

# Combined Expression of Pancreatic Duodenal Homeobox 1 and Islet Factor 1 Induces Immature Enterocytes to Produce Insulin

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Immature rat intestinal stem cells (IEC-6) given the ability to express the transcription factor, pancreatic duodenal homeobox 1 (Pdx-1), yielded YK cells. Although these cells produced multiple enteroendocrine hormones, they did not produce insulin. Exposure of YK cells to 2 nmol/l betacellulin yielded BYK cells that showed the presence of insulin expression in cytoplasm and that secreted insulin into culture media. By examining the mechanism of differentiation in BYK cells, we found that another transcription factor, islet factor 1 (Isl-1) was newly expressed with the disappearance of Pax-6 expression in those cells after exposure to betacellulin. These results indicated that combined expression of Pdx-1 and Isl-1 in IEC-6 cells was required for the production of insulin. In fact, overexpression of both Pdx-1 and Isl-1 in IEC-6 cells (Isl-YK-12, -14, and -15 cells) gave them the ability to express insulin without exposure to betacellulin. Furthermore, implantation of the Isl-YK-14 cells into diabetic rats reduced the animals' plasma glucose levels; glucose levels dropped from 19.4 to 16.9 mmol/l 1 day after the injection of cells. As expected, the plasma insulin concentrations were 2.7 times higher in the diabetic rats injected with Isl-YK-14 cells compared to in controls. In summary, our results indicated that immature intestinal stem cells can differentiate into insulin-producing cells given the ability to express the transcription factors Pdx-1 and Isl-1. *Diabetes* 51:1398–1408, 2002

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BYK cell, YK cells treated with betacellulin; Cdx-2, caudal-related homeodomain protein 2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GK, glucokinase; HNF, hepatocyte nuclear factor; Isl-1, islet factor 1; Isl-YK cells, YK cells transfected with Isl-1; Ngn-3, neurogenin 3; PBST, PBS containing 0.3% Triton X; Pdx-1, pancreatic duodenal homeobox 1; RIA, radioimmunoassay; STZ, streptozotocin.

**D**uring embryonic development of the pancreas in rodents, the primordial pancreatic lineage is derived from endodermal cells of the primitive foregut (1,2). Anatomically, this process begins with the dorsal and ventral budding of the primitive gut epithelium (3,4), leading to the formation of the pancreas. Recent studies have allowed us to better understand this complex process at the cellular level. Although our knowledge is incomplete, we know that the early stage of pancreatic development requires transient expression of the signaling proteins, Sonic and Indian, two members of the hedgehog family of proteins (5,6). However, the actions of the hedgehog proteins by themselves are insufficient because added mesenchymal factors are required to induce this process (7,8). In addition, several homeodomain and basic helix-loop-helix transcription factors, including islet factor-1 (Isl-1), Nkx6.1, Pax4, Pax6, and NeuroD/Beta2 play important roles in the differentiation of the pancreatic endocrine cells (9,10). These transcription factors are expressed during early or late stages of pancreatic differentiation. Their absence does not interfere with the initial steps, but does affect later developmental stages (11).

Added to the preceding list of transcription factors is pancreatic duodenal homeobox 1 (Pdx-1), another important homeodomain protein. Pdx-1 is expressed in the pluripotent precursor cells of the dorsal and ventral pancreatic buds that arise from the duodenal endoderm. Recent studies have shown Pdx-1 to be an essential factor for pancreatic islet cell differentiation, especially in cells of  $\beta$ -cell lineage (12,13). In adults, Pdx-1 is found in both pancreatic islet  $\beta$ -cells and duodenal epithelial cells (14). Pdx-1 activates not only the expression of insulin, but also that of amylin, pancreatic polypeptide, and somatostatin genes in the pancreatic islet cells (15,16).

Knockout mice lacking Pdx-1 have a very small pancreas and a marked reduction in enteroendocrine cell numbers, most notably in the villi of the proximal duodenum (12). Although reduced in numbers, the presence of both pancreatic and intestinal cells in Pdx-1-deficient animals suggest that Pdx-1 is not the sole factor underlying the differentiation of these cells. Consistent with this hypothesis, recent evidence has shown that betacellulin, a growth factor found in the pancreas and intestine, is also required for pancreatic cell formation (18). Studies have

demonstrated that betacellulin treatment of the amylase-secreting pancreatic cell line, AR42J, imparts to it the ability to secrete insulin (19). These findings underlie our hypothesis that immature intestinal cells may be induced to differentiate into either enteroendocrine or pancreatic endocrine cells when given the ability to express Pdx-1. Furthermore, we postulated that the cells treated in this fashion should be able to express insulin.

To test these hypotheses, we chose an immature intestinal crypt cell line, IEC-6, derived from the small intestine of a normal rat (20). The characteristics of IEC-6 include undifferentiated morphology and limited expression of intestinal cell genes. Overexpression of a caudal-related homeodomain protein, Cdx-2, facilitated differentiation of IEC-6 cells into either goblet-like or absorptive enterocyte-like cells (21). In contrast, the expression of Pdx-1 enabled the IEC-6 cells to express multiple enteroendocrine hormones (22). In addition, we showed that IEC-6 cells exposed to both IGF-I and insulin triggered differentiation. The differentiated cells acquired the ability to synthesize and secrete transforming growth factor- $\beta$ 1 (23). The capacity of the IEC-6 cells to differentiate after selected manipulations provides an ideal model for our studies.

The results of our studies showed that IEC-6 cells expressed and secreted insulin when given the ability to express Pdx-1. The expression of Isl-1 mRNA plays a central role in mediating this process, because IEC-6 cells transfected with both Pdx-1 and Isl-1 also expressed insulin without exposure to betacellulin.

## RESEARCH DESIGN AND METHODS

**Cell lines and culture condition.** Immature rat intestinal stem cells, IEC-6 (passage 11; American Type Culture Collection, Rockville, MD), a nontransformed line, were maintained in culture, as previously described (22). The IEC-6 cells were grown in T-150 flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% dialyzed fetal bovine serum (FBS), 5.6 mmol/l D-glucose, 44.0 mmol/l NaHCO<sub>3</sub>, 0.1 g/l streptomycin, and 10<sup>5</sup> units/l penicillin G. These cells were transfected with or without Pdx-1 and/or Isl-1 and then seeded onto a six-well 0.5- $\mu$ m micropore filter (Falcon Cell Culture Insert; Becton Dickinson, Rutherford, NJ), and grown for 4 days in DMEM containing 5% FBS before manipulations. The cells were washed twice with PBS before being used for histologic and molecular biological analyses.

**Stable transfection.** The complete coding sequence of the rat Isl-1 or mouse Pdx-1 cDNA was inserted separately into the plasmid vector, pcDNA3, to yield pcDNA3-Pdx-1 or pcDNA3-Isl-1, respectively. The two clones had differential antibiotic resistance to neomycin (G418) and hygromycin (Calbiochem-Novabiochem, La Jolla, CA), respectively. First, 3  $\mu$ g of the pcDNA3-Pdx-1 (neomycin resistant vector) or empty vector (pcDNA3) were transfected into IEC-6 cells by electroporation at 250 V and 975  $\mu$ F using the Gene Pulser (BioRad, Osaka City, Japan). Then, 50 of the neomycin resistant clones were isolated and labeled as YK cells. The number designation was used to identify individual clones. Clones YK-12, -14, and -15 were randomly cloned from isolates as representative of the Pdx-1-expressing cells. In this study, we tested all three clones to probe for representative features. The same procedure was used to establish Isl-1-expressing cells by transfection of 3  $\mu$ g pcDNA3-Isl-1 into IEC-6 cells and then selecting for hygromycin resistance. Again, three randomly selected isolates—IS-1, IS-2, and IS-3—were chosen for the studies. Furthermore, Isl-1-YK cells that expressed both Pdx-1 and Isl-1 were established by transfecting 3  $\mu$ g of pcDNA3-Isl-1 into YK-12, -14, and -15 cells that already expressed Pdx-1. These cells should then have had double antibiotic resistance and were selected in medium containing 0.6 mg/ml of G418 and 0.2 mg/ml of hygromycin and then screened for Pdx-1 and Isl-1 expression using Northern blot analysis. No difference in growth was observed between YK and IEC-6 cells containing empty vectors. The YK cells grown on micropore filter became trabecular in shape and showed strong positive staining of Pdx-1 in the nucleus (22). The effect of betacellulin was examined by the daily addition of 2 nmol/l of the IFG-1 for 10 days to cells seeded on micropore filters.

**RNA extraction, Northern blot, and RT-PCR methods.** Total RNA was extracted from cells using a procedure previously described (24). The extracted product was used as a template in reactions containing RT (New England Biolabs) and the 3' primer of interest to synthesize the first strand of the cDNA. RT-PCR analysis was done as previously described (24). The first-strand cDNA was used in subsequent PCR analyses, with the oligonucleotide primers listed below for each one of the genes of interest

somatostatin: 5') TCTCTGCTGCCTGGGACCT, 3') GCCAAGAAGTACTTG GCCAGTTC

insulin: 5') TGCCAGGCTTTGTCAAACAGCACC, 3') CTCCACCCAGCTCCA GTT

PP: 5') TGAACAGAGGGCTCAATACG, 3') AGACAGAAGGGAGGCTACAAA TCC

Nkx6.1: 5') CCGGTATTACACCACTTGGC, 3') AGCGCAAAGGGGCTGCCT TTTT

Isl-1: 5') GCGGAGGATGGGCTTTTCTG, 3') TTCTGCTTTTCGTTGAGCACAG neuroD: 5') CTGGCCAAGAAGTACTACATCTGG, 3') GGAGTAGGGATGCACCG GGAA

Pax4: 5') CTCACGGAGCCTTAAGGTATC, 3') TGAATCTGGATACTGCCACCG Pax6: 5') GTCTACCAGCCAATTCCACAGC, 3') CAGCCATTTCTCTTTCTTT CCT

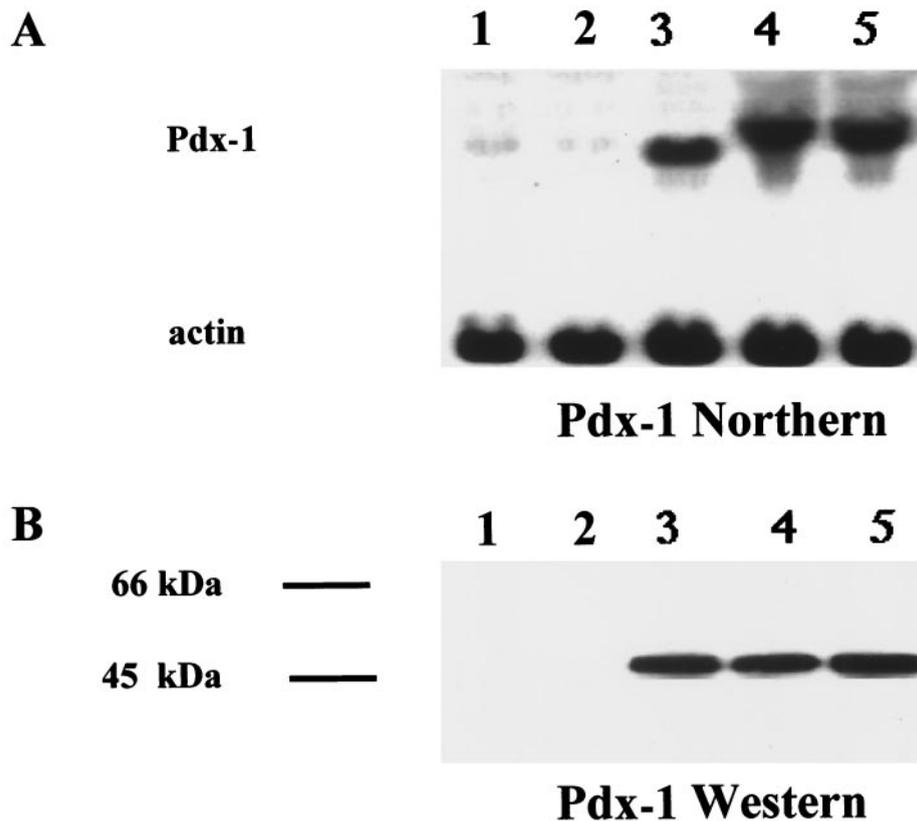
The identity of the PCR products was confirmed by size after agarose gel electrophoresis and nucleotide sequence analysis. Total RNA was extracted from neonatal rat intestine, neonatal rat pancreas, IEC-6 cells, Isl-1-transfected IEC-6 cells, YK cells, YK cells exposed to 2 nmol/l betacellulin, and Isl-1 transfected YK cells. A 10- $\mu$ g aliquot of each sample of total RNA was used for Northern blot analysis. The transferred blots were hybridized with a <sup>32</sup>P-labeled fragment of Pdx-1 (772–1,134), Isl-1 (341–576), or insulin II (56–238) cDNA. The signal arising from actin mRNA served as the internal control to evaluate differences among various samples.

**Western blot analysis.** Relative amounts of Pdx-1 and Isl-1 proteins in the cells were measured using Western blot analysis, according to a procedure previously described (24). Specifically, 5  $\mu$ g cellular lysate were separated by standard SDS-PAGE and then transferred to polyvinylidene fluoride membrane. Specific antibodies against Pdx-1 (25) or Isl-1 (26) protein were used to probe the blot. The antibody-protein complex was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**Immunohistochemical analysis and electron microscopy.** The cells were stained as previously described (22). In brief, the cells were fixed for 2 h with 4% paraformaldehyde, and washed for 4 days with 0.1 mol/l PBS containing 0.3% triton X (PBST) then incubated with an anti-insulin antibody (26) diluted to 1:5,000 in PBST. Immunocytochemical staining was achieved using the avidin-biotin complex method and ABC-nickel reactions (22). For immunofluorescence double staining, the fixed cells were incubated with a mixture of antibodies against Pdx-1 (25) and insulin diluted to 1:5,000 in PBST or a mixture of antibodies against Isl-1 (26) and insulin diluted to 1:5,000 in PBST. The cells were then incubated with the species-specific secondary antibodies conjugated to either fluorescein isothiocyanate or Texas Red. The positive reaction was observed under fluorescence microscopy (Olympus IX70; Olympus, Osaka, Japan) and the images were taken with a charge-coupled device camera (Cool SNAP/HQ; Nippon Roper, Osaka, Japan). Specificity of the positive staining was examined by an immunocytochemical absorption study. Electron microscopic studies of secretory granules were accomplished by incubating cells for 1 h with 1% OsO<sub>4</sub> in 0.1 mol/l PBS at 4°C. The specimen was embedded in epoxy resin and cut into ultra-thin sections. These samples were stained with 2% uranyl acetate and Reynold's solution and then observed under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

**Insulin release into culture media.** Pdx-1-expressing YK-14 cells were plated onto six-well dishes (Corning Glass Works, Corning, NY) at a density of 1  $\times$  10<sup>6</sup> cells per well and allowed to grow for 6 days with DMEM containing 5% FCS and 2 nmol/l betacellulin. DMEM containing betacellulin was replaced each day. The cells were washed three times for 10 min each in DMEM with 0.1% BSA and 5.6 mmol/l glucose. After being washed, the cells were incubated with DMEM containing 5.6 or 22.0 mmol/l glucose and 0.1% BSA for 12 h. The medium was collected and analyzed for its insulin content with a radioimmunoassay (RIA) requiring a specific antibody against insulin (27). The protein content in the cell was analyzed by Bradford assay (BioRad Laboratories). Insulin secretion was normalized based on the total protein in each sample.

**Implantation of insulin-secreting Isl-YK-14 cells into peritoneal cavity.** Male SD rats, age 8 weeks, were used in the implantation studies. Rats were rendered diabetic with a single intravenous injection of streptozotocin (STZ) (Sigma, Madrid, Spain) at a dosage of 50 mg/kg body wt. Blood glucose was measured between 1700 and 1800 in 8-h-fasted animals using the glucose oxidase method (Glucose-Pro; Sanwa, Kyoto, Japan). Then, 6 days after the STZ injection, five diabetic rats were implanted with Isl-YK-14 cells. Prepara-



**FIG. 1.** Expression of Pdx-1 mRNA and protein in stably transfected IEC-6 cells. **A:** Northern blot analysis of Pdx-1 mRNA in IEC-6 or YK cells. Total RNA extracted from wild-type IEC-6 (*lane 1*), IEC-6 containing empty vector (*lane 2*), IEC-6 cells stably transfected with the Pdx-1 cDNA isolate YK-12 (*lane 3*), YK-14 cells (*lane 4*), and YK-15 cells (*lane 5*). The blot shown is representative of three independent experiments. The abundance of actin mRNA (lower band) reflects an equal loading of RNA in each lane. **B:** Western blot of Pdx-1. The content of each lane is identical to that in **A**.

tion of cells for implantation involved washing and resuspending cells in PBS supplemented with 5.6 mmol/l glucose at  $5 \times 10^6$  cells/ml. The animals were placed under general anesthesia using ketamine hydrochloride (95 mg/kg, i.p.) and injected with  $5 \times 10^6$  cells in the peritoneal cavity. The concentration of blood glucose and insulin were measured on a daily basis.

**Statistic analysis.** Results are given as means  $\pm$  SD, unless otherwise stated. Scheffe's multiple comparison test was used to determine the significance of any differences among more than two groups, and the unpaired Student's *t* test was used to determine the significance of any differences between two groups.

## RESULTS

**IEC-6 stably transfected with Pdx-1 expressed high levels of mRNA and protein.** We have recently established a cell line, YK, derived from wild-type IEC-6 cells stably transfected with Pdx-1 cDNA (22). In culture, these cells form a trabecular network and have the capacity to differentiate into cells that express several hormones, including serotonin, somatostatin, gastrin, and cholecystokinin (22). Three separate clones—YK-12, -14, and -15 from the pool of YK cells—were chosen for analysis. The results showed expression of Pdx-1 mRNA and protein in YK-12, -14, and -15 isolates (Fig. 1A and B, lanes 3, 4, and 5, respectively), but not in wild-type IEC-6 or cells carrying empty vector. The expression of actin mRNA was detected in both YK and IEC-6 cells (Fig. 1A, lanes 1 and 2). Pdx-1 protein was detected only in YK cells, and not IEC-6 cells, as a 46-kDa band (Fig. 1B). The apparent molecular mass of Pdx-1 is 46 kDa and differs from its cDNA estimated value of 31 kDa likely because of translocation, as described in a recent report (28). These studies showed

clearly that IEC-6 cells stably transfected with Pdx-1 cDNA express both the mRNA and the protein.

**Exposure of YK cells to betacellulin induces insulin expression.** The process of differentiation leading to the formation of pancreatic cells requires specific transcription factors and extrinsic signals, as noted above. This knowledge prompted us to postulate that immature intestinal cells transfected with Pdx-1 might acquire the ability to express insulin after exposure to betacellulin. We tested this hypothesis using YK cells exposed to 2 nmol/l betacellulin. YK cells treated with betacellulin (BYK cells) were examined for the expression of hormones somatostatin, pp, insulin, and glucagon using RT-PCR (Fig. 2). When the three isolates BYK-12, -14, and -15 were examined, they revealed the same finding (only data from BYK-14 are shown). YK-14 cells (Fig. 2, lane 5) expressed pp and somatostatin, whereas the BYK-14 clone newly expressed insulin in addition to pp and somatostatin (*lane 6*) but not glucagon. The identity of the 182-bp RT-PCR product was presumed to be that of insulin cDNA, verified by nucleotide sequencing. In contrast, wild-type IEC-6 cells or those transfected with empty vector (Fig. 2, lanes 3 and 4, respectively) did not express any of the mRNA-encoding hormones of interest. In addition, treatment of IEC-6 cells with betacellulin did not cause them to produce insulin or alter expression of the other hormones (data not shown). As expected, rat pancreatic cells (Fig. 2, lane 2) expressed mRNAs for all four hormones.

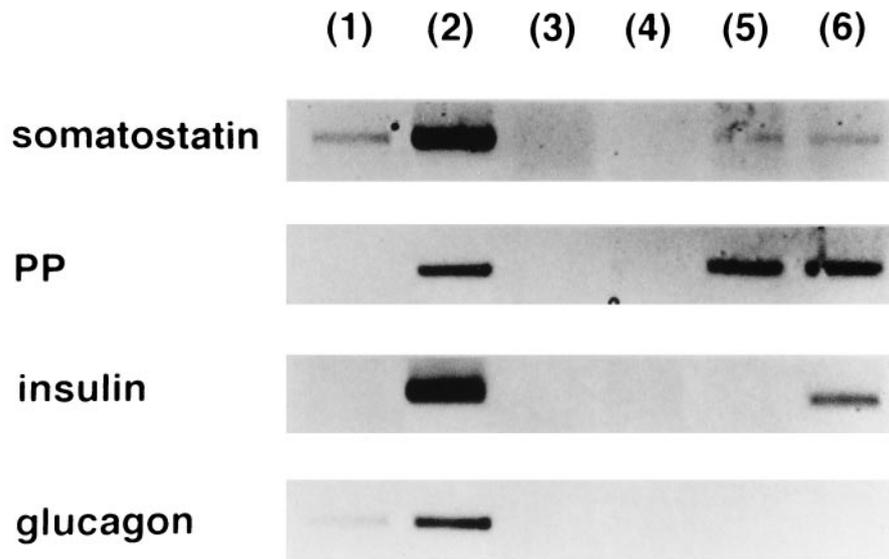


FIG. 2. Effects of Pdx-1 overexpression and/or betacellulin on hormone expression in IEC-6 cells. RT-PCR analysis was used to detect the presence of somatostatin, PP, insulin, or glucagon mRNA in total RNA isolated from neonatal rat intestine (lane 1), pancreas (lane 2), IEC-6 cells (lane 3), IEC-6 cells containing empty vector (lane 4), YK-14 cells (lane 5), and YK-14 cells treated with 2 nmol/l betacellulin (lane 6). Similar data obtained using YK-12 and -15 cells are not shown.

To further demonstrate that BYK isolates express insulin, the cells were immunostained for the presence of the hormone. Immunoreactivity for insulin was observed in the cytoplasm of trabecular-shaped BYK-14 cells (Fig. 3B, indicated by arrows). In agreement with results of RT-PCR (Fig. 2), no insulin was found in untreated YK-14 cells (Fig. 3A). Next, we examined the ultrastructure of BYK-14 cells (Fig. 4B [left and right]) and YK-14 cells (Fig. 4A [left and right]) using electron microscopy. Results showed the presence of secretory granules in the cytoplasm of both cell types. Whereas YK-14 cells contained secretory granules of pleomorphic shapes (Fig. 4A, right), those of BYK-14 cells were round in shape with an electron-dense core (Fig. 4B, right). Findings similar to those in BYK-14 cells were also observed in BYK-12 and BYK-15 isolates (data not shown). The addition of these findings to those of RT-PCR demonstrated that Pdx-1-expressing YK isolates derived from IEC-6 cells exposed to betacellulin enabled these cells to express insulin.

**Insulin release from BYK cells into medium is not regulated by glucose.** Glucose-regulated expression of the insulin gene is a key feature of this gene in pancreatic cells. To test whether glucose induced the expression of insulin in BYK cells, the cells were incubated in medium containing either 5.6 or 22.0 mmol/l D-glucose and then assayed for their concentration of insulin using RIA. Results showed that the expression of insulin was not detected in YK-14 cells (Table 1). However, after exposure of the same cells to betacellulin, insulin ( $106.2 \pm 6.6$  nmol/l) was secreted into the cell culture medium. The concentration of insulin in the medium was the same in the presence of 5.6 or 22.0 mmol/l glucose (data not shown). These results demonstrated that exposure of the Pdx-1-expressing IEC-6 cells (YK cells) to betacellulin induces the cells to express and secrete insulin, although this response is not regulated by the glucose concentration in the medium.

**Betacellulin induces expression of Isl-1.** We previously showed that IEC-6 cells express a variety of transcription

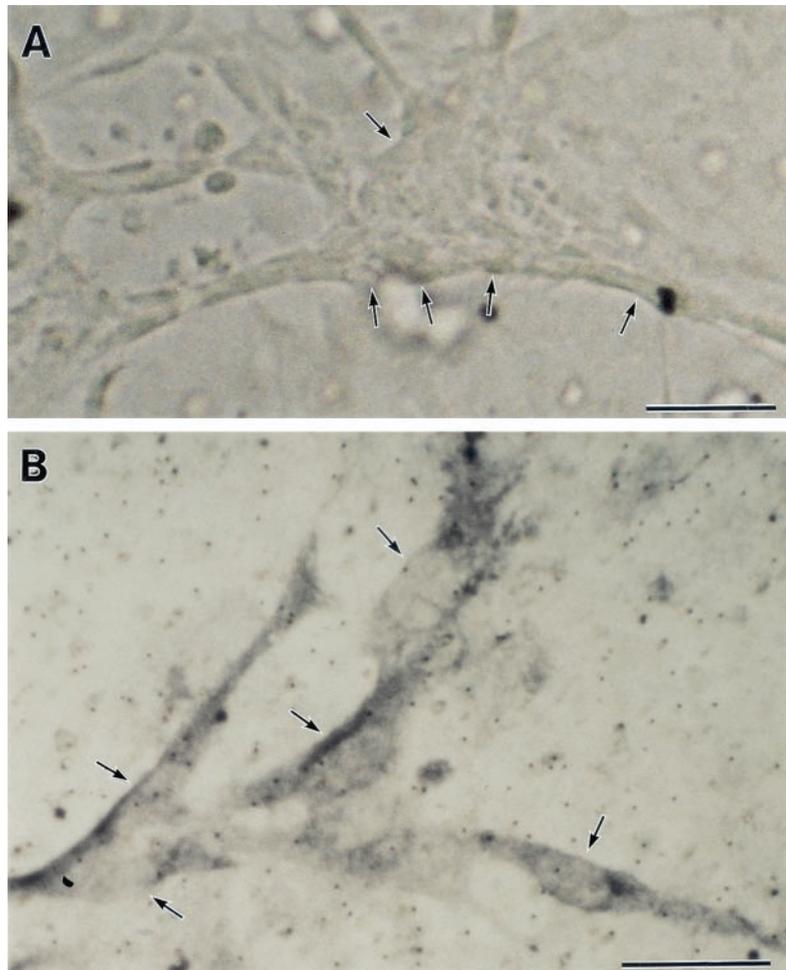
factors, including hepatocyte nuclear factor (HNF)-1 $\alpha$ , -3 $\alpha$ , -3 $\beta$ , and -4 $\alpha$  and NeuroD/Beta2 (22). We then postulated that in the presence of betacellulin, the expression of added transcription factors that enable BYK cells to produce insulin is probably different than in untreated Pdx-1-expressing cells. To examine this possibility, we used RT-PCR to probe for the expression of several transcription factors known to be important for pancreatic  $\beta$ -cell differentiation. The results showed that two Pdx-1-expressing cells, isolates YK-12 and -14 (Fig. 5, lanes 4 and 5, respectively), contained mRNAs encoding NeuroD/Beta2, Pax6, and Nkx6.1, but not mRNAs for Isl-1 and Pax4. After exposure of the YK-12, -14, and -15 cells to betacellulin (Fig. 5, lanes 6–8), we detected Isl-1 expression with the disappearance of Pax6 expression. As expected, rat pancreas expressed all factors (lane 1), but the wild-type IEC-6 cells transfected with empty vector (lane 2) or those exposed to betacellulin (lane 3) expressed none of the preceding factors except NeuroD/Beta2. These findings demonstrate that betacellulin induces the expression of Isl-1 in YK cells and that this event appears to be important in enabling the expression of insulin in these cells.

**Expression of Isl-1 in YK cells enables insulin expression without exposure to betacellulin.** If the expression of Isl-1 in YK cells is critical for the expression of insulin, then overexpression of Isl-1 in Pdx-1-expressing IEC-6 cells might mimic the effects of betacellulin treat-

TABLE 1  
Insulin released by YK-14 cells with and without exposure to betacellulin

Condition	Insulin concentration (nmol/l)
Without betacellulin	<0.1
With betacellulin	$106.2 \pm 6.6^*$

Data are mean and mean  $\pm$  SD ( $n = 4$ ). YK-14 cells were exposed to 2 nmol/l betacellulin for 6 days in cultured medium containing 5.6 mmol/l glucose. The spent culture medium was assayed for insulin concentration using RIA. \* $P < 0.01$  vs. cells without betacellulin.



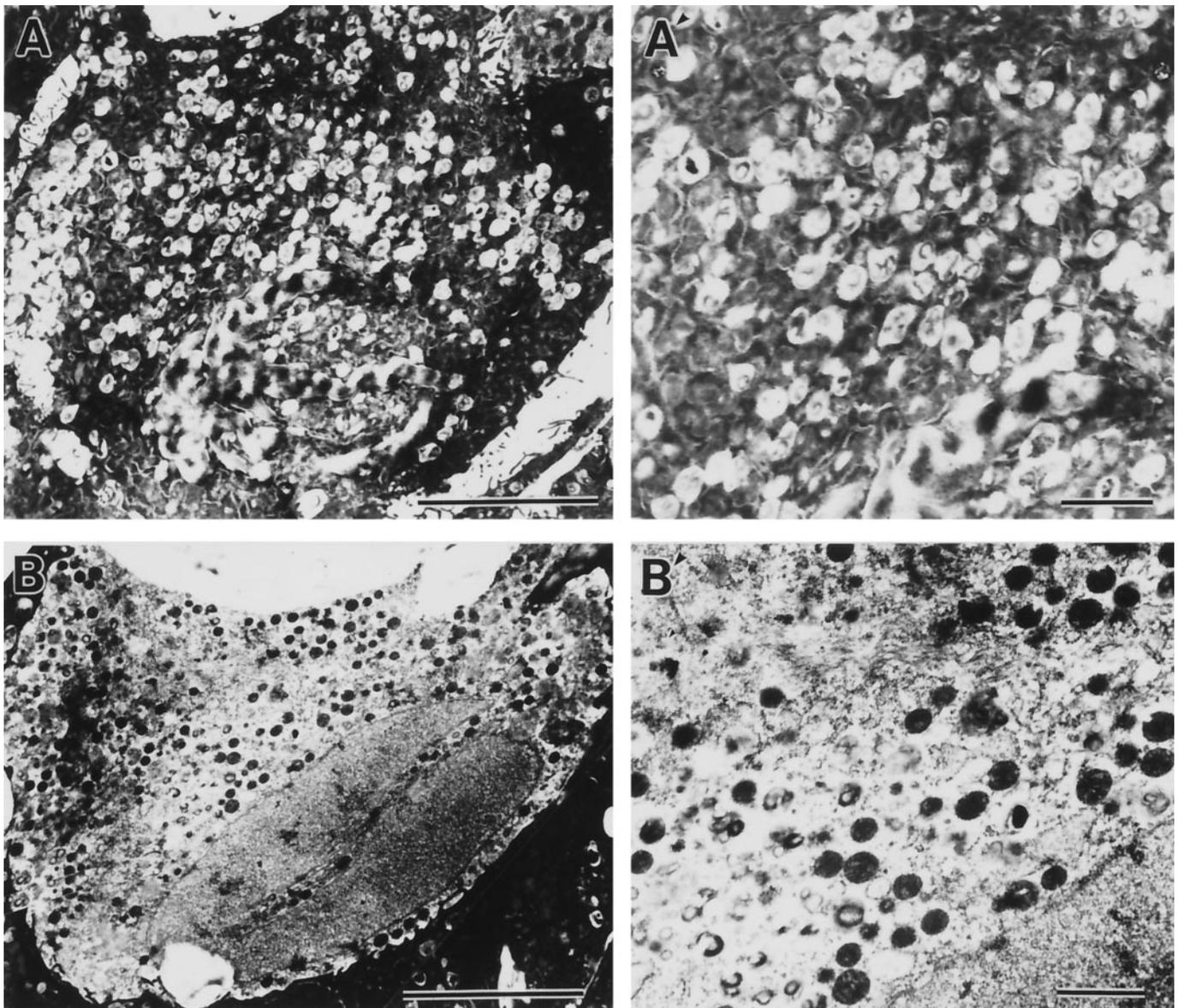
**FIG. 3.** Immunocytochemical staining for insulin in YK and BYK cells. **A:** Lack of staining for insulin in YK-14 cells. **B:** Presence of positive staining for insulin in the cytoplasm of trabecular-shaped BYK-14 cells (arrows). Data shown are representative of three independent experiments using YK-12 and -15 cells. Bar = 25  $\mu$ m.

ment. Therefore, we stably transfected YK-12, -14, and -15 clones with Isl-1 cDNA and then tested the isolates for the presence of Isl-1 expression using Northern and Western blot analysis. The results (Fig. 6) showed that neither wild-type IEC-6 nor YK-12 cells expressed Isl-1 mRNA (Fig. 6A, lanes 1 and 2) or protein (Fig. 6B, lanes 1 and 2). However, in cells transfected with Isl-1 cDNA, both Isl-1 mRNA and protein were observed (Fig. 6B, lanes 5–7). As expected, IEC-6 cells transfected with Isl-1 cDNA also expressed both Isl-1 mRNA and protein (lanes 3 and 4).

Next we tested for the presence of insulin mRNA in YK cells transfected with Isl-1 (Isl-YK cells) and BYK cells using Northern blot analysis. The results showed that, as expected, insulin mRNA was detected in the pancreatic cells (Fig. 6C, lane 1), but not in the rat intestinal cells (Fig. 6C, lane 2). Similarly, the mRNA was not present in wild-type IEC-6 cells (Fig. 6C, lane 3), IEC-6 cells transfected with Isl-1 (Fig. 6C, lane 4), or YK-14 cells (Fig. 6C, lane 5). However, insulin mRNA was present in BYK-14 and -15 cells (Fig. 6C, lanes 6 and 7) and Isl-YK-12, -14, and -15 cells (Fig. 6C, lanes 8–10). Although not shown here, the abundance of insulin mRNA in the cells and protein concentration in the medium did not change in BYK cells after exposure to 5.6 or 22.0 mmol/l D-glucose. These findings show that equipping the Pdx-1-expressing YK

cells with the ability to make Isl-1 enables the cells to produce insulin mRNA without the need for treatment with betacellulin.

**Pdx-1, Isl-1, and insulin expression in Isl-YK cells.** Next, we searched for co-localization of Pdx-1, Isl-1, and insulin expression in Isl-YK-14 cells using immunofluorescence double staining. In Isl-YK-14 cells, positive staining for insulin was localized to the cytoplasm (Fig. 7B and E). Consistent with the transcriptional role of Pdx-1, positive staining for this protein was found mainly in the nucleus but also minimally in cytoplasm (Fig. 7A). Insulin and Pdx-1 staining co-localized to Isl-YK-14 cells (Fig. 7C). Similarly, positive staining for Isl-1 appeared in the nucleus of Isl-YK-14 cells (Fig. 7D), and this staining co-localized with that for insulin in Isl-YK-14 cells (Fig. 7F). The same studies using Isl-YK-12 and -15 cells showed the identical findings (results not shown). These observations demonstrated that the expression of Pdx-1, Isl-1, and insulin co-localize in Isl-YK cells. As expected, positive staining of Isl-1 was also observed in the nucleus of IEC-6 cells transfected with Isl-1 cDNA alone (Fig. 7G), but the staining for insulin (Fig. 7H) was not present in these cells. **Isl-YK-14 cells express insulin in diabetic rats.** To determine whether Isl-YK-14 cells could produce insulin in vivo, we implanted these cells into the peritoneal cavity of



**FIG. 4.** Electron microscopic analysis of YK-14 and BYK-14 cells. The electron micrographs show the presence of numerous secretory granules in the cytoplasm of both YK-14 (A) and BYK-14 cells (B). At higher magnification the YK-14 cells contain secretory granules with pleomorphic shapes (A'), whereas BYK-14 cells contain secretory granules that have round shapes with electron dense cores (B'). The data shown are representative of three independent experiments; studies using YK-12 and -15 cells are not shown. Bars = 1  $\mu$ m.

diabetic rats. Rats were rendered diabetic, on day 0, with a single injection of STZ, and the appearance of diabetes was monitored over the ensuing 5 days. On day 6 of the study, the animals were injected with Isl-YK-14 cells and then monitored for glucose levels. In animals injected with YK-14 cells (Fig. 8A), the mean glucose continued to climb from 19.3 up to 21.8 mmol/l spanning days 7–9 of the study. In contrast, the rats implanted with the insulin-producing Isl-YK-14 cells showed a rapid decrease in blood glucose levels from 19.4 mmol/l on day 6 to 16.9 mmol/l on day 7, a value that was significantly lower ( $P < 0.05$ ) than in animals injected with YK-14 cells. Consistent with the reduction in glucose, the plasma insulin concentration was 2.7-fold higher in rats carrying the Isl-YK-14 cells ( $P < 0.05$ ) than in rats with YK-14 cells on day 7 of the study (Fig. 8B). Similar results were obtained using Isl-YK-15 cells. These findings show that Isl-YK cells produced

functionally active insulin after being injected into diabetic rats.

#### DISCUSSION

Pancreatic cell differentiation from progenitor cells requires both intrinsic and extrinsic influences, such as transcription and growth factors. In this report, we used the cell line IEC-6, derived from rat immature intestinal crypt cells, to test whether these cells can be induced to express insulin. Our previous studies showed that IEC-6 cells given the ability to express a transcription factor, Pdx-1, undergo differentiation into enteroendocrine cells. In culture, these cells form a trabecular network, acquire features of endocrine cells, and synthesize several enteroendocrine hormones, but not insulin (22). After exposure of the Pdx-1-expressing IEC-6 cells (YK cells) to 2

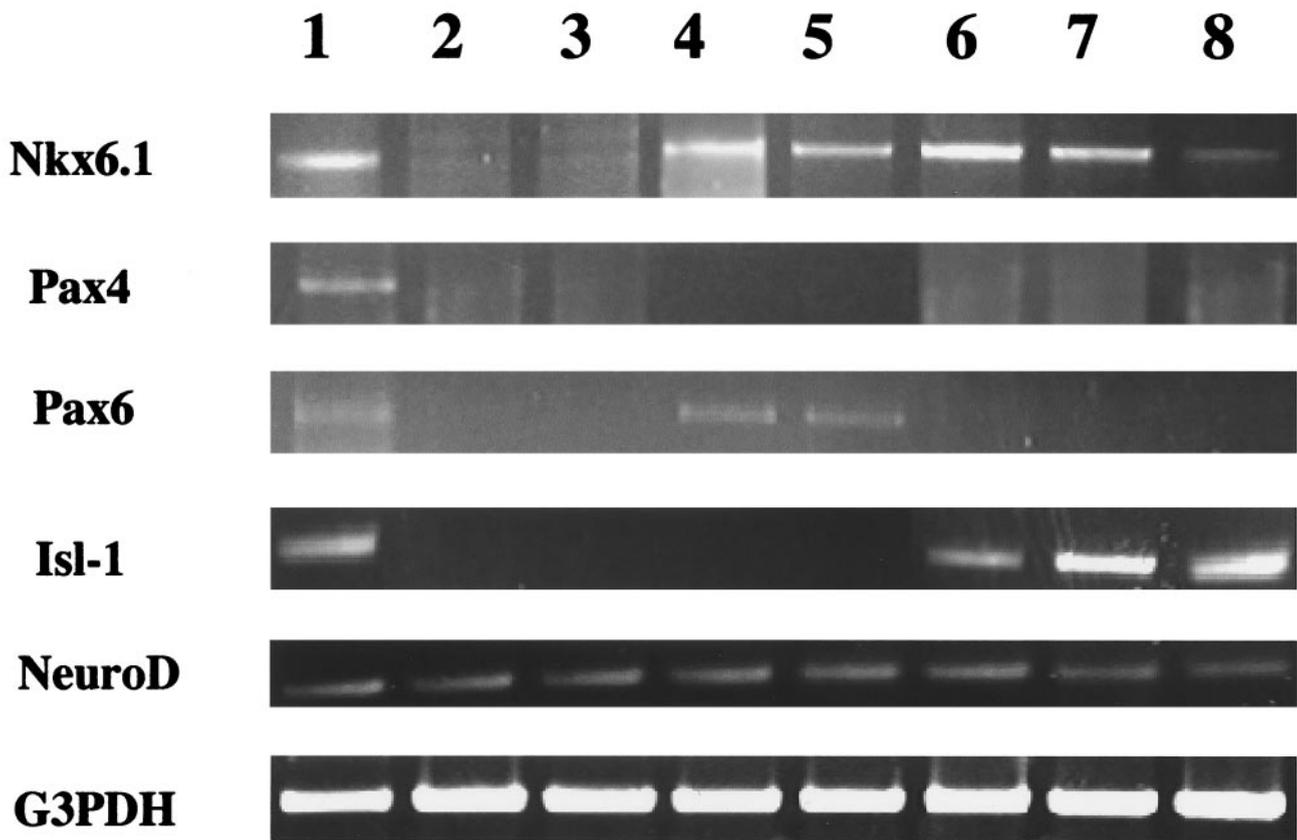


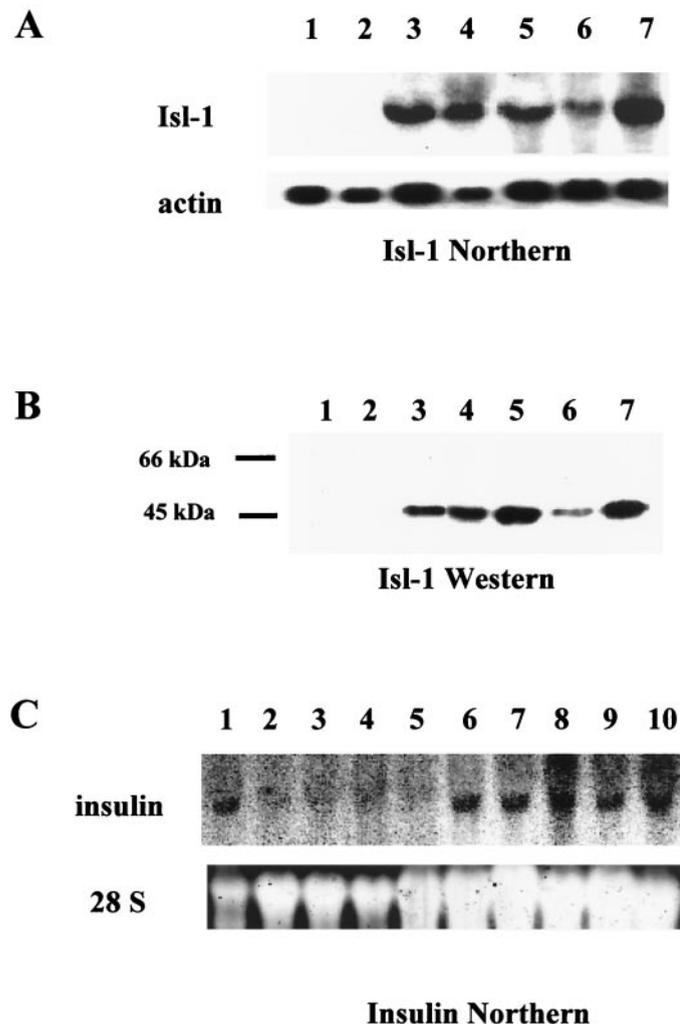
FIG. 5. Effects of Pdx-1 overexpression and/or betacellulin on expression of selected transcription factors. RT-PCR was used to probe for the presence of NeuroD, Isl-1, Nkx6.1, Pax6, Pax4, and G3PDH in total RNA isolated from neonatal rat pancreas (lane 1), IEC-6 cells containing empty vector (lane 2), IEC-6 cells with empty vector exposed to betacellulin (lane 3), YK-14 cells (lane 4), YK-15 cells (lane 5), BYK-12 cells (lane 6), BYK-14 cells (lane 7), and BYK-15 cells (lane 8).

nmol/l betacellulin, these cells synthesized insulin and secreted this hormone into the culture medium. However, the regulation of secreted insulin from the cells in response to glucose concentrations was not observed. Insulin gene expression is known to require the participation of several transcription factors (29,30); in this study, betacellulin treatment of YK cells yielded BYK cells, which have the ability to synthesize insulin. The BYK cells expressed transcription factor(s) that differed from those of YK cells. This finding prompted us to examine whether the specific transcription factors were important for insulin gene expression in the BYK cells. Results of RT-PCR using total RNA from the insulin-producing BYK cells revealed the presence of sequences encoding; NeuroD/Beta2, Isl-1, and Nkx6.1. Among the transcription factors tested, only Isl-1 was detected in the BYK and not in the YK cells before treatment with betacellulin. These results suggest that the combined expression of Pdx-1 and Isl-1 enables intestinal stem cells to produce insulin.

Recent studies *in vitro* and *in vivo* have suggested that enteroendocrine cells may share a common lineage with those of the intestinal epithelium, such as the absorptive enterocytes, goblet, or Paneth cells. The origin of both the enteroendocrine and intestinal epithelial cells is believed to arise from a primitive intestinal stem cell located in the intestinal crypt layers (31). Published studies have shown that two transcription factors, *cdx-2* and Pdx-1, may be important in deciding the pathway leading to differentiation and thus the formation of absorptive enterocytes or

pancreatic cells, respectively (32). The overexpression of *cdx-2* in IEC-6 cells resulted in the formation of absorptive enterocytes or goblet cells (33). In contrast, cellular expression of Pdx-1, a homeotic selector in IEC-6 cells, led to the formation of pancreatic  $\beta$ -cells (22). The presence of Pdx-1 enables these immature enterocytes to produce multiple enteroendocrine hormones, such as cholecystokinin, pp, gastrin, serotonin, somatostatin, and chromogranin A, but not insulin (22). Although Pdx-1 is an important factor for cellular expression of insulin, we found that this transcription factor alone is not sufficient to induce expression of the gene in intestinal stem cells. It appears that the combined expression of Pdx-1 and Isl-1 is required to trigger insulin gene expression.

We found that the presence of Isl-1 appears to be necessary for the expression of insulin in BYK cells. Differentiation leading to the formation of enteroendocrine cells is associated with the expression of many transcription factors, including NeuroD/Beta2, Pax4, Pax6, Nkx6.1, and Isl-1 (9,10,34). Despite the expression of these factors in addition to neurogenin 3 (Ngn-3; data not shown) in the Pdx-1-expressing YK cells, they did not produce insulin. However, the induction of Isl-1 expression in the YK cells enabled the production of insulin. These observations are consistent with the idea that cellular expression of both Pdx-1 and Isl-1 is necessary for the cells to produce insulin. In fact, this role of Isl-1 in insulin expression is consistent with our recent data showing that a human brain tumor arising from neuroec-



**FIG. 6.** Insulin mRNA expression in BYK and Isl-YK cells. **A:** Northern blot studies using total RNA isolated from IEC-6 cells (*lane 1*), YK-14 cells (*lane 2*), and IEC-6 cells transfected with Isl-1 cDNA alone. Clone1 (*lane 3*), clone2 (*lane 4*), and Isl-YK-12, -14, and -15 cells (*lanes 5-7*, respectively) were hybridized with the radio-labeled cDNA probes encoding Isl-1 or actin. **B:** Western blot containing cell lysate from cells designated in **A** probed with Isl-1 antibody. **C:** 10  $\mu$ g total RNA were obtained from neonatal rat pancreas (*lane 1*), neonatal rat intestine (*lane 2*), IEC-6 cells (*lane 3*), IEC-6 cells transfected with Isl-1 cDNA (*lanes 4 and 5*, different clones), BYK-14 cells (*lane 6*), BYK-15 cells (*lane 7*), and Isl-YK-12, -14, and -15 cells (*lanes 8-10*, respectively). Those RNA fractions were separated by electrophoresis and transferred to a membrane and hybridized with the radio-labeled cDNA probes encoding insulin.

toderm cells produced insulin without the expression of Pdx-1. The insulin-producing brain tumor cells also expressed Isl-1, Ngn-3, and NeuroD/Beta2 (26) without expression of Pdx-1. These findings suggest that Isl-1, NeuroD/Beta2 and/or Ngn-3 may play important roles during the production of insulin, more so than Pdx-1 and especially in nonislet cells. In support of this possibility, the Isl-1 knockout mouse was found to have no dorsal pancreatic mesenchyme or differentiated islet cells, despite normal expression of Pdx-1 in these animals (7). In addition, Isl-1, which is one of the LIM homeodomain proteins, may synergistically activate the insulin mRNA transcription rate through binding with basic helix-loop-helix proteins (35,36). Together, these experiments and

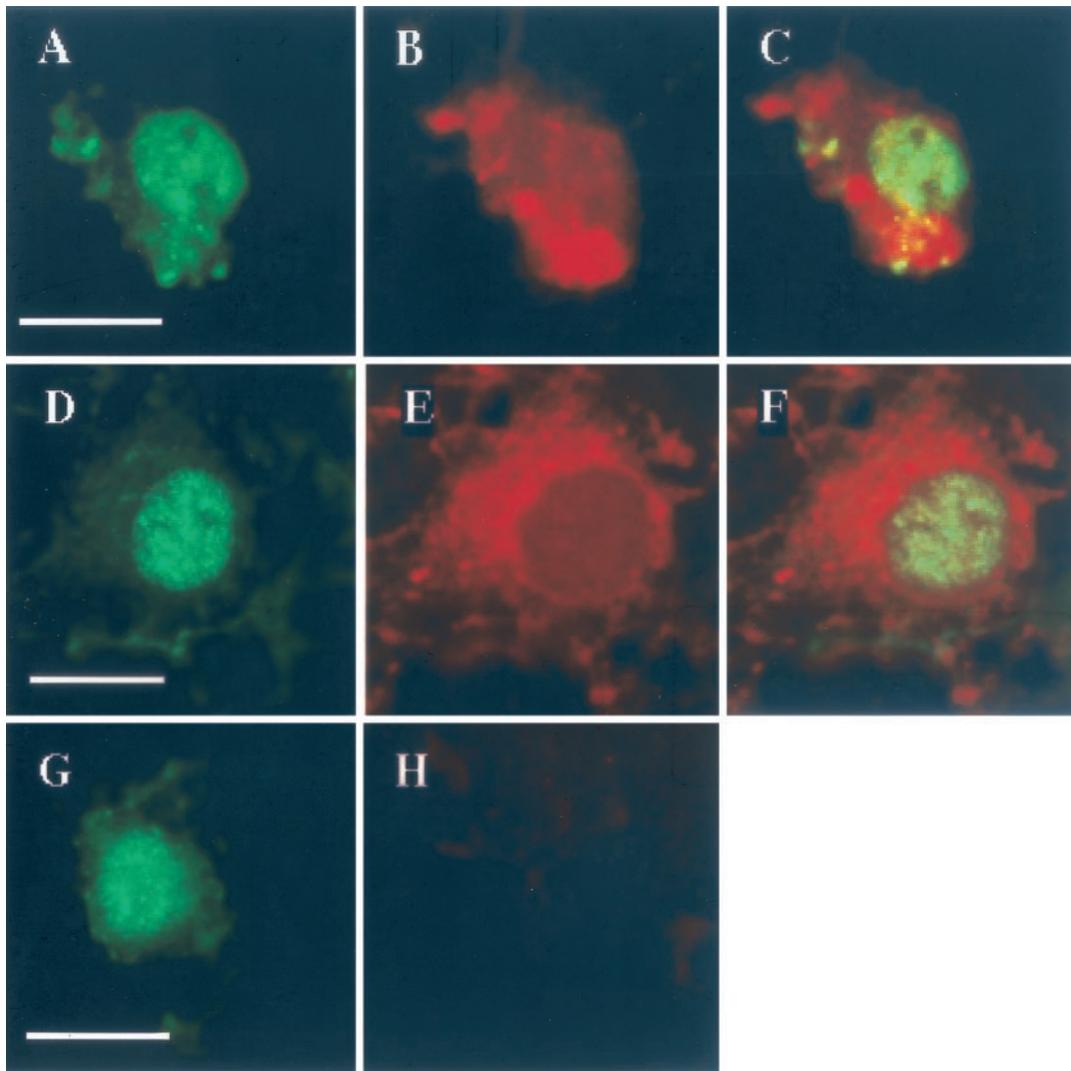
along with our results suggest that Isl-1 is a key factor for the differentiation of cells that produce insulin.

With regard to Pax6, the expression of this gene was found in YK cells, but then disappeared in BYK and Isl-YK cells. Pax-6 is required for the development of pancreatic endocrine cells, duodenal cells positive for gastric inhibitory peptide, and gastrin- and somatostatin-producing cells in the stomach (37). Above all, glucagon gene expression is shown to require this gene expression (38). In fact, although the promoter of glucagon contains two AT-rich sequences that are recognized by the homeodomain containing Cdx-2 and Pax-6 (39), these genes have been shown to bind directly to each other and transactivate synergistically the glucagon gene via their interaction with the G1 element (40). Therefore, our data show that the lack of glucagon expression observed in BYK cells or Isl-YK cells may suggest these cells are more characteristic of  $\beta$ - rather than  $\alpha$ -cells.

The finding that both BYK and Isl-YK cells produce multiple hormones except glucagons may point to the lack of terminal differentiation. A potential explanation arises from the known abilities of the transcription factors Pdx-1 and Isl-1 to regulate the expression of the insulin, somatostatin (41), and pp genes (42). To understand how cells restrict their function to the production of insulin, it will be necessary to further understand the control of the noninsulin genes and possibly identify factor(s) that repress their expression. For example, the transcription factor, Pax4 is a potential candidate. This protein is a paired-domain, PD-containing transcription factor that plays an important role in pancreatic  $\beta/\delta$ -cell development (43). Overexpression of Pax-4 represses the basal transcription of several genes, including Pdx-1 (44), and this action exerts a negative effect on cellular differentiation leading to the formation of  $\alpha$ -cells, but at the same time permits the formation of  $\beta$ -cells (43). Therefore, the absence of Pax-4 may underlie the ability of these cells to express multiple hormones.

Another feature of BYK and Isl-YK cells is the absence of insulin production in response to glucose concentrations in the media. Previous studies have shown that control of the insulin promoter requires at least two different *cis*-acting motifs designed E and A (45), bound by Pdx-1 (28) and Isl-1 (30) *in vitro*. These motifs are not unique to the insulin gene and are also found in other pancreatic  $\beta$ -cell genes encoding glucokinase (GK) (46), GLUT2 (47), and Kir. The expression of GK, GLUT2, and Kir is present in both rat pancreatic and Isl-YK cells (data not shown). The high-km GLUT2 and GK have key roles in regulating the rate of glucose flux in  $\beta$ -cells (48) and presumably generate the necessary signals for glucose-regulated insulin secretion (49). However, insulin released from BYK cells into the cell culture medium containing a high concentration of glucose (22.0 mmol/l) was not different from that exposed to low concentrations (5.6 mmol/l) of glucose. The lack of glucose-regulated expression of insulin in the BYK cells did not parallel that present in rat pancreatic cells and will need further studies using Isl-YK cells.

The significance of our findings is better understood when viewed in the light of recent developments in improving treatment for diabetes. The goal of producing surrogate cells that have the ability to produce and secrete



**FIG. 7.** Double staining for Pdx-1/Is1-1 and insulin in Isl-YK-14 cells. Cells probed with separate antibodies that recognize Pdx-1 (A) and Is1-1 (D) showed presence of staining for these proteins, mainly in the nuclei. As expected, insulin staining is found in the cytoplasm (B and E). The co-expression of Pdx-1 and Is1-1 enabled the Isl-YK-14 cells to express insulin, as noted by the co-localization of these proteins in the same cell (C and F). In IEC-6 cells transfected with Is1-1 cDNA alone (G), the presence of the protein is demonstrated by staining, but insulin is not found in these cells (H). Bars = 30  $\mu$ m.

insulin is an attractive approach for treating the disease. For example, the introduction of an adenovirus containing Pdx-1 into animals has been shown to facilitate the expression of the insulin gene in liver and ameliorate STZ-induced hyperglycemia (50). The results of other studies have demonstrated glucose-regulated expression of insulin in animals infected with an adeno-associated virus carrying a modified insulin gene fused to the promoter sequence of L-type pyruvate kinase (51). More recently, glucose regulation of a chimeric gene comprised of an insulinotropic polypeptide promoter fused to the insulin gene was introduced into gastric K cells (52). These animals successfully acquired glucose-induced insulin secretion.

Our data represent a small but significant step in developing a novel approach to modifying intestinal stem cells and equipping them with the ability to produce and secrete insulin. For cells to produce insulin, they must express Pdx-1 after treatment with betacellulin or be given the ability to co-express Is1-1. Immature intestinal cells treated

in this fashion produce insulin, but the expression and secretion of the hormone is not regulated by glucose. Glucose-regulated expression of insulin in the cells is the focus of future studies and is essential for further development of the use of this approach to treat diabetes.

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## Implantation with ISL-YK-14 cells

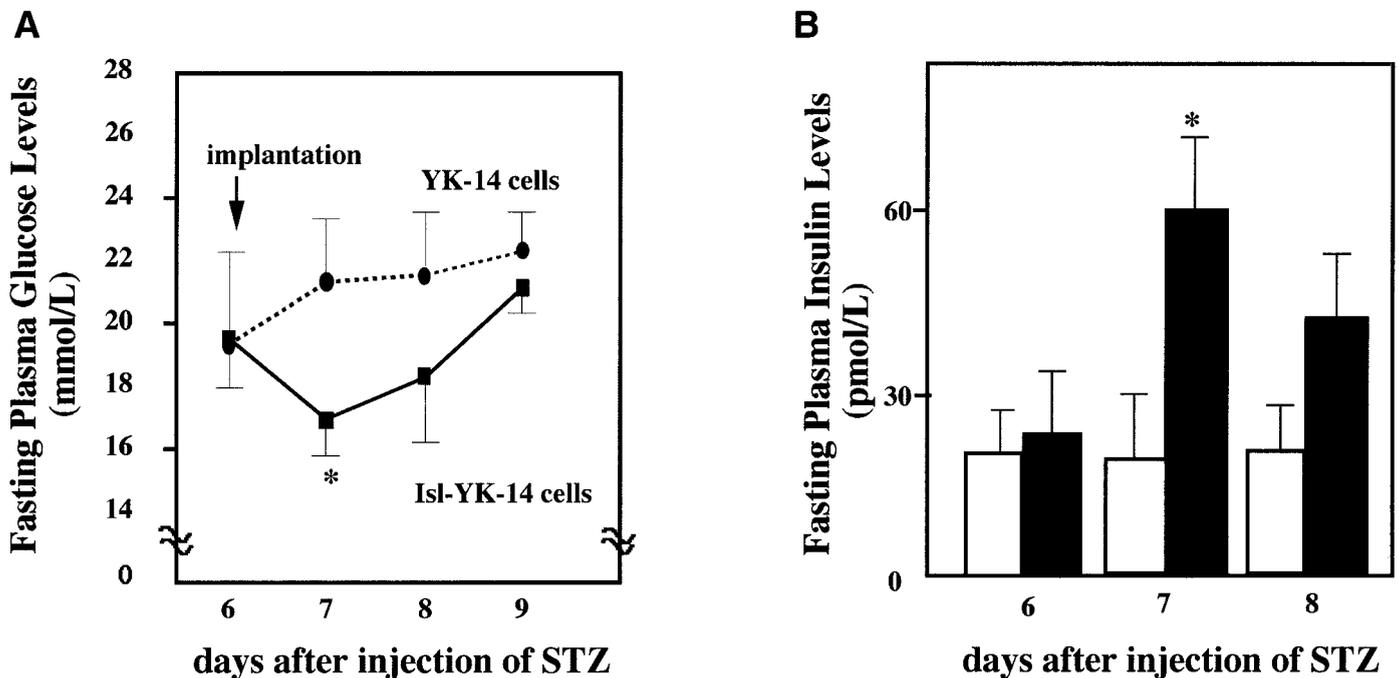


FIG. 8. The changes of plasma glucose and insulin concentrations after implantation with Isl-YK-14 cells. Rats were treated with STZ to induce diabetes and followed for 5 days before being separated into two groups. Fasting plasma glucose (A) and insulin (B) levels are shown. On day 6, one group was injected intraperitoneally with  $5 \times 10^6$  Isl-YK-14 cells (□ and ■, respectively) and the second group that served as control was injected intraperitoneally with the same number of YK-14 cells (● and □, respectively). Results are given as means  $\pm$  SD ( $n = 5$ ). \* $P < 0.05$  denotes a significant difference when compared with values of the control injected rats on the same day.

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### REFERENCES

- Pictet RL, Rall LB, Phelps P, Rutter WJ: The neural crest and the origin of the insulin-producing and other gastrointestinal hormone producing cells. *Science* 191:191–192, 1976
- Fontaine J, Le Douarin NM: Analysis of endoderm formation in the avian blastoderm by the use of quail-chick chimaeras: the problem of the neuroectodermal origin of the cells of the APUD series. *J Embryol Exp Morphol* 41:209–222, 1977
- Sander M, German MS: The  $\beta$  cell transcription factors and the development of the pancreas. *J Mol Med* 75:327–340, 1997
- Eklund H: Transcribing pancreas. *Diabetes* 47:1817–1823, 1998
- Hebrok M, Kim SK, Melton DA: Notocord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev* 12:1705–1713, 1998
- Kim SK, Melton DA: Pancreas development is promoted by cyclopamine, a Hedgehog signalling inhibitor. *Proc Natl Acad Sci U S A* 95:13036–13041, 1998
- Ahlgren U, Pfaff S, Jessel TM, Eklund T, Eklund H: Independent requirement for ISL-1 in the formation of the pancreatic mesenchyme and islet cells. *Nature* 385:257–260, 1997
- Lammert E, Cleaver O, Melton D: Induction of pancreatic differentiation by signals from blood vessels. *Science* 294:564–567, 2001
- St Ogne L, Wehr R, Gruss P: Pancreas development and diabetes. *Curr Opin Genet Dev* 9:295–300, 1999
- Mutoh H, Fung BP, Naya FJ, Tsai MJ, Nishitani J, Leiter AB: The basic helix-loop-helix transcription factor BETA2/NeuroD is expressed in mammalian enteroendocrine cells and activates secretin gene expression. *Proc Natl Acad Sci U S A* 94:3560–3564, 1997
- Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis Elhund H: Notch-signalling controls pancreatic cell differentiation. *Nature* 400:877–881, 1999
- Jonsson J, Carlsson L, Thomas E, Eklund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
- Stoffers DA, Zinkin NT, Stanojevic PA, Clarke WL, Habener JF: Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nat Genet* 15:106–110, 1997
- Miller CP, McGehee RE, Habener JF: IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. *EMBO J* 13:1145–1156, 1994
- Watada H, Kajimoto Y, Kaneto Y, Matsuoka T, Fujitani Y, Miyazaki J, Yamasaki Y: Involvement of the homeodomain-containing transcription factor PDX-1 in islet amyloid polypeptide gene transcription. *Biochem Biophys Res Commun* 229:746–751, 1996
- Itkin-Ansari P, Demeterco C, Bossie S, Tour DD, Beattie GM, Movassat J, Mally MI, Hayek A, Levine F: PDX-1 and cell-cell contact act in synergy to promote delta-cell development in a human pancreatic endocrine precursor cell line. *Mol Endocrinol* 14:814–822, 2000
- Seno M, Toda H, Kosaka M, Sasada R, Igarashi K, Shing Y, Folkman J, Ueda E, Yamada H: Human betacellulin, a member of the EGF family dominantly expressed in pancreas and small intestine, is fully active in a monomeric form. *Growth Factors* 13:181–191, 1996
- Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I: Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J Clin Invest* 97:1647–1654, 1996
- Quaroni A, May RJ: Establishment and characterization of intestinal epithelial cell cultures. *Methods Cell Biol* 21B:404–427, 1980
- Lorentz O, Duluc I, Arcangelis AD, Simon-Assmann P, Keding M, Freund JN: Key role of the Cdx2 homeobox gene in extracellular matrix-mediated intestinal cell differentiation. *J Cell Biol* 139:1553–1565, 1997
- Yamada S, Kojima H, Fujimiya M, Nakamura T, Kashiwagi A, Kikkawa R: Differentiation of immature enterocytes into enteroendocrine cells by Pdx-1 overexpression. *Am J Physiol* 281:G229–G236, 2001
- Kojima H, Hidaka H, Matsumura K, Fujita Y, Nishio Y, Maegawa H, Haneda M, Yasuda H, Fujimiya M, Kikkawa R, Kashiwagi A: Concerted regulation of early enterocyte differentiation by insulin-like growth factor I, insulin, and transforming growth factor- $\beta$ 1. *Proc Assoc Am Physicians* 110:197–206, 1998
- Nakamura T, Robichaud AF, Kikkawa R, Kashiwagi A, Kojima H, Fujimiya

- M, Wong NCW: Transcription factors and age related decline in apolipoprotein AI expression. *J Lipid Res* 40:1709–1718, 1999
25. Watada H, Kajimoto Y, Miyagawa J, Hanafusa T, Hamaguchi K, Matsuoka T, Yamamoto K, Matsuzawa Y, Kawamori R, Yamasaki Y: PDX-1 induces insulin and glucokinase gene expressions in alphaTC1 clone 6 cells in the presence of betacellulin. *Diabetes* 45:1826–1831, 1996
  26. Nakamura T, Kishi A, Nishio Y, Maegawa H, Egawa K, Norman CWW, Kojima H, Fujimiya M, Arai R, Kashiwagi A, Kikkawa K: Insulin production in a neuroectodermal tumor that expresses ISL-1 but not Pdx-1. *J Clin Endocrinol Metab* 86:1795–1800, 2001
  27. Mashima H, Yamada S, Tajima T, Seno M, Yamada H, Takeda J, Kojima I: Gene expressed during the differentiation of pancreatic AR42J cells into insulin-secreting cells. *Diabetes* 48:304–309, 1999
  28. Macfarlan WM, McKinnon CM, Felton-Edkins ZA, Cragg H, James RF, Docherty K: Glucose stimulates translocation of the homeodomain transcription factor PDX1 from the cytoplasm to the nucleus in pancreatic beta-cells. *J Biol Chem* 274:1011–1016, 1999
  29. Ohlsson H, Karlsson K, Edlund T: IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J* 12:4251–4259, 1993
  30. Karlsson O, Thor S, Norberg T, Ohlsson H, Edlund T: Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* 344:879–882, 1990
  31. Cornell JS, Meister A: Glutathione and gamma-glutamyl cycle enzymes in crypt and villus tip cells of rat jejunal mucosa. *Proc Natl Acad Sci U S A* 73:420–422, 1976
  32. Helena E: How to get there from the gut? *Curr Opin Biol* 11:663–668, 1999
  33. Suh E, Traber PG: An intestine-specific homeobox gene regulates proliferation and differentiation. *Mol Cell Biol* 16:619–625, 1996
  34. Mirmira RG, Watada H, German MS: Beta-cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. *J Biol Chem* 275:14743–14751, 2000
  35. German MS, Wang J, Chadwick RB, Rutter WJ: Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. *Genes Dev* 6:2165–2176, 1992
  36. Johnson JD, Zhang W, Rudnick A, Rutter WJ, German MS: Transcriptional synergy between LIM-homeodomain proteins and basic helix-loop-helix proteins: the LIM2 domain determines specificity. *Mol Cell Biol* 17:3488–3496, 1997
  37. Larsson LI, St-Onge L, Hougaard DM, Sosa-Pineda B, Gruss P: Pax 4 and 6 regulate gastrointestinal endocrine cell development. *Mech Dev* 79:153–159, 1998
  38. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P: Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* 387:406–409, 1997
  39. Ritz-Laser B, Estreicher A, Klages N, Saule S, Philippe J: Pax-6 and Cdx-2/3 interact to activate glucagon gene expression on the G1 control element. *J Biol Chem* 274:4124–4132, 1999
  40. Hussain MA, Lee J, Miller CP, Habener JF: POU domain transcription factor brain 4 confers pancreatic alpha-cell-specific expression of the proglucagon gene through interaction with a novel proximal promoter G1 element. *Mol Cell Biol* 17:7186–7194, 1997
  41. Vallejo M, Penchuk L, Habener JF: Somatostatin gene upstream enhancer element activated by a protein complex consisting of CREB, Isl-1-like, and alpha-CBF-like transcription factors. *J Biol Chem* 267:12876–12884, 1992
  42. Harrison KA, Thaler J, Pfaff SL, Gu H, Kehrl JH: Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlx9-deficient mice. *Nat Genet* 23:71–75, 1999
  43. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P: The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature* 386:399–402, 1997
  44. Fujitani Y, Kajimoto Y, Yasuda T, Matsuoka TA, Kaneto H, Umayahara Y, Fujita N, Watada H, Miyazaki JI, Yamasaki Y, Hori M: Identification of a portable repression domain and an E1A-responsive activation domain in Pax4: a possible role of Pax4 as a transcriptional repressor in the pancreas. *Mol Cell Biol* 19:8281–8291, 1999
  45. German M, Ashcroft S, Docherty K, Edlund H, Edlund T, Goodison S, Imura H, Kennedy G, Madsen O, Melloul D: The insulin gene promoter: a simplified nomenclature. *Diabetes* 44:1002–1004, 1995
  46. Shelton KD, Franklin AJ, Khoor A, Beechem J, Magnuson MA: Multiple elements in the upstream glucokinase promoter contribute to transcription in insulinoma cells. *Mol Cell Biol* 12:4578–4589, 1992
  47. Bonny C, Roduit R, Gremlich S, Nicod P, Thorens B, Waeber G: The loss of GLUT2 expression in the pancreatic beta-cells of diabetic db/db mice is associated with an impaired DNA-binding activity of islet-specific trans-acting factors. *Mol Cell Endocrinol* 135:59–65, 1997
  48. Motoyoshi S, Shirota T, Araki E, Sakai K, Kaneko K, Motoshima H, Yoshizato K, Shirakami A, Kishikawa H, Shichiri M: Cellular characterization of pituitary adenoma cell line (AtT20 cell) transfected with insulin, glucose transporter type 2 (GLUT2) and glucokinase genes: insulin secretion in response to physiological concentrations of glucose. *Diabetologia* 41:1492–1501, 1998
  49. Matschinsky FM: A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 45:223–241, 1995
  50. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seiffers R, Kopolovic J, Kaiser N, Karasik A: Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat Med* 6:568–572, 2000
  51. Lee HC, Kim SJ, Kim KS, Shin H, Yoon JW: Remission in models of type I diabetes by gene therapy using a single-chain insulin analogue. *Nature* 408:483–488, 2000
  52. Cheung AT, Dayanandan B, Lewis JT, Korbitt GS, Rajotte RV, Bryer-Ash M, Boylan MO, Wolfe MM, Kieffer TJ: Glucose-dependent insulin release from genetically engineered K cells. *Science* 8;290:1959–62, 2000