling elements coupled to PI3K should prove informative, as will further investigation of the transcriptional factors by which PI3K links to tissue-specific gene regulation.

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References

- Alpert, S., D. Hanahan, and G. Teitelman. 1988. Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell*. 53:259-308.
- Backer, J.M., M.G. Myers, Jr., X.J. Sun, D.J. Chin, S.E. Shoelson, M. Miralpeix, and M.F. White. 1993. Association of IRS-1 with the insulin receptor and the phosphatidylinositol 3-kinase: formation of binary and ternary signaling complexes in intact cells. *J. Biol. Chem.* 268:8204-8212.
- Beattie, G.M., F. Levine, M.I. Mally, T. Otonkoski, J.S. O'Brien, D.R. Salomon, and A. Hayek. 1994. Acid B-galactosidase: a developmentally regulated marker of endocrine cell precursors in the human fetal pancreas. *J. Clin. Endocrinol. Metab.* 78:1232-1240.
- Beattie, G.M., J.S. Rubin, M.I. Mally, T. Otonkoski, and A. Hayek. 1996a. Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes*. 45:1223-1228.
- Beattie, G.M., T. Otonkoski, A.D. Lopez, and A. Hayek. 1996b. Functional β -cell mass after transplantation of human fetal pancreatic cells: differentiation or proliferation? *Diabetes*. 46:244-248.
- Brinkmann, V., H. Foroutan, M. Sachs, K.M. Weidner, and W. Birchmeier. 1995. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. *J. Cell Biol.* 131:1573-1586.
- Brown, W.J., D.B. De Wald, S.D. Emr, H. Plutner, and W.E. Balch. 1995. Role for phosphatidylinositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. *J. Cell Biol.* 130:781-796.
- Busca, R., C. Bertolotto, J.P. Ortonne, and R. Ballotti. 1996. Inhibition of the phosphatidylinositol 3-kinase/p70S6-kinase pathway induces B16 melanoma cell differentiation. *J. Biol. Chem.* 271:31824-31830.
- Cantley, L.C., K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell*. 64:281-302.
- Carpenter, C.L., and L.C. Cantley. 1996. Phosphoinositide kinases. *Curr. Opin.*

- Cell Biol.* 8:153–158.
- Cordier-Bussat, M., C. Morel, and J. Philippe. 1995. Homologous DNA sequences and cellular factors are implicated in the control of glucagon and insulin gene expression. *Mol. Cell Biol.* 15:3904–3916.
- De Tata, V., A. Ptasznik, and V.J. Cristofalo. 1993. Effect of the tumor promoter phorbol 12-myristate 13-acetate (PMA) on proliferation of young and senescent WI-38 human diploid fibroblasts. *Exp. Cell Res.* 205:261–269.
- Eberle, M., A.E. Traynor-Kaplan, L.A. Sklar, and J. Norgauer. 1990. Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? *J. Biol. Chem.* 265:16725–16728.
- Gao, Z.-y., R.J. Konrad, H. Collins, F.M. Matschinsky, P.L. Rothenberg, and B.A. Wolf. 1996. Wortmannin inhibits insulin secretion in pancreatic islets and B-TC3 cells independent of its inhibition of phosphatidylinositol 3-kinase. *Diabetes.* 45:854–862.
- Graziani, A., D. Gramaglia, L.C. Cantley, and P.M. Comoglio. 1991. The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. *J. Biol. Chem.* 266:22087–22090.
- Harbeck, M.C., D.C. Louie, J. Howland, B.A. Wolf, and P.L. Rothenberg. 1996. Expression of insulin receptor mRNA and insulin receptor substrate 1 in pancreatic islet β -cells. *Diabetes.* 45:711–717.
- Hempstead, B.L., S.J. Rabin, L. Kaplan, S. Reid, L.F. Parada, and D.R. Kaplan. 1992. Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron.* 9:883–896.
- Henderson, E., and R. Stein. 1994. c-jun inhibits transcriptional activation by the insulin enhancer, and the insulin control element is the target of control. *Mol. Cell Biol.* 14:655–662.
- Herrera, P.L., J. Huarte, F. Sanvito, P. Meda, L. Orci, and J.D. Vassali. 1991. Embryogenesis of the murine endocrine pancreas: early expression of the pancreatic peptic gene. *Development (Camb.)*. 113:1257–1265.
- Hiles, I.D., M. Otsu, S. Volinia, M.J. Fry, I. Gout, R. Dhand, G. Panayotou, F.R. Larrea, A. Thompson, and N.F. Totty. 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell.* 70:419–429.
- Hu, P., A. Mondino, E.Y. Skolnik, and J. Schlessinger. 1993. Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. *Mol. Cell Biol.* 13:7677–7688.
- Hu, Q., A. Klippel, A.J. Muslin, W.J. Fantl, and L.T. Williams. 1995. Ras-dependent induction of cellular responses by constitutive active phosphatidylinositol 3-kinase. *Science (Wash. DC)*. 268:100–102.
- Inagaki, N., T. Maekawa, T. Sudo, S. Ishii, Y. Seino, and H. Imura. 1992. c-Jun represses the human insulin promoter activity that depends on multiple cAMP response elements. *Proc. Natl. Acad. Sci. USA.* 89:1045–1049.
- Josson, J., L. Carlsson, T. Edlund, and H. Edlund. 1994. Insulin-promoter-factor 1 (IPF1) is required for pancreas development in mice. *Nature (Lond.)*. 371:606–609.
- Kimura, K., S. Hattori, Y. Kabuyama, Y. Shizawa, J. Takayanagi, S. Nakamura, S. Toki, Y. Matsuda, K. Onodere, and Y. Fukui. 1994. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 269:18961–18967.
- Koranyi, L., D.E. James, E.W. Kraegen, and M.A. Permutt. 1992. Feedback inhibition of insulin gene expression by insulin. *J. Clin. Invest.* 89:432–436.
- Kruse, F., S.D. Rose, G.H. Swif, R.E. Hammer, and R.J. McDonald. 1993. An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer. *Genes Dev.* 7:774–786.
- Leonardo, M.J., and D. Baltimore. 1989. NF- κ B: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell.* 58:227–229.
- Miller, C.P., R.E. McGehee, and J.F. Habener. 1994. IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1145–1156.
- Nakanishi, H., K.A. Brewer, and J.H. Exton. 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 268:13–16.
- Newgard, C.B., and J.D. McGarry. 1995. Metabolic coupling factors in pancreatic β -cell signal transduction. *Annu. Rev. Biochem.* 64:689–719.
- Oberg, C., J. Waltenberger, L. Claesson-Welsh, and M. Welsh. 1994. Expression of protein tyrosine kinases in islet cells: possible role of the Fk-1 receptor for β -cell maturation from duct cells. *Growth Fact.* 10:115–126.
- Okada, T., L. Sakuma, Y. Fukui, O. Hazeki, and M. Ui. 1994. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 269:3563–3567.
- Otonkoski, T., G.M. Beattie, M.I. Mally, C. Ricordi, and A. Hayek. 1993. Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J. Clin. Invest.* 92:1459–1466.
- Otonkoski, T., V. Cirulli, G.M. Beattie, M.I. Mally, G. Soto, J.S. Rubin, and A. Hayek. 1996. A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic β -cell growth. *Endocrinology.* 137:3131–3139.
- Philippe, J., W.L. Chick, and J.F. Habener. 1987a. Multipotential phenotypic expression of genes encoding peptide hormones in clonal insulinoma cell lines. *J. Clin. Invest.* 79:351–358.
- Philippe, J., D.J. Drucker, W.L. Chick, and J.F. Habener. 1987b. Transcriptional regulation of genes encoding insulin, glucagon, and angiotensinogen by sodium butyrate in a rat islet cell line. *Mol. Cell Biol.* 7:560–563.
- Powis, G.R., M.M. Bonjouklian, A. Berggren, R. Gallegos, C. Abraham, L. Ashendel, W.F. Zalkow, J. Matter, G. Dodge, G. Gridey, and C.J. Vlahos. 1994. Wortmannin a potent and selective inhibitor of phosphatidylinositol 3-kinase. *Cancer Res.* 54:2419–2423.
- Ptasznik, A., A.E. Traynor-Kaplan, and G.M. Bokoch. 1995. G-protein coupled chemoattractant receptors regulate Lyn tyrosine kinase Shc adapter protein signaling complexes. *J. Biol. Chem.* 270:19969–19973.
- Ptasznik, A., E.R. Prossnitz, D. Yoshikawa, A. Smrcka, A.E. Traynor-Kaplan, and G.M. Bokoch. 1996. A tyrosine kinase signaling pathway accounts for the majority of phosphatidylinositol 3,4,5-trisphosphate formation in chemoattractant-stimulated human neutrophils. *J. Biol. Chem.* 271:25204–25207.
- Pulverer, B.J., J.M. Kyriakis, J. Avruch, E. Nikolakaki, and J.R. Woodgett. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature (Lond.)*. 353:670–672.
- Saad, M.J., F. Folli, E. Araki, N. Hashimoto, P. Csermely, and C.R. Kahn. 1994. Regulation of insulin receptor, insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-F442A adipocytes. Effects of differentiation, insulin and dexamethasone. *Mol. Endocrinol.* 8:545–557.
- Sandler, S., A. Andersson, A. Schnell, A. Mellgren, J. Tollemar, H. Borg, B. Petersson, C.G. Groth, and C. Hellerstrom. 1985. Tissue culture of human fetal pancreas. Development and function of B cells in vitro and transplantation of explants to nude mice. *Diabetes.* 34:1113–1119.
- Sandler, S., A. Andersson, O. Korsgren, J. Tollemar, B. Petersson, C.G. Groth, and C. Hellerstrom. 1989. Tissue culture of human fetal pancreas. Effects of nicotinamide on insulin production and formation of islet-like cell clusters. *Diabetes.* 38(Suppl1):168–171.
- Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nurnberg, et al. 1995. Cloning and characterization of a G-protein-activated human phosphatidylinositol 3-kinase. *Science (Wash. DC)*. 269:690–693.
- Teitelman, G., and J.K. Lee. 1987. Cell lineage analysis of pancreatic islet cell development: glucagon and insulin cells arise from catecholaminergic precursors present in the pancreatic duct. *Dev. Biol.* 121:454–466.
- Thomas, S.M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J.S. Brugge. 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell.* 68:1031–1036.
- Toker, A., M. Meyer, K.K. Reddy, J.R. Falck, R. Aneja, S. Aneja, A. Parra, D.J. Burns, L.M. Ballas, and L.C. Cantley. 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PI-3,4-P2 and PI-3,4,5-P3. *J. Biol. Chem.* 269:32358–32367.
- Tomasz, M., R. Lipman, D. Chowdhury, J. Pawlak, G.L. Verdine, and K. Nakanishi. 1987. Isolation and structure of a covalent cross-link adduct between Mitomycin C and DNA. *Science (Wash. DC)*. 235:1204–1208.
- Touhara, K., B.E. Hawes, T. Biesen, and R.J. Lefkowitz. 1995. G protein β - γ subunits stimulate phosphorylation of Shc adapter protein. *Proc. Natl. Acad. Sci. USA.* 92:9284–9287.
- Traynor-Kaplan, A.E., A.L. Harris, B.L. Thompson, P. Taylor, and L.A. Sklar. 1988. An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature (Lond.)*. 334:353–356.
- Vlahos, C.J., W.F. Matter, K.Y. Hui, and R.F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269:5241–5248.
- Watada, H., Y. Kajimoto, J. Miyagawa, T. Hanafusa, K. Hamaguchi, T. Matsuo, K. Yamamoto, Y. Matsuzawa, R. Kawamori, and Y. Yamasaki. 1996. PDX-1 induces insulin and glucokinase gene expressions in TC1 clone 6 cells in the presence of betacellulin. *Diabetes.* 45:1826–1831.
- Wennstrom, S., A. Siegbahn, Y. Koutaro, A.K. Arvidsson, C.H. Heldin, S. Mori, and L. Claesson Welsh. 1994. Membrane ruffling and chemotaxis transduced by the PDGF B-receptor require the binding site for phosphatidylinositol 3-kinase. *Oncogene.* 9:651–660.
- Wymann, M.P., G. Bulgarelli-Leva, M.J. Zvelebil, L. Pirola, B. Vanhaesebroeck, M.D. Waterfield, and G. Panayotou. 1996. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell Biol.* 16:1722–1733.
- Yonemura, Y., T. Yakashima, and K. Miwa. 1984. Amelioration of diabetes mellitus in partially depancreatized rats by poly-(ADP-ribose)synthetase inhibitors. Evidence of islet β -cell regeneration. *Diabetes.* 33:401–405.