PDX-1 Induces Differentiation of Intestinal Epithelioid IEC-6 Into Insulin-Producing Cells

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A homeodomain containing transcription factor PDX-1 can induce β-cell–specific gene expressions in some non–β-cells and may therefore be useful for future diabetes gene/cell therapy. Among the potential target organs or tissues for transcription factor–mediated induction of β-cell–like differentiation are the intestinal epithelial cells. They have certain merits over other tissues and organs in terms of accessibility for gene delivery and of similarity in developmental background to the pancreatic primordium. In this study, we used an intestinal epithelium–derived cell line, IEC-6 cells, and investigated the possible effects of PDX-1 expression in those cells. By exogenous expression of the PDX-1 gene, IEC-6 cells started expressing multiple β-cell–specific genes such as amylin, glucokinase, and Nkx6.1, which were not found in the original IEC-6 cells. Insulin gene expression, which was missing initially even in the PDX-1–transfected IEC-6 cells, became detectable when the cells were transplanted under the renal capsule of a rat. When the PDX-1$^+$ IEC-6 cells were kept in vitro, treatment with betacellulin could also confer insulin gene expression to them. Although insulin secretory granules became visible by electron microscopy, they were secreted regardless of glucose concentration. The in vivo or in vitro inductions of the insulin gene expression were not observed in the PDX-1$^-$ IEC-6 cells. Thus, our present observations demonstrate the potency of intestinal epithelial cells as a tool for diabetes gene/cell therapy and provide further support for the potency of PDX-1 in driving β-cell–like differentiation in non–β-cells. *Diabetes* 51:2505–2513, 2002

In recent years, attention has been focused on the possibility of gene or cell therapy of diabetes mellitus using artificially prepared non–β-cell–derived β-cells. Among the potentially useful factors for the induction of β-cell differentiation in non–β-cells is a homeodomain-containing transcription factor, PDX-1. PDX-1, which is also known as IDX-1/IPF-1/STF-1, was originally identified as an insulin gene regulating transcription factor but was later shown to have more extensive roles in regulating pancreas development and maintaining β-cell function (1–4). Gene disruption of PDX-1 inhibits the maturation and outgrowth of the pancreatic bud, resulting in lack of the whole pancreas (4). Also, when this disruption occurs in insulin-producing cells, maturation of the pancreatic β-cells is inhibited, suggesting an essential role for PDX-1 in early and late pancreas development (4,5).

In agreement with the phenotypes of the knockout animals, recent gain-of-function experiments have revealed the potential of PDX-1 in conferring some β-cell–like features to non–β-cells. We and others have recently shown that the exogenous expression of PDX-1 in two lines of glucagon-producing cells can induce the expression of β-cell–specific genes, including the insulin gene, although one cell line required treatment with betacellulin to activate the insulin gene (6,7). Also, more recently, Ferber et al. (8) showed that PDX-1 can endow some cells in the liver with pancreatic β-cell characteristics in vivo using recombinant adenovirus-mediated gene delivery. These data have highlighted the potential usefulness of PDX-1 as a reprogramming factor of non–β-cells toward β-cell–like cells that can be used in diabetes cell/gene therapy.

With relatively good accessibility in terms of gene delivery, the small intestinal epithelium has been considered as a potential target of gene/cell therapy in general (9). This is especially true for the case of diabetes gene/cell therapy, because the intestinal epithelium and pancreatic β-cells have similar developmental backgrounds: after formation of the primitive gut tube, most epithelial cells in the gut tube give rise to the gastrointestinal tract, including the small intestine, and restricted groups of cells located in the posterior foregut give rise to ventral and dorsal pancreatic buds (10,11). Considering the importance of the network formed by multiple transcription factors in β-cell differentiation, the similarity in developmental background is likely to contribute favorably to successful induction of β-cell–like differentiation.

Another positive factor is that small intestinal epithelial cells are composed of both differentiated and undifferentiated cell populations. The latter cells, which are present in the crypt region, are known to serve as stem cells and differentiate into intestinal epithelium cells and endocrine

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ABC, avidin-biotin complex; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; KRH, Krebs-Ringer-HEPES; TGF, transforming growth factor.

cells. Even in the adult intestine, stem cells should remain active and supply enough cells to compensate for a very rapid turnover of intestinal epithelium cells with a mean cell duration time of 2–3 days (12,13). Because some organ-specific stem cells have been shown to retain a transdifferentiation potential, the presence of such stem cells in the intestine makes it a more promising target for diabetes gene/cell therapy (9).

In this study, we show that intestinal crypt-like IEC-6 cells (14) have the potency to induce the expression of multiple β-cell–specific genes such as amylin/islet amyloid polypeptide and glucokinase in response to exogenously introduced PDX-1. Furthermore, the insulin gene expression was switched on in the PDX-1–expressing IEC-6 cells when they were kept under the renal capsule, where the pancreatic bud epithelium can differentiate into mature endocrine cells (15). It is interesting that betacellulin treatment in vitro could mimic the effect of under-renal-capsule transplantation. Together with earlier reports, our results demonstrate the potency of PDX-1 as a β-cell differentiation factor and support the potential usefulness of intestinal epithelial cells for generating artificial β-cells.

RESEARCH DESIGN AND METHODS

Cell culture. The rat intestinal epithelioid cell line, IEC-6 cells, was obtained from Riken Gene Bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (vol/vol) heat-inactivated FCS, 1% penicillin, and 1% streptomycin. The rat insulinoma cell line RINm5F (a gift from Dr. K. Yokono, Kobe University, Kobe, Japan) was grown in RPMI-1640 containing 10% (vol/vol) FCS and 1% penicillin-streptomycin. The mouse primitive endoderm-resembling cell line JC44 (16) (a gift from Dr. B.A. Fenderson, Thomas Jefferson University, Philadelphia, PA) was cultured in the same medium as RINm5F. The mouse yolk sac endoderm-derived cell line YSE (17) (a gift from Dr. M.C. Yoder, Indiana University, Bloomington, IN) was maintained in the same medium as IEC-6 cells. MIN6 cells were cultured in DMEM supplemented with 25 mmol/l glucose, 50 mg/l penicillin, 50 mg/l streptomycin, and 15% heat-inactivated FCS.

For examination of the effects of various growth factors, cells were cultured on a Lab-Tek chamber slide (Nalge Nunc International, Rochester, NY) for 4 days in a nonserum medium (DMEM containing 5 μg/ml transferrin, 20 nmol/l progesterone, and 30 mmol/l selenious acid) and 2 nmol/l transforming growth factor (TGF)-α, epidermal growth factor (EGF) (Wakunaga Pharmaceutical, Hiroshima, Japan), IGF-1 (a gift from Fujisawa Pharmaceutical, Osaka, Japan), or betacellulin (a gift from Dr. Reiko Sasada, Takeda Pharmaceutical, Tsukuba, Japan).

For measurement of insulin secretion in response to glucose, arginine, or calcium ionophore A23187 (Sigma, St. Louis, MO), 1×10^5 cells were plated in a six-well plate and kept for 2 days with (PDX-1$^+$ IEC-6 and PDX-1$^-$ IEC-6 cells) or without (PDX-1$^+$ IEC-6 and MIN6 cells) 1 nmol/l betacellulin. After washing twice with Krebs-Ringer-HEPES (KRH) containing 128 mmol/l NaCl, 5 mmol/l KCl, 2.7 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l Na_2HPO_4, 20 mmol/l HEPES, and 2.8 mmol/l glucose and 60-min preincubation with the same KRH medium, the insulin secretion was measured by incubation at 37°C for 30 min (MIN6 cells) and 120 min (PDX-1$^+$ IEC-6 and PDX-1$^-$ IEC-6 cells) in medium with appropriate secretagogues. After the incubation, supernatant was collected and used for enzyme-linked immunosorbent assay (ELISA) for insulin as described below.

Preparation of stable transfectants. For the generation of stable transfectants, IEC-6 cells were replated in 10-cm plates 24 h before transfection. Ten micrograms of the PDX-1 expression plasmid carrying neomycin phosphotransferase gene, pcDNA3-IPF1 (18), was transfected into cells by a lipofection method using lipofectAMINE reagent (Life Technologies, Tokyo, Japan) under the conditions recommended by the manufacturer. Forty-eight hours after transfection, the cells were transferred to 600 μg/ml G418-containing medium (Sigma). G418-resistant colonies appeared ~4 weeks after transfection, and the resistant clones were subcloned and used for additional analyses.

Northern blot analyses. Northern blot analyses followed a standard protocol using 10 μg of total RNA (18). A mouse PDX-1 cDNA (nucleotide 772-1134) was used as a probe.

Preparation of nuclear extracts, Western blot analyses, and gel mobility shift analyses. Nuclear extracts were prepared following the procedure described by Sadowski and Gilman (19). Western blot analyses were performed as described previously using PDX-1 antibody (7).

Gel-shift analyses were performed as described previously using human insulin A3 element as a probe (7). The sequence of the sense strand of the probe was 5′-CCCCTGGTTAAGACTCTAATGACCCGCTGG-3′. In some of the binding assays, 5 μl of preimmune serum or anti–PDX-1 antiserum (18) was added to the 20-μl binding mixture 30 min before the addition of the DNA probes.

RT-PCR. Total RNA extraction and subsequent RT-PCR were performed as described previously (20). One microgram of total RNA was loaded for RT, and 1 μl of the reaction mixture was used for subsequent PCR. The sequence of primers used for PCR of β-actin, insulin, glucokinase, GLUT2, and islet amyloid polypeptide (amylin) mRNAs was described elsewhere (20). For verification of the results for insulin and GLUT2, other sets of primers that were used by Jensen et al. (21) were also used. The primers for somatostatin, Isl1, NeuroD, and Nkx6.1 mRNAs were also as described by Jensen et al. (21). Those for HNF-3β mRNA were as described by Duncan et al. (22), and those for Kir6.2 mRNA were described by Wulfsen et al. (23). The PCR conditions were denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The number of PCR cycles for β-actin cDNA amplifications was 25; that for HNF-3β, amylin, somatostatin, Isl1, and NeuroD was 33; and that for Kir6.2, insulin, glucokinase, and Nkx6.1 was 35. The PCR products were separated on an agarose gel and visualized with ethidium bromide.

Under-renal-capsule transplantation. PDX-1–expressing IEC-6 cells were suspended in prewarmed DMEM medium containing 0.6% agarose and 10% FCS. Droplets (10 μl, 20–30 cells) of the cell suspension solution were placed on a Petri dish (ϕ 60 mm) and allowed to solidify. Liquid medium (DMEM, 10% FCS) was added, and the cells were cultured for 1 week to form aggregates. To calculate cell numbers at this point, we heated a Petri dish containing 5–10 droplets to 42°C to melt the agarose, which was then treated with 0.05% trypsin and 0.02% EDTA, then subjected to cell counting. This revealed that each cell aggregate contained 51 cells on average after 1-week culture in vitro, allowing us, in turn, to estimate the total cell numbers contained in an agarose droplet (1,000–1,400 cells per droplet). Male Sprague-Dawley rats (20–26 weeks old) were anesthetized and subjected to under-renal-capsule transplantation. Through a small midline incision in the abdomen, both kidneys were exposed and a small tear was made in the renal capsule. Five gel droplets containing cell aggregates were inserted and placed on the bare area of a kidney. After 2 or 8 weeks, the rats were killed to harvest the kidney and pancreas for subsequent investigation.

Immunohistochemistry. For the staining of cultured cells, the cells were placed on a Lab-Tek chamber slide and cultured for 3 days in appropriate medium. After rinsing with PBS three times, the cells were fixed with acetone/methanol. For staining of the harvested kidney, the tissues were fixed overnight in 4% paraformaldehyde. Fixed tissues were processed routinely for paraffin embedding, and ~5-μm sections were prepared and mounted on slides. Each slide was treated with the H_2O_2 solution to inactivate endogenous peroxidase. Detection of insulin and PDX-1 was done by the avidin-biotin complex (ABC) method using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). After rehydration and blocking, the mounted sections were incubated for 30 min with guinea pig polyclonal anti-insulin antibody (Dako, Glostrup, Denmark) diluted 1:3,000 or PDX-1 antiserum (7) diluted 1:5,000 in PBS containing 1% bovine serum albumin. They were then incubated for 30 min with biotinylated anti–guinea pig IgG (Vector Laboratories) diluted 1:200 or biotinylated anti-rabbit IgG. The sections were then incubated with ABC reagent for 30 min, and positive reactions were visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride substrate (Zymed Laboratories, San Francisco, CA).

ELISA for insulin. Insulin was extracted from the kidney or pancreas as described elsewhere (24). Insulin concentration was measured using a Lebis insulin ELISA kit (Shibayagi, Gunma, Japan).

Electron microscopic examination for PDX-1$^+$ IEC-6 cells. The cells grown on the culture dish were fixed with 1% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 2 h. After the cells were rinsed with the phosphate buffer, they were postfixed with 2% OsO_4 for 2 h at 4°C, rinsed with distilled water, and stained en bloc with 1% uranyl acetate for 1 h. The cells were then dehydrated in graded concentrations of ethanol and embedded in Epok 812 (Okensyoji, Tokyo, Japan). Thin sections were cut, stained with both 4% uranyl acetate and lead citrate, and examined in an electron microscope (H-300; Hitachi Seisakusyo, Hitachi, Japan).

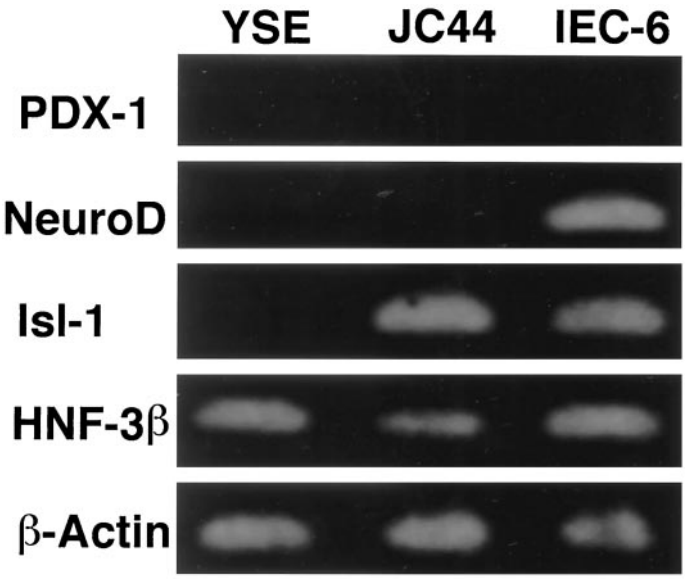

FIG. 1. Expression of islet cell–related transcription factors in IEC-6 cells. Total RNA was isolated from the rat intestinal epithelioid cell line IEC-6, primitive endoderm-resembling cell line JC44, and mouse yolk sac endoderm-derived cell line YSE and used for RT-PCR for the detection of PDX-1, NeuroD, Isl1, and HNF-3β mRNA. β-Actin mRNA was also amplified as a positive control. The sequence information of the primers used for RT and PCR is described in RESEARCH DESIGN AND METHODS. Similar results were obtained in at least three independent experiments.

RESULTS

Preparation of IEC-6 clones stably expressing PDX-1. To evaluate the potential of intestinal crypt cell–like IEC cells to differentiate to β-cells, we first examined the expressions of islet cell–related transcription factors in the cells. As shown in Fig. 1, RT-PCR analyses revealed that IEC-6 cells express HNF-3β (25), Isl1 (26), and NeuroD (27) but lack PDX-1 and Nkx6.1 (28). In contrast, yolk sac endoderm-derived YSE cells (17) and primitive endoderm-derived JC44 cells (16) did not show a comparable expression pattern (Fig. 1). Thus, the expression pattern of IEC-6 cells is consistent with the previous observation obtained with murine intestinal tissues (29) and retains some similarity to pancreatic β-cells.

IEC-6 cells were transfected with PDX-1 expression plasmid, and stable transformants were selected according to G418 resistance. In total, 71 independent clones were obtained, and 28 arbitrarily chosen clones were subjected to Northern blot analyses. As a result, 18 PDX-1–positive clones and 10 PDX-1–negative clones were identified (data not shown). Among them, we randomly chose three positive (PDX-1$^+$ IEC-6; clones A, B, and C) and three negative (PDX-1$^-$ IEC-6; clones D, E, and F) and used them for additional experiments.

As shown in Fig. 2*A*, PDX-1$^+$ IEC-6 cells expressed a 1.8 kb–long PDX-1 mRNA derived from the exogenously introduced gene. Although it was less abundant than the 2.3-kb PDX-1 mRNA originally expressed in β-cell–derived βTC1 cells, the protein amounts of PDX-1 were comparable to those expressed in βTC1 cells (Fig. 2*B*). Immunocytochemical staining with anti–PDX-1 antibody showed that PDX-1 is localized exclusively in the nuclei in PDX-1$^+$ IEC-6 cells (Fig. 2*C*). This observation was confirmed by Western blot analyses using isolated cytosolic fractions (data not shown). Gel mobility shift assays using the A3 region of the insulin gene promoter as a probe identified a specifically binding protein in the nuclear extract of the PDX-1$^+$ IEC-6 cells, and the DNA-protein complex was recognized by a PDX-1 antibody (Fig. 2*D*). In agreement with the similarity in the amounts of PDX-1 protein in the cells, the intensity of the retarded bands was almost comparable to that for βTC1 cells (Fig. 2*D*, *lanes 3* and *7*). These results thus suggested that the exogenously expressed PDX-1 functioned properly in IEC-6 cells in terms of its protein amount, nuclear localization, and DNA binding activity.

Induction of amylin, glucokinase, and Nkx6.1 expressions in PDX-1–positive IEC-6 cells. To investigate the changes of gene expression patterns caused by the exogenous PDX-1 expression in IEC-6 cells, we performed RT-PCR analyses. As presented in Fig. 1, Isl1, NeuroD, and HNF-3β mRNA were detectable even in parental IEC-6 cells and also were expressed in PDX-1$^+$ IEC-6 cells (Fig. 3). In contrast, there was no mRNA for β-cell–specific genes such as insulin, amylin, and glucokinase expressed in parental IEC-6 cells. However, after stable transfection of PDX-1–expressing plasmids, they started expressing amylin, glucokinase, and Nkx6.1 cells (Fig. 3). None of the three clones of PDX-1$^-$ IEC-6 (clones D, E, and F) expressed any of those three genes, suggesting that the phenomenon depends specifically on the introduction of PDX-1 into the cells rather than a nonspecific spontaneous conversion of the cell character during gene manipulation and culturing of the cells in vitro. The gene expressions for insulin (Fig. 3), GLUT2, somatostatin, and Kir6.2 (data not shown) remained undetectable even in PDX-1$^+$ IEC-6 cells.

Induction of insulin production in PDX-1–expressing IEC-6 cells kept under the renal capsule. During embryonic development, the expression of PDX-1 is initiated in the epithelium of the foregut shortly before the evagination of the pancreatic bud and is maintained in the epithelium of the pancreatic bud during early pancreatic development. The PDX-1–expressing pancreatic bud epithelium is known to form islet-like clusters of endocrine cells when separated from the mesenchymal tissue and transplanted under a renal capsule (15). Considering the similarity between the pancreatic bud epithelium and PDX-1–expressing IEC-6 cells, we investigated the possibility of under-renal-capsule transplantation facilitating differentiation toward the endocrine pancreas in PDX-1$^+$ IEC-6 cells.

Figure 4 illustrates the results of immunostaining of a rat kidney into which either PDX-1$^+$ IEC-6 cells or PDX-1$^-$ IEC-6 cells had been transplanted 2 weeks earlier. Both PDX-1–expressing and –nonexpressing IEC-6 cells remained alive, but the cell aggregates did not show any evident increase in volume, suggesting hardly any cell growth under the renal capsule. At 8 weeks after transplantation, the cell aggregates had decreased in mass, indicating enhanced cell death.

Even after transplantation, the PDX-1 expression remained absent in PDX-1$^-$ IEC-6 cells, whereas PDX-1 remained in the nuclei in PDX-1$^+$ IEC-6 cells, suggesting that the under-renal-capsule transplantation did not affect the expression or localization of PDX-1 in IEC-6 cells. It is

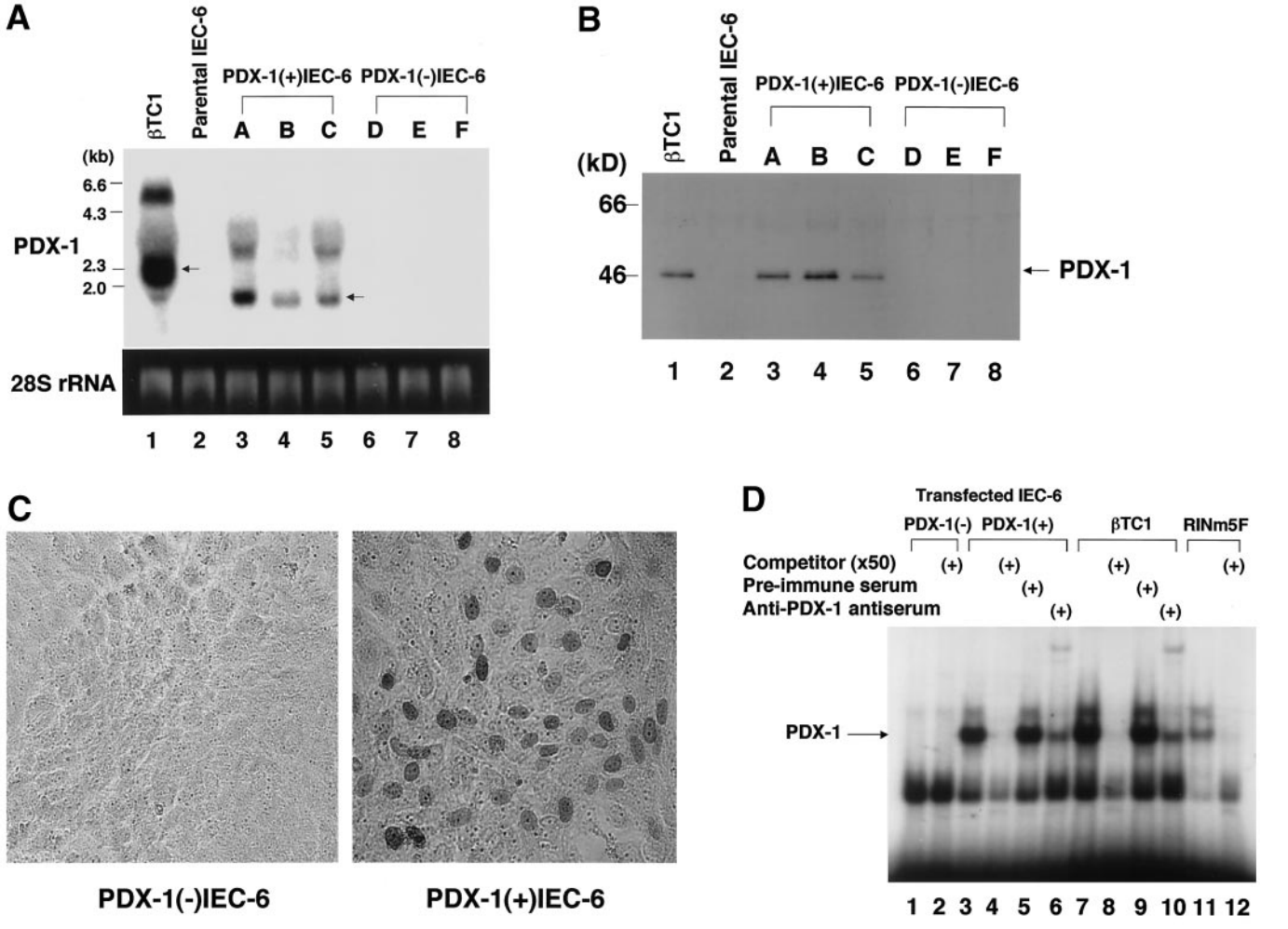

FIG. 2. Expression and function of PDX-1 in stably transfected IEC-6 cells. Three PDX-1$^+$ IEC-6 clones (A, B, and C) and three PDX-1$^-$ IEC-6 clones (D, E, and F) were randomly chosen after an initial screening by Northern blot analyses (data not shown) and subjected to detailed evaluation of PDX-1 expression and function. *A*: Northern blot analysis was performed using 10 μg of total RNA isolated from βTC1 cell (*lane 1*), parental IEC-6 cells (*lane 2*), three independent PDX-1$^+$ IEC-6 clones (clone A, *lane 3*; clone B, *lane 4*; clone C, *lane 5*), and three independent PDX-1$^-$ IEC-6 clones (clone D, *lane 6*; clone E, *lane 7*; clone F, *lane 8*). A PDX-1 cDNA was used as a probe. *B*: Western blot analysis used the same set of cells after isolation of 5 μg of nuclear extracts. The antibody used was an anti–PDX-1 antibody (7). *C*: Immunocytochemical staining for PDX-1 was performed to determine the intracellular localization of exogenously introduced PDX-1. Left and right panels depict PDX-1$^-$ IEC-6 (clone D) and PDX-1$^+$ IEC-6 (clone A) cells, respectively, which were allowed to react with anti–PDX-1 antibody. Similar results were obtained with other clones of PDX-1$^-$ IEC-6 (clones E and F) and PDX-1$^+$ IEC-6 (clones B and C) cells. *D*: Gel mobility shift analysis was performed using nuclear extracts isolated from PDX-1$^-$ IEC-6 cells (clone D; *lanes 1* and *2*) and PDX-1$^+$ IEC-6 cells (clone A; *lanes 3–6*), βTC1 cells (*lanes 7–10*), and RINm5F (*lanes 11* and *12*). The human insulin A3 element was used as a binding probe. Where indicated, 100-fold unlabeled double-strand DNA (*lanes 4* and *8*), 2 μg of preimmune serum (*lanes 5* and *9*), or 2 μg of anti–PDX-1 antiserum (*lanes 6* and *10*) were added to the binding mixtures. Similar results to clone D and to clone A were obtained with other clones of PDX-1$^-$ IEC-6 (clones E and F) and of PDX-1$^+$ IEC-6 cells (clones B and C), respectively.

interesting that intense staining for insulin was observed in >50% of the transplanted PDX-1$^+$ IEC-6 cells (Fig. 4). In contrast, no insulin-expressing cells were found in the transplanted PDX-1$^-$ IEC-6 cells. To estimate the amount of insulin expressed in the transplanted cells, we measured the insulin amount in a whole kidney into which ~5,000–7,000 cells were transplanted. In agreement with the absence of insulin staining, the transplantation of PDX-1$^-$ IEC-6 cells did not increase the insulin content of a kidney (Table 1). In contrast, the kidney that received transplanted PDX-1$^+$ IEC-6 cells revealed a significant increase in the insulin content. These results suggest that the under-renal-capsule transplantation or other treatment that can mimic it may be effective for enhancing β-cell–like function in PDX-1–transfected intestinal epithelioid cells.

Induction of insulin production in PDX-1–expressing IEC-6 cells by betacellulin. The possible involvement of various growth factors in pancreatic differentiation and regeneration has been suggested (30,31). This may also be the case with the differentiation of PDX-1–expressing IEC-6 cells because the space under the renal capsule, where PDX-1$^+$ IEC-6 cells could become insulin-positive, is known to be rich in growth factors (32). Accordingly, we examined the effects of growth factors on the enhancement of β-cell–like differentiation in IEC-6 cells.

As shown in Figs. 5*b* and 6*A*, the addition of betacellulin to the medium induced insulin production in PDX-1$^+$ IEC-6 cells. This induction was not observed when betacellulin was added to PDX-1$^-$ IEC-6 cells (Fig. 5*f*) or parental IEC-6 cells (data not shown). Other growth factors, such as TGF-α, EGF, and IGF-1, had no effect on the gene expression in both PDX-1$^+$ IEC-6 (Fig. 5*c–e*) and PDX-1$^-$ IEC-6 cells. According to the results of Western blot analyses, the cytosolic insulin content of the betacel-

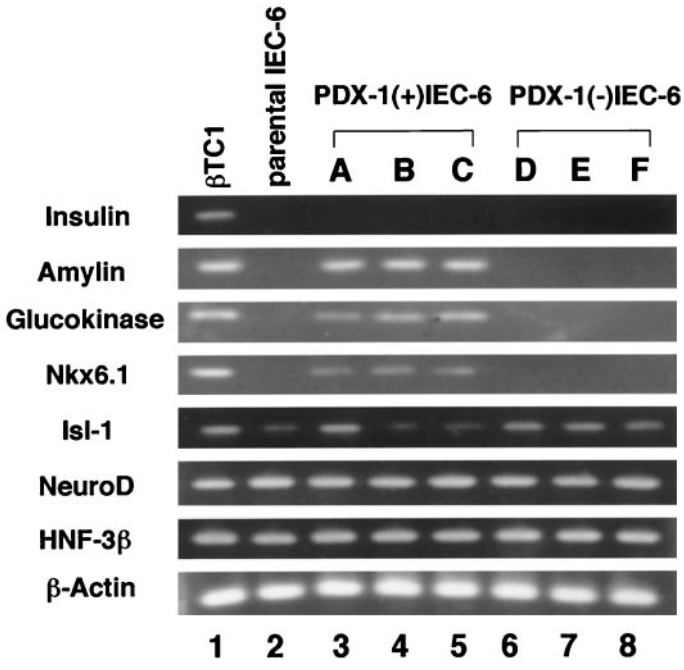

FIG. 3. Expression of β-cell–specific genes in PDX-1–expressing IEC-6 cells. RT-PCR was performed for the detection of insulin, amylin, glucokinase, Nkx6.1, Isl-1, NeuroD, and HNF3β. β-Actin mRNA was also amplified as a positive control. Total RNA isolated from βTC1 cells (*lane 1*), parental IEC-6 cells (*lane 2*), three independent PDX-1$^+$ IEC-6 clones (clone A, *lane 3*; clone B, *lane 4*; clone C, *lane 5*), and three independent PDX-1$^-$ IEC-6 clones (clone D, *lane 6*; clone E, *lane 7*; clone F, *lane 8*) were used. References for the primers are described in RESEARCH DESIGN AND METHODS. Similar results were obtained in at least three independent experiments.

lulin-treated PDX-1$^+$ IEC-6 cells was ~10% of that of βTC1 cells (data not shown).

The betacellulin treatment also induced somatostatin mRNA expression in PDX-1$^+$ IEC-6 cells according to results of RT-PCR analyses (Fig. 6*B*). However, immunocytochemical staining could not detect somatostatin protein in the cells (data not shown), possibly because of its low expression rate and/or constant secretion. Together with previous reports (33,34), our present data support the unique role of betacellulin in enhancing the β/δ-cell–like phenotype in non–β-cells.

Formation of secretory granules in betacellulin-treated PDX-1$^+$ IEC-6 cells. Both the betacellulin-treated PDX-1$^+$ IEC-6 cells and parental IEC-6 cells grew in monolayer and looked similar in appearance (data not shown). For more detailed observation, we performed electron microscopic analyses. Inconsistent with the induction of insulin expression in the cells, there were many small secretory granules in PDX-1$^+$ IEC-6 cells that had been treated with betacellulin (Fig. 7*B*). Some of the secretory granules were located close to or almost in contact with the plasma membrane, suggesting that they were to be secreted by exocytosis. This contrasts with the betacellulin-untreated PDX-1$^+$ IEC-6 cells, which totally lacked secretory granules although they had as well developed rough endoplasmic reticulum and Golgi apparatus (Fig. 7*A*). The parental IEC-6 cells, even when betacellulin was added to them, had no secretory granules (data not shown). In addition to the appearance of secretory gran-

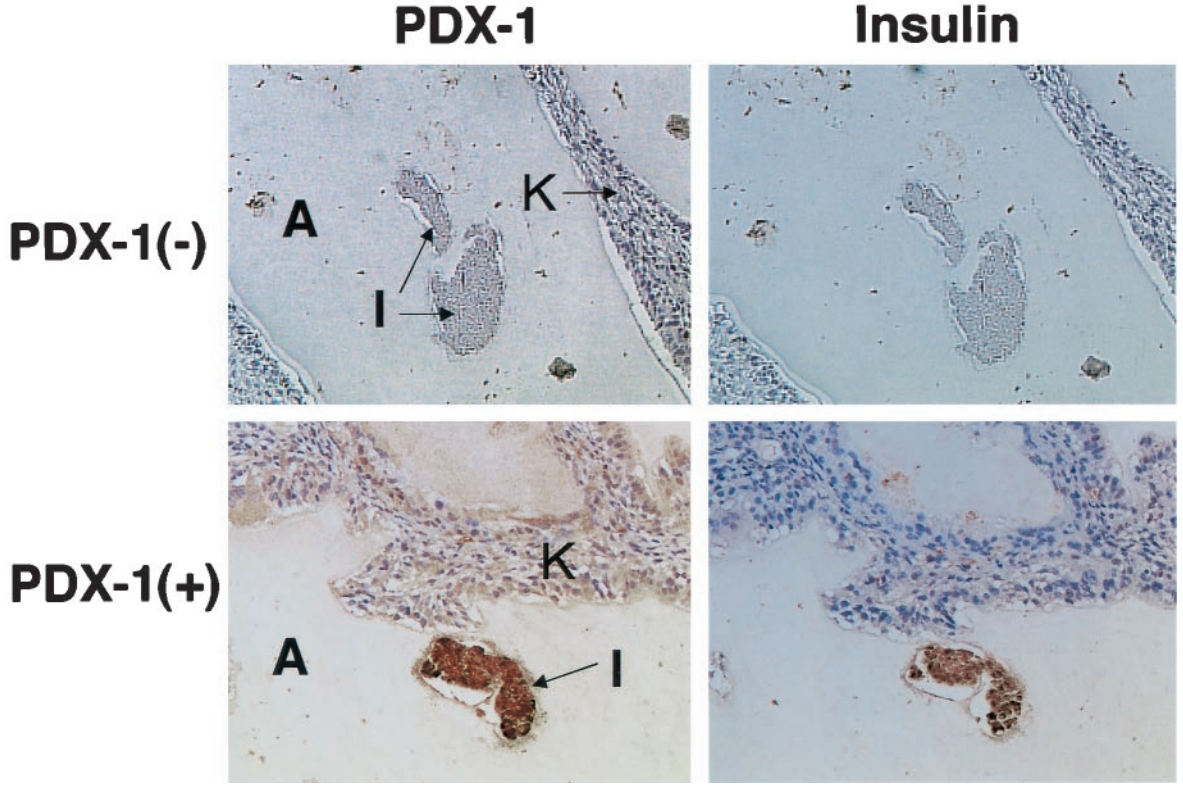

FIG. 4. Insulin staining in PDX-1$^+$ IEC-6 cells transplanted under a renal capsule. PDX-1$^-$ IEC-6 (clone D) and PDX-1$^+$ IEC-6 cells (clone A) were transplanted under a renal capsule of an adult rat and left for 2 weeks. After this period, the kidney was isolated and immunostaining for PDX-1 and insulin was performed. Upper panels represent the results for PDX-1$^-$ IEC-6 cells stained with anti–PDX-1 antibody (left) and anti-insulin antibody (right), and lower panels represent results for PDX-1$^+$ IEC-6 cells stained with anti–PDX-1 antibody (left) and anti-insulin antibody (right). Similar results to clone D and to clone A were obtained with other clones of PDX-1$^-$ IEC-6 (clones E and F) and of PDX-1$^+$ IEC-6 (clones B and C) cells, respectively. I, transplanted IEC-6 cells; K, kidney; A, agarose.

TABLE 1
Insulin content in PDX-1$^+$ IEC-6 cells kept under the renal capsule*

	Agarose	IEC-6	PDX-1$^+$ IEC-6	Pancreas
Immunoreactive insulin (ng/mg protein)	0.03 ± 0.01	0.03 ± 0.01	6.18 ± 1.04	1832 ± 231
n	5	6	6	4

Data are normalized with respect to the protein concentration expressed as means ± SE. *Insulin was extracted from the kidney that had received transplanted cell aggregates of parental IEC-6 cells or PDX-1$^+$ IEC-6 cells or agarose (no cells) as a control.

ules, there was an expansion of lysosomes in the betacellulin-treated cells (Fig. 7*B*). Although its physiological meaning is not known, a similar observation was previously obtained when IEC-6 cells were treated with glucocorticoids (35).

Effects of glucose or other secretagogues on insulin secretion from betacellulin-treated PDX-1$^+$ IEC-6 cells. We investigated whether PDX-1$^+$ IEC-6 cells treated with betacellulin can secrete insulin in response to glucose. As shown in Fig. 8*A*, the betacellulin-treated PDX-1$^+$ IEC-6 cells secrete insulin, whereas the betacellulin-untreated PDX-1$^+$ IEC-6 cells (Fig. 8*A*) or betacellulin-treated PDX-1$^-$ IEC-6 cells (data not shown) did not. However, in contrast to β-cell–derived MIN6 cells (Fig. 8*B*), the betacellulin-treated PDX-1$^+$ IEC-6 cells did not show any increase in insulin secretion even when the cells were exposed to a higher concentration of glucose. Also, neither arginine nor calcium ionophore A23187 enhanced insulin secretion. Thus, the betacellulin-treated PDX-1$^+$ IEC-6 cells expressed and secreted insulin but lacked response to glucose concentration or other secretagogues.

DISCUSSION

In the early developmental process of vertebrates, the definitive endoderm gives rise to all digestive organs, including the pancreas. Whereas most epithelial cells in the gut tube become part of the gastrointestinal tract, including small intestine, some groups of cells located in the posterior foregut give rise to ventral and dorsal pancreatic buds, and finally both buds become fused to give rise to the pancreas (10,11). Considering such developmental similarity between the pancreas and intestine and the high potential for transdifferentiation that has been shown for some tissue/organ-specific stem cells, the intestinal crypt cells may provide a useful tool for diabetes gene/cell therapy. The good accessibility for gene delivery is also an advantage of the intestinal cells.

The IEC-6 cell is derived from neonatal rat ileum and seems to retain its potential to respond to exogenously introduced transcription factors and to differentiate. Suh et al. (36) reported that conditional expression of an intestine-specific homeodomain-containing transcription factor, Cdx2/3 (37–39), in IEC-6 cells led to differentiation

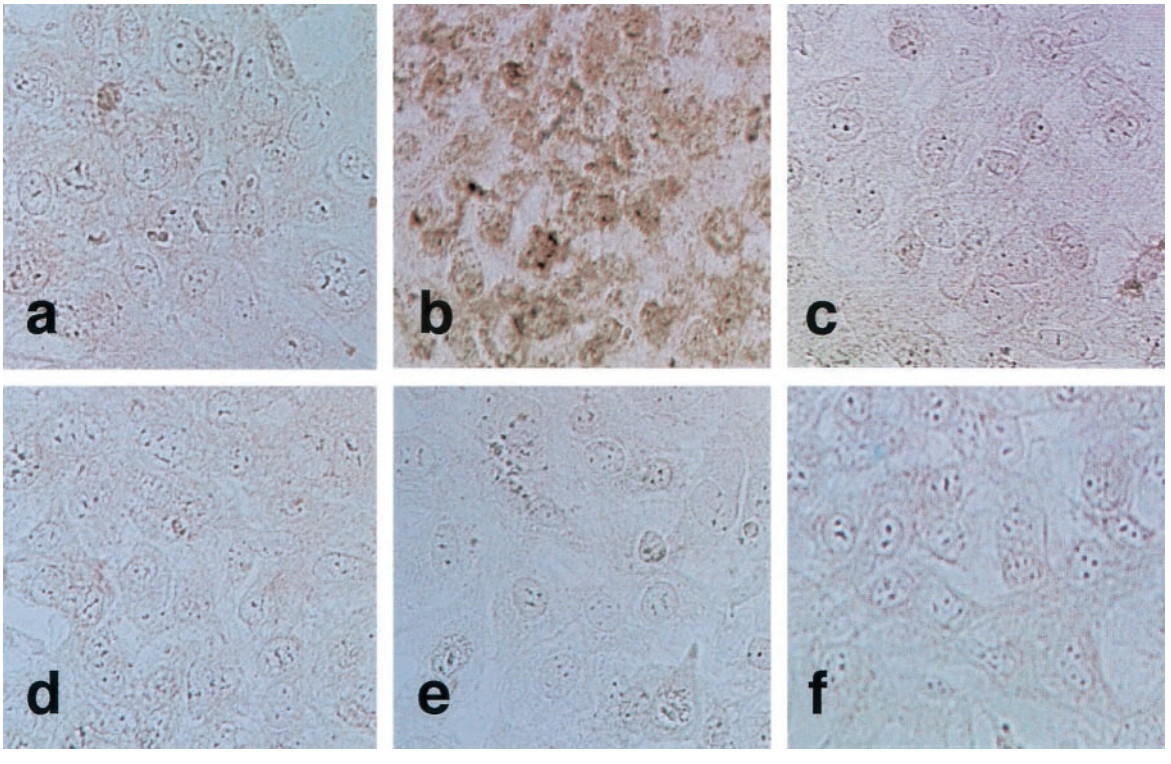

FIG. 5. Immunocytochemistry for insulin in PDX-1$^+$ IEC-6 cells with betacellulin. Insulin immunostaining was performed with PDX-1$^+$ IEC-6 cells (clone A) that were untreated (*a*) or treated for 4 days with 2 nmol/l betacellulin (*b*), 2 nmol/l TGF-α (*c*), 2 nmol/l EGF (*d*), or 2 nmol/l IGF-1 (*e*). Similar results were also obtained with other PDX-1$^+$ IEC-6 clones (clones B and C). As a control, the result obtained with PDX-1$^-$ IEC-6 cells (clone D) treated with betacellulin is shown in *f*. Similar negative results were also obtained with other PDX-1$^-$ IEC-6 clones (clones E and F).

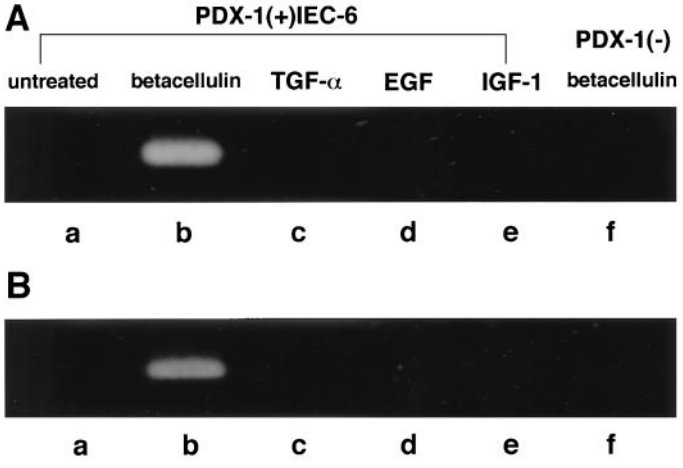

FIG. 6. Effects of growth factors on expression of insulin and somatostatin in PDX-1$^+$ IEC-6 cells. Insulin (*A*) and somatostatin (*B*) gene expression were examined by RT-PCR analyses in the same set of PDX-1$^+$ IEC-6 cells treated with various growth factors. RT-PCR analyses were done using total RNA isolated from PDX-1$^+$ IEC-6 cells (clone A) that were untreated (*lane a*) or treated for 4 days with 2 nmol/l betacellulin (*lane b*), 2 nmol/l TGF-α (*lane c*), 2 nmol/l EGF (*lane d*), or 2 nmol/l IGF-1 (*lane e*). As a control, the result obtained with PDX-1$^-$ IEC-6 cells treated with betacellulin is shown in *lane f*. Similar results were obtained in at least three independent experiments.

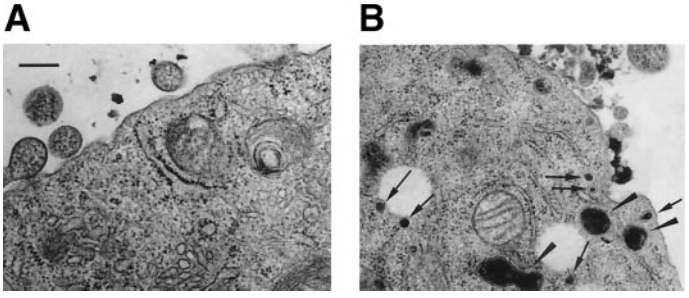

FIG. 7. Electron microscopic examination of PDX-1$^+$ IEC-6 cells. *A*: PDX-1$^+$ IEC-6 cells in the absence of betacellulin. *B*: PDX-1$^+$ IEC-6 cells in the presence of betacellulin. Both cells have ordinary cell organelles, such as rough endoplasmic reticulum and mitochondria. Secretory granules, however, can be seen only in the cells kept in the presence of betacellulin (arrows). Note that some of those granules are located close to plasma membrane. Lysosomes (arrowheads), which are rare in appearance in *A*, are also conspicuous in *B*. Bar = 0.5 μm.

into goblet-like or enterocyte-like cells. In this study, we showed that exogenously introduced PDX-1 conferred some pancreatic β-cell phenotype to the transfected cells. In contrast, whereas developmentally more primitive YSE cells and JC44 cells lack some of the islet-cell–related transcription factors that are expressed in IEC-6 cells, exogenously introduced PDX-1 did not induce any changes in the expressions of the β-cell–specific genes in those two cell lines (S.Y. and Y.K., unpublished results), suggesting that intestinal epithelioid IEC-6 cells retain some feature that facilitates β-cell–like differentiation. Also, being able to differentiate in multiple directions, IEC-6 cells possess some stem cell–like characteristic that is also seen in the crypt-type intestinal cells in vivo (14).

Previous studies showed that PDX-1 is a direct activator of several β-cell–specific genes, such as insulin, amylin, glucokinase, GLUT2, and Nkx6.1 (18,40–42). In this study, we found that the PDX-1–transfected IEC-6 cells expressed glucokinase, amylin, and Nkx6.1 but not GLUT2. This agrees with our previous observation with glucagon-producing αTC1 cells showing that PDX-1 can also induce glucokinase gene expression but not GLUT2 (7). However, apart from these gain-of-function experiments, the loss-of-function experiment done by Ahlgren et al. (5) showed that β-cell–specific gene disruption of PDX-1 caused the decrease of GLUT2, Nkx6.1, and amylin but not glucokinase. These observations tempt us to assume that PDX-1 is necessary but not sufficient for GLUT2 gene expression, whereas it is sufficient but not necessary for the glucokinase gene expression. This is possible because a family of homeodomain-containing transcription factors is known to be expressed in pancreatic β-cells, and they may bind to

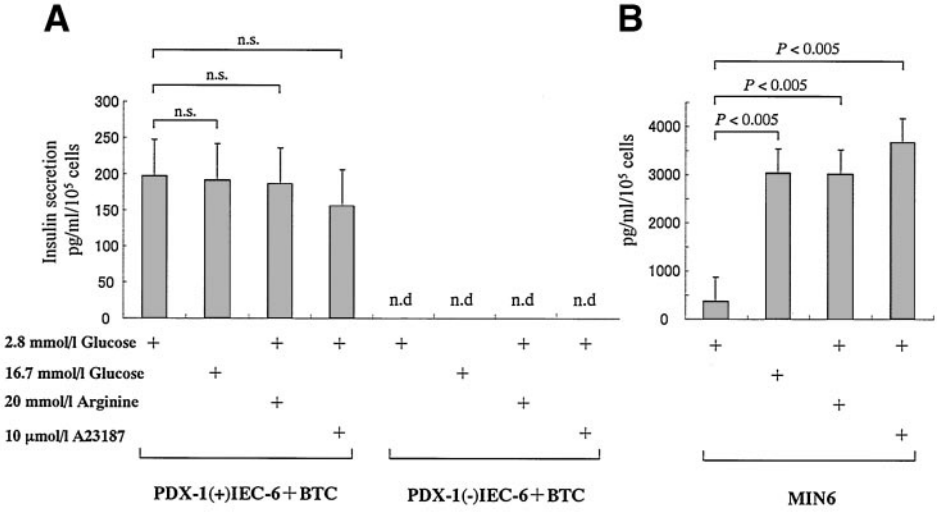

FIG. 8. Evaluation of glucose-responsive insulin secretion. Insulin secretion in response to glucose, arginine, or calcium ionophore A 23187 was investigated in betacellulin-treated PDX-1$^+$ IEC-6 cells (clone A) and betacellulin-treated PDX-1$^-$ IEC-6 cells (clone D) (*A*). As control, data were also obtained with MIN6 cells (*B*). The cells were kept for 2 h (PDX-1$^+$ IEC-6 and PDX-1$^-$ IEC-6 cells) or 30 min (MIN6 cells) in medium containing 2.8 or 16.7 mmol/l glucose. Where indicated, 20 mmol/l arginine or 10 μmol/l calcium ionophore A23187 was added to medium containing 2.8 mmol/l glucose. The insulin concentrations in the medium were expressed as means ± SE (pg/ml per 10^5 cells) of at least four experiments. Another PDX-1$^+$ IEC-6 clone (clone B) gave similar results to clone A. BTC, betacellulin; n.d., not detectable; n.s., not significant.

the AT-rich motif, to which PDX-1 binds, and compensate for some of the PDX-1 function in its absence. Although speculative, it is possible that no other homeodomain factors can substitute for PDX-1 in terms of the activation of the GLUT2 gene, for example, as a result of the involvement of a co-factor that can associate with PDX-1 but not with other homeodomain transcription factors.

Gittes et al. (15) previously showed that the epithelium of isolated pancreatic bud in the embryo forms mature islets when kept under the renal capsule. It was this observation that prompted us to do a similar trial using the PDX-1–transfected IEC-6 cells in the present study. Because the epithelial cells of the pancreatic bud share some common features with the PDX-1–transfected IEC-6 cells, such as the developmental origin being the gut and the expression of PDX-1, we considered that the under-renal-capsule transplantation may also work for the PDX-1–expressing IEC-6 cells. Our present results proved this hypothesis, supporting the potential usefulness of the space as a place for in vivo induction of β-cell differentiation. The detailed mechanism underlying the phenomenon needs to be examined, especially focusing on the possible involvement of diffusible mediators such as growth factors.

An ELISA experiment revealed that the insulin amount detected in the kidney after transplantation of PDX-1$^+$ IEC-6 cells was still far less than that in the pancreas (Table 1). However, there is a great difference in numbers of insulin-producing cells between the kidney, to which PDX-1$^+$ IEC-6 cells were transplanted, and the pancreas: 5,000–7,000 transplanted PDX-1$^+$ IEC-6 cells in the kidney versus roughly 1 million β-cells in the rat pancreas. This indicates that the insulin content per single PDX-1$^+$ IEC-6 cell (0.58–0.81 pg) was comparable to that of a β-cell (1.2–2.0 pg).

As a clue to identifying the factors that induce the insulin expression in the PDX-1–expressing IEC-6 cells, we screened the possible effects of various growth factors that may exert some roles in the pancreas. Among them, betacellulin was originally identified from the conditioned medium of β-cell–derived βTC3 cells as a growth-promoting factor having structural characteristics of the EGF family members (31). We previously reported that exogenously expressed PDX-1 induces glucokinase and insulin gene expressions in α-cell–derived αTC1.6 cells in the presence of betacellulin (7). In addition, betacellulin with the aid of activin A could convert the amylase-secreting pancreatic acinar cell–like AR42J cells into insulin-secreting cells (33). Together with the present results revealing the potential of betacellulin in inducing endogenous insulin gene expression in the intestinal cell–derived IEC-6 cells, these data indicate that betacellulin is likely to be a potent and widely functional inducer of β-cell differentiation. A recent study that demonstrated the in vivo effect of betacellulin on promotion of the islet neogenesis in alloxan-perfused pancreas supports this idea (34).

In electron microscopy, secretory granules were visible in the cytoplasm of the betacellulin-treated PDX-1$^+$ IEC-6 cells, supporting the idea that the cells acquired some neuroendocrine cell characteristics. However, the granules seemed to be secreted even when the cells were kept under low glucose concentrations. One possible cause for this is the lack of Kir6.2 expression in the cells. Kir6.2 is an essential component of ATP-sensitive potassium channel, and disruption of its gene in mice causes total loss of glucose responsiveness in insulin secretion (43). Although other factors involved in glucose-responsive insulin secretion might also be in effect, it may be worthwhile to restore Kir6.2 expression in PDX-1$^+$ IEC-6 cells to see whether it can confer glucose responsiveness.

During preparation of this manuscript, Yamada et al. (44) reported a similar observation using the same set of intestinal cells and the same transcription factor. In that study, they grew the PDX-1$^+$ IEC-6 cells on micropore filters and found that two types of cells developed: trabecular-shaped and non–trabecular-shaped. According to them, the trabecular-shaped cells showed strong positive staining for PDX-1 in the nucleus, whereas the non–trabecular-shaped cells showed only weak cytoplasm staining for PDX-1. This obviously contrasts with our present results that found PDX-1 localized exclusively in the nuclei of PDX-1$^+$ IEC-6 cells (Fig. 2*C*). This difference may depend on the difference in culture condition or on possible clonal variation of the parental IEC-6 cells used in each experiment. However, apart from that, their study proved the potential of PDX-1 in inducing some enteroendocrine cell–like characteristics but no insulin expression (44). Therefore, their observation is largely consistent with the phenotype of our PDX-1$^+$ IEC-6 cells, which was observed before the under-renal-capsule transplantation or the betacellulin treatment. Indeed, our PDX-1$^+$ IEC-6 cells also expressed some enteroendocrine markers, such as cholecystokinin and gastrin (S.Y. and Y.K., unpublished observations).

In conclusion, our present observations demonstrated the potency of PDX-1 in inducing the expression of β-cell–characteristic genes in intestinal crypt cell–like IEC-6 cells. Because of the accessibility of intestinal tissues from brain-death donors, ex vivo transfection of a transcription factor would be a reasonable step to try toward diabetes cell therapy. Also, with a relatively facile in vivo gene delivery, the intestinal epithelium may be a good target for transcription factor–mediated in vivo induction of β-cell differentiation. Toward the long-term goal of diabetes gene/cell therapy, however, efforts need to be focused on acquiring stable glucose-responsive insulin secretion in engineered intestinal epithelium cells. Establishment of an efficient system for gene delivery to the intestine would also be necessary for future clinical use.

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