

PDX-1/VP16 Fusion Protein, Together With NeuroD or Ngn3, Markedly Induces Insulin Gene Transcription and Ameliorates Glucose Tolerance

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Diabetes is the most prevalent and serious metabolic disease, and the number of diabetic patients worldwide is increasing. The reduction of insulin biosynthesis in pancreatic β -cells is closely associated with the onset and progression of diabetes, and thus it is important to search for ways to induce insulin-producing cells in non- β -cells. In this study, we showed that a modified form of the pancreatic and duodenal homeobox factor 1 (PDX-1) carrying the VP16 transcriptional activation domain (PDX-1/VP16) markedly increases insulin biosynthesis and induces various pancreas-related factors in the liver, especially in the presence of NeuroD or neurogenin 3 (Ngn3). Furthermore, in streptozotocin-induced diabetic mice, PDX-1/VP16 overexpression, together with NeuroD or Ngn3, drastically ameliorated glucose tolerance. Thus PDX-1/VP16 expression, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. This approach warrants further investigation and may have utility in the treatment of diabetes. *Diabetes* 54: 1009–1022, 2005

The pancreatic and duodenal homeobox factor 1 (PDX-1), a member of the homeodomain-containing transcription factor family (also known as IDX-1/STF-1/IPF1) (1–3), is expressed in the pancreas and duodenum and plays a crucial role in pancreas development (4–14), β -cell differentiation (15–26), and maintenance of normal β -cell function by regulating several β -cell-related genes (14,27–37). At early stages of embryonic development, PDX-1 is initially expressed in the gut region when the foregut endoderm becomes com-

mitted to common pancreatic precursor cells (1,4–7,11,13). During pancreas development, PDX-1 expression is maintained in precursor cells that coexpress several hormones; later, its expression is restricted to β -cells (1–3,5,6,11,13,28). Mice homozygous for a targeted mutation in the PDX-1 gene are apancreatic and develop fatal perinatal hyperglycemia (4), and heterozygous PDX-1-deficient mice have impaired glucose tolerance (12); both of these findings suggest the crucial role PDX-1 plays in pancreas development. In clinical manifestations, mutations in PDX-1 are known to cause maturity-onset diabetes of the young (MODY) (38).

NeuroD, a member of the basic helix-loop-helix (bHLH) transcription factor family, also known as BETA2, is expressed in pancreatic and intestinal endocrine cells and neural tissue. NeuroD also plays an important role in pancreas development and the regulation of insulin gene transcription (39–41). It has been reported that the insulin-enhancer elements E-box and A-box play an important role in regulating cell-specific expression of the insulin gene (42,43), and that NeuroD's binding to the E-box as well as PDX-1's binding to the A-box are very important for insulin gene transcription. Mice homozygous for the null mutation in NeuroD have a striking reduction in the number of β -cells, develop severe diabetes, and die perinatally (40). In clinical manifestations, mutations in NeuroD cause MODY (44). Neurogenin 3 (Ngn3) is also a bHLH transcription factor that binds to the E-box and is involved in pancreas development (45–50). Transgenic mice overexpressing Ngn3 early in their development show a marked increase in endocrine cell formation, indicating that Ngn3 induces differentiation of islet cell precursors (46,47). In contrast, mice with targeted disruption of Ngn3 have no endocrine cells (48).

Insulin plays a crucial role in maintaining blood glucose levels; insulin facilitates glucose uptake into muscle and adipose tissue and suppresses gluconeogenesis in the liver. In addition, insulin signaling has a functional role in β -cells themselves; it has been reported that insulin signaling is crucial for insulin gene expression and glucose-stimulated insulin secretion (49–52). In the diabetic state, however, hyperglycemia (53–60) and subsequent production of reactive oxygen species (61–65) decrease insulin gene expression and secretion, accompanied by reduced PDX-1 expression and DNA binding activity. Although pancreas and islet transplantation efficiently restore normoglycemia, such treatment requires lifelong immunosup-

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ABC, avidin-biotin complex; Ad, adenovirus; ALT, alanine aminotransferase; AST, aspartic acid aminotransferase; bHLH, basic helix-loop-helix; DAB, 3,3'-diaminobenzidine tetrahydrochloride; GFP, green fluorescent protein; MODY, maturity-onset diabetes of the young; Ngn3, neurogenin 3; PDX-1, pancreatic and duodenal homeobox factor 1; PFU, plaque forming unit; STZ, streptozotocin; TBS, Tris-buffered saline.

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pressive therapy and is limited by tissue supply (66,67). Therefore, it is important to search for ways to enhance insulin gene transcription and induce insulin-producing cells. Although the pancreas and liver arise from adjacent regions of the endoderm in embryonic development, the liver is a potential target for diabetes gene therapy (16,19,41,68–73). Moreover, it has been shown recently that a modified form of XIHbox8, the *Xenopus* homolog of PDX-1, carrying the VP16 transcriptional activation domain from the herpes simplex virus, efficiently induces insulin gene expression in the liver of the tadpole (72,73). Thus, although PDX-1 expression itself is obviously important, as has been demonstrated by various studies, it is likely that PDX-1 requires the recruitment of coordinately functioning transcription factors or cofactors to fully exert its function.

In this study, we showed that PDX-1/VP16 expression, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance in diabetic animals, implying that this combination is useful for replacing the reduced β -cell function found in diabetes.

RESEARCH DESIGN AND METHODS

Gene transfection and luciferase assays. HepG2 cells were grown in Eagle's minimum essential medium supplemented with 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin sulfate in a humidified atmosphere of 5% CO₂ at 37°C. The insulin 2 promoter-reporter (firefly luciferase) plasmid (1.0 μ g) containing 5'-flanking sequences of the rat insulin 2 promoter region (–238 bp to 3 bp) (42,74) and 0.5 μ g of the pSV- β -galactosidase control vector (Promega) were cotransfected with 1.0 μ g of the PDX-1, PDX-1/VP16, NeuroD, and/or Ngn3 expression plasmids (or empty vectors) using LipofectAMINE reagent (Life Technologies). The insulin 2 promoter-reporter (firefly luciferase) plasmid containing A3-box– or E1-box–mutated 5'-flanking sequences of the rat insulin 2 promoter region (–238 bp to 3 bp) (42,74) was similarly transfected. The A3-box is the most important PDX-1 binding site for insulin gene transcription, and the rat insulin 2 promoter has only one E-box to which NeuroD is known to bind. The PDX-1/VP16 (amino acid 410–490) fusion plasmid was made by PCR using the VP16 plasmid (kindly provided by Dr. Daniel S. Kessler, University of Pennsylvania School of Medicine) and rat PDX-1 expression plasmid as templates; the resulting clones were verified by sequencing. Cells were harvested for luciferase and β -galactosidase assays 48 h after transfection. Preparations of cellular extracts were assayed using a luciferase assay system (Promega). For the luciferase assay, light emission was measured with a Monolight 3010 Luminometer (Pharmingen, San Diego, CA), and β -galactosidase assays were performed with the β -galactosidase enzyme assay system (Promega). The luciferase results were normalized with respect to transfection efficiency as assessed from the results of the β -galactosidase assays.

Preparation of recombinant adenoviruses expressing PDX-1, PDX-1/VP16, NeuroD, and Ngn3. Recombinant adenoviruses expressing PDX-1, PDX-1/VP16, NeuroD, and Ngn3 were prepared using the AdEasy system (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center) (75). In brief, the encoding region of PDX-1, PDX-1/VP16, NeuroD, and Ngn3 was cloned into a shuttle vector pAdTrack-CMV. To produce an homologous recombination, 1.0 μ g of linearized plasmid containing PDX-1, PDX-1/VP16, NeuroD, or Ngn3 and 0.1 μ g of the adenoviral backbone plasmid pAdEasy-1 were introduced into electrocompetent *E. coli* BJ5183 cells by electroporation (2,500 V, 200 Ohms, 25 μ FD). The resultant plasmids were then retransformed into *E. coli* XL-Gold Ultracompetent Cells (Stratagene, La Jolla, CA). The plasmids were linearized with *Pac I* and then transfected into the adenovirus packaging cell line 293 using LipofectAMINE (Invitrogen, Carlsbad, CA). The cell lysate was collected from 293 cells 10 days after transfection and added to a fresh batch of 293 cells. When most of the cells were killed by the adenovirus infection and detached, the cell lysate was obtained. This process was repeated three times. The control adenovirus expressing green fluorescent protein (Ad-GFP) was prepared in the same manner. The adenovirus titers were further increased up to 1×10^{10} plaque forming units (PFU)/ml using the Adeno-X Virus Purification Kit (Clontech). The virus titers were estimated using the Adeno-X Titer Kit (Clontech).

Induction of hyperglycemia by streptozotocin and treatment with recombinant adenovirus. C57BL/6 male mice (age 8 weeks; Japan SLC, Hamamatsu, Japan) were made diabetic by streptozotocin injection (STZ; 220 mg/kg, i.p.; Sigma) freshly dissolved in citrate buffer (pH 4.5). Mice were injected with 100 μ l of Ad-PDX-1, Ad-PDX-1/VP16, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml) into the cervical vein 1 week after STZ injection. We adjusted the total volume of the injected adenovirus; for example, when PDX-1 (100 μ l) was combined with NeuroD or Ngn3 (100 μ l), the PDX-1 alone control consisted of PDX-1 (100 μ l) and GFP (100 μ l). Although it is easier to inject the adenovirus via the tail vein rather than the cervical vein, in our experiments we injected the adenovirus via the cervical vein to ensure there was no leakage during the injection. After the adenovirus injection, nonfasting blood glucose levels were measured regularly with a portable glucose meter (Precision QID; Medisense, St. Charles, MA) after snipping the tail. To measure plasma insulin levels, nonfasting blood samples were collected into heparinized capillary tubes and plasma insulin levels were determined using an Insulin-EIA Test Kit (Glazyme).

Glucose tolerance tests. After being fasted overnight, mice were injected with glucose (1.0 g/kg body wt, i.p.). Blood samples were taken at various time points (0–120 min), and blood glucose levels were determined as described above.

RT-PCR analysis. Total RNA was extracted from frozen tissues using Trizol (Invitrogen). After quantifying RNA by spectrophotometry, 2.5 μ g of RNA were heated at 85°C for 3 min and then reverse-transcribed into cDNA in a 25- μ l solution containing 200 units of Superscript II RNase H-RT (Invitrogen), 50 ng random hexamers (Invitrogen), 160 μ mol/l dNTP, and 10 mmol/l dithiothreitol. The reaction consisted of 10 min at 25°C, 60 min at 42°C, and 10 min at 95°C. Polymerization reactions were performed with a PerkinElmer 9700 Thermocycler (Norwalk, CT) using a 50- μ l reaction volume containing 3 μ l of cDNA (20 ng RNA equivalents), 5 units of AmpliTaq Gold DNA polymerase (PerkinElmer), 1.5 mmol/l MgCl₂, 160 μ mol/l cold dNTPs, and 10 pmol of appropriate oligonucleotide primers. The oligonucleotide primers were as follows: insulin 1 (370 bp), GAC CAG CTA TAA TCA GAG ACC (forward), AGT TGC AGT TCT CCA GCT G (reverse); insulin 2 (388 bp), AGC CCT AAG TGA TCC GCT ACA A (forward), AGT TGC AGT TCT CCA GCT G (reverse); glucokinase (islet type; 208 bp), TGG ATG ACA GAG CCA GGA TGG (forward), ACT TCT GAG CCT TCT GGG GTG (reverse); SUR-1 (267 bp), CCA GAC CAA GGG AAG ATT CA (forward), GTC CTG TAG GAT GAT GGA CA (reverse); Kir6.2 (218 bp), CCT GAG GAA TAT GTG CTG AC (forward), CAC AGG AAG GAC ATG GTG AA, (reverse); glucagon (205 bp), ACA GAG GAG AAC CCC AGA TC (forward), CAT CAT GAC GTT TGG CAA TG (reverse); somatostatin (226 bp), AGT TTC TGC AGA AGT CTC TGG (forward), AAG TTC TTG CAG CCA GCT TTG (reverse); and pancreatic polypeptide (194 bp), ACA GGA TGG CCG TCG CAT ACT (forward), GGC CTG GTC AGT GTG TTG ATG (reverse). The thermal cycle profile used a 10-min denaturing step at 94°C followed by 32 cycles (1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C) and an extension step of 10 min at 72°C. The products were then separated by agarose gel electrophoresis.

Northern blot analysis. Total RNA (10 μ g) isolated from freeze-clamped liver tissues were electrophoresed on 1.0% formaldehyde-denatured agarose gel in $1 \times$ MOPS running buffer, and then transferred overnight to a Hybond-N⁺ membrane (Amersham, Arlington Heights, IL). The insulin probe was labeled with [α -³²P]dCTP using the Rediprime labeling system kit (Amersham). After being hybridized overnight with a ³²P-labeled probe at 42°C, the membranes were washed in $2 \times$ sodium chloride–sodium phosphate EDTA buffer, 0.1% SDS at 42°C. Kodak XAR film was exposed with an intensifying screen at –80°C.

Western blot analysis. Whole-cell extracts obtained from various tissues were fractionated by 10% SDS-PAGE and transferred to reinforced cellulose nitrate membrane (Optitrans BA-S85; Schleicher & Schuell). After blocking, the membranes were incubated at 4°C overnight in Tris-buffered saline (TBS; 50 mmol/l Tris-HCl, 150 mmol/l NaCl) containing a 1:500 dilution of GFP antibody (Living Colors AV peptide antibody; Clontech) and then incubated for 1 h at room temperature in TBS containing a 1:1,000 dilution of anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad). Immunoreactive bands were visualized by incubation with LumiGLO (Cell Signaling) and exposure to light-sensitive film.

Immunohistochemical analyses. The mice were anesthetized using sodium pentobarbital. After a midline abdominal incision was made, liver tissues were removed from the mice and fixed overnight with 4% paraformaldehyde in PBS buffer. Fixed tissues were routinely processed for paraffin embedding and ~4- μ m sections were prepared and mounted on slides. Before being incubated with antibodies, the mounted sections were rinsed three times with PBS. To detect PDX-1, NeuroD, and Ngn3, the avidin-biotin complex (ABC) method was performed using the Vectastain ABC Kit (Vector Laboratories,

Burlingame, CA). After being treated with Target Retrieval Solution (Dako) at 90°C for 5 min, the mounted sections were incubated overnight with rabbit anti-PDX-1 antiserum (30), goat anti-NeuroD antibody (N-19; Santa Cruz), and goat anti-Ngn3 antibody (D-15; Santa Cruz) diluted 1:1,000 in PBS containing 1% BSA. This was followed by a 1-h incubation with biotinylated anti-rabbit IgG (for PDX-1) or anti-goat IgG (for NeuroD and Ngn3) (Vector Laboratories) diluted 1:200. The sections were then incubated with ABC reagent for 1 h, and positive reactions were visualized by incubation with the peroxidase substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB; Zymed Laboratories, San Francisco, CA). To detect insulin, the mounted sections were incubated overnight with guinea pig polyclonal anti-insulin antibody (Dako, Glostrup, Denmark) diluted 1:1,000 in PBS containing 1% BSA and then incubated for 1 h at room temperature with goat anti-guinea pig IgG (1:200; Alexa 546; Molecular Probes). To detect C-peptide, the ABC method was performed using a Vectastain ABC Kit. The mounted sections were incubated overnight with goat anti-C-peptide antiserum (Linco Research, St. Charles, MO) diluted 1:100 in PBS containing 1% BSA. This was followed by a 1-h incubation with biotinylated anti-goat IgG diluted 1:200. The sections were then incubated with ABC reagent for 1 h, and positive reactions were visualized by incubation with the peroxidase substrate solution containing DAB.

Electron microscopy. Liver tissues were fixed with 2% OsO₄ at 4°C for 2 h. After being dehydrated with ascending concentrations of ethanol, the tissues were embedded in Quetol 812 resin. Ultra-thin sections (80–90 nm) mounted on copper grids were stained with aqueous uranyl acetate for 15 min and Reynolds' lead citrate for 5 and 3 min, respectively, then viewed using a Hitachi H-300 electron microscope.

RESULTS

Adenovirus-mediated expression of PDX-1, NeuroD, and Ngn3 induces insulin gene expression in the liver and ameliorates glucose tolerance in diabetic animals. To evaluate the possible effect of ectopic expression of PDX-1, NeuroD, and Ngn3 in the liver, we prepared PDX-1-, NeuroD-, and Ngn3-expressing adenoviruses (Ad-PDX-1, Ad-NeuroD, and Ad-Ngn3) and a control adenovirus (Ad-GFP) and delivered each adenovirus to 8-week-old male C57BL/6 mice. Figure 1A (*upper panel*) shows a representative liver after exposure to the adenovirus. As seen by GFP in this fluorescent micrograph of a liver, many cells were infected with the adenovirus. To confirm that infected adenoviruses can express the target proteins in the liver, we performed immunostaining for PDX-1, NeuroD, and Ngn3. As shown in Fig. 1A (*middle panels*), PDX-1, NeuroD, and Ngn3 were clearly detected in the liver after infection with Ad-PDX-1, Ad-NeuroD, and Ad-Ngn3, respectively. These three transcription factors were not detected at all in the liver without each adenovirus infection or by an immunostaining without each primary antibody (data not shown). In addition, it is known that PDX-1 and NeuroD are expressed in mature pancreatic islets and that Ngn3 is transiently expressed at the embryonic stage during pancreas development. As expected, PDX-1 and NeuroD, but not Ngn3, were clearly detected in mouse mature pancreatic islets by immunostaining using the same antibodies (Fig. 1A, *lower panels*). To confirm that the adenovirus was infected only in the liver, we examined GFP expression in various tissues (brain, heart, lung, liver, spleen, pancreas, kidney, fat, and muscle). As shown in Fig. 1B, GFP was expressed in the liver, but not in any other tissues, indicating that the adenovirus was infected only in the liver. In addition, after injection of each adenovirus, we examined the expression of PDX-1, NeuroD, and Ngn3 in various tissues. Without the adenovirus injection, PDX-1 and NeuroD were detected only in the pancreas (endogenous PDX-1 and NeuroD were detected) and Ngn3 was not detected in any

tissue. After each adenovirus injection, expression of these three transcription factors was increased only in the liver, but not in any other tissues, indicating that the adenoviruses were infected only in the liver (data not shown).

Insulin 2 gene expression was detected 3 days after the injection of Ad-PDX-1 (Fig. 2A), whereas insulin 1 gene expression was not detected at all (data not shown). Similar results were obtained after treatment with Ad-NeuroD or Ad-Ngn3. Also, larger amounts of insulin 2 gene expression were detected in the liver 3 days after the infection of Ad-PDX-1 plus Ad-NeuroD or Ad-Ngn3. Insulin 2 gene expression was still detected 14 days after adenovirus injection, although expression levels were slightly attenuated. In addition, we thought that insulin production in the liver might be easily induced by such transcription factors when pancreatic β -cells are destroyed and/or blood glucose levels are high. To examine this possibility, we compared the induction of insulin mRNA expression in nondiabetic C57BL/6 mice and STZ-induced diabetic mice. However, there was no difference in the induction of insulin mRNA expression by such transcription factors between nondiabetic C57BL/6 mice (Fig. 2A) and STZ-induced diabetic mice (Fig. 2B).

To examine whether hepatic insulin production induced by PDX-1, NeuroD, and/or Ngn3 is capable of controlling blood glucose levels in diabetic mice, we injected 220 mg/kg STZ into C57BL/6 mice and 1 week later treated the mice with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or control Ad-GFP. When PDX-1 was ectopically induced in the liver, insulin 2 gene expression was clearly observed (Fig. 2B), whereas insulin 1 gene expression was not detected at all (data not shown). In addition, larger amounts of insulin 2 gene expression were induced in the liver by the overexpression of PDX-1 plus NeuroD or Ngn3. Furthermore, 3 days after adenovirus injection, blood glucose levels were moderately decreased by PDX-1 alone (Fig. 3A) and more significantly decreased by overexpression of PDX-1 plus NeuroD or Ngn3 (Fig. 3B). After that, however, blood glucose levels gradually increased.

Overexpression of a modified form of PDX-1 carrying the VP16 transcriptional activation domain, together with NeuroD or Ngn3, markedly increases insulin gene promoter activity in HepG2 cells and is more effective than wild-type PDX-1. It has been shown recently that a modified form of XIHbox8, the *Xenopus* homolog of PDX-1 carrying the VP16 transcriptional activation domain from herpes simplex virus, efficiently induces insulin gene expression in the liver (72,73). To evaluate the effect of PDX-1, PDX-1/VP16, NeuroD, and Ngn3 expression on insulin gene transcription, we examined insulin gene promoter activity in HepG2 cells after transient transfection of each expression plasmid. As shown in Fig. 4A, basal insulin promoter activity was very low in HepG2 cells but moderately increased after the transfection of PDX-1, NeuroD, or Ngn3 and was more clearly increased by overexpression of PDX-1 plus NeuroD or Ngn3. Also, although PDX-1/VP16 exerted only a slightly more obvious effect on the insulin promoter compared with wild-type PDX-1, PDX-1/VP16 together with NeuroD or Ngn3 dramatically increased insulin promoter activity (nearly 300-fold increase).

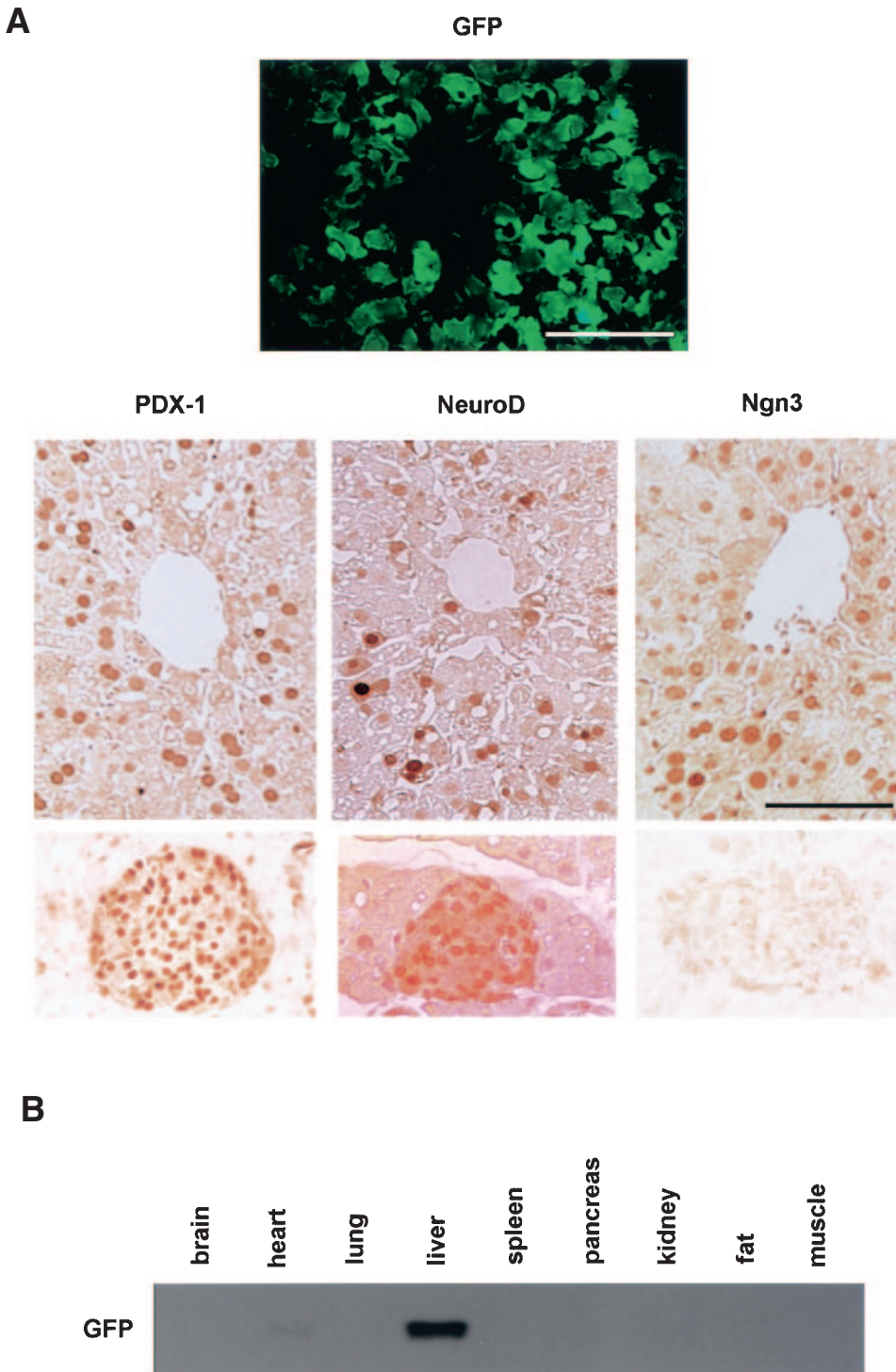


FIG. 1. Adenovirus-mediated expression of PDX-1, NeuroD, and Ngn3 in the liver. **A:** Male C57BL/6 mice were injected with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml) into the cervical vein. Each panel shows a representative liver after exposure to each adenovirus. As seen with GFP (*upper panel*), many cells in the liver were infected after exposure to Ad-GFP. PDX-1, NeuroD, or Ngn3 protein expression was also clearly detected in the liver after infection with Ad-PDX, Ad-NeuroD, or Ad-Ngn3 (*middle panels*). Bar, 50 μ m. In addition, it is known that PDX-1 and NeuroD are expressed in mature pancreatic islets and that Ngn3 is transiently expressed at the embryonic stage during pancreas development. As expected, PDX-1 and NeuroD, but not Ngn3, were clearly detected in mouse mature pancreatic islets by immunostaining using the same antibodies (*lower panels*). **B:** Male C57BL/6 mice were injected with Ad-GFP into the cervical vein. We then obtained whole cell extracts from various tissues (brain, heart, lung, liver, spleen, pancreas, kidney, fat, and muscle) and performed Western blot analysis with anti-GFP antibody.

In addition, A3-box- and E1-box-mutated insulin promoter activities were also increased by overexpression of PDX-1/VP16 plus NeuroD or Ngn3, but the extent of the increase was much less obvious compared with their effects on wild-type insulin promoter activity (Fig. 4B). The A3-box is the most important PDX-1 binding site for insulin gene transcription, and the rat insulin 2 promoter has only one E-box to which NeuroD is known to bind. These results suggest that PDX-1/VP16 plus NeuroD or Ngn3 exerts synergistic effects on insulin gene transcription in an A-box- and E-box-dependent manner.

Adenoviral PDX-1/VP16 expression, together with NeuroD or Ngn3, markedly induces insulin and other various pancreas-related factors. To examine the additional effect of VP16 on PDX-1-mediated induction of insulin gene expression, we prepared an adenovirus expressing the PDX-1/VP16 fusion protein (Ad-PDX-1/VP16) and delivered the adenovirus to 8-week-old male C57BL/6 mice. As shown in Fig. 5A, 3 days after Ad-PDX-1/VP16 injection, insulin 1 and 2 were both detected by RT-PCR, although insulin 1 was not detected by the expression of wild-type PDX-1 in the absence of VP16. In addition, larger

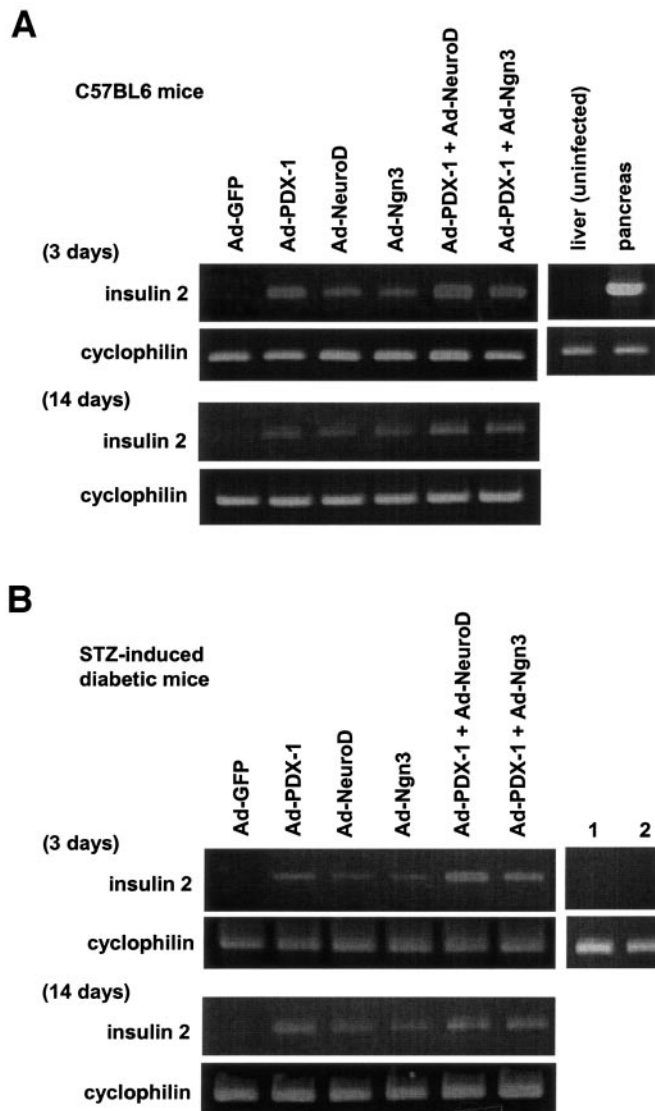


FIG. 2. Adenoviral expression of PDX-1, NeuroD, and Ngn3 in the liver induces insulin gene expression. **A:** Male C57BL/6 mice were injected with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml). Then, 3 (*upper panel*) and 14 (*lower panel*) days after the injection we examined insulin mRNA expression by RT-PCR. Insulin mRNA expression in the liver and pancreas without adenovirus treatment is also shown (*right panel*). **B:** Male C57BL/6 mice were made diabetic with STZ and injected with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml) 1 week later. Then 3 (*upper panel*) and 14 (*lower panel*) days after injection, we examined insulin mRNA expression by RT-PCR. Similar results were obtained in three independent experiments. Insulin mRNA expression in the liver (*lane 1*) and pancreas (*lane 2*) after STZ administration are also shown (*right panel*).

amounts of insulin gene expression were detected by PDX-1/VP16 in the presence of NeuroD or Ngn3. The expression of insulin 1 and 2 mRNA could still be clearly detected 14 days after the adenovirus injection. Similar results were obtained when STZ-induced diabetic mice were treated with Ad-PDX-1/VP16 in the absence or presence of Ad-NeuroD or Ad-Ngn3 (Fig. 5B). We further evaluated insulin mRNA levels by Northern blot analysis. Insulin mRNA was detected 3 (Fig. 6A, *lane 7*) and 14 (Fig. 6B) days after Ad-PDX-1/VP16 injection. In addition, larger amounts of insulin gene expression were detected by treatment with Ad-PDX-1/VP16 plus Ad-NeuroD (*lane 7*) or with Ad-Ngn3 (*lane 9*). After treatment with Ad-

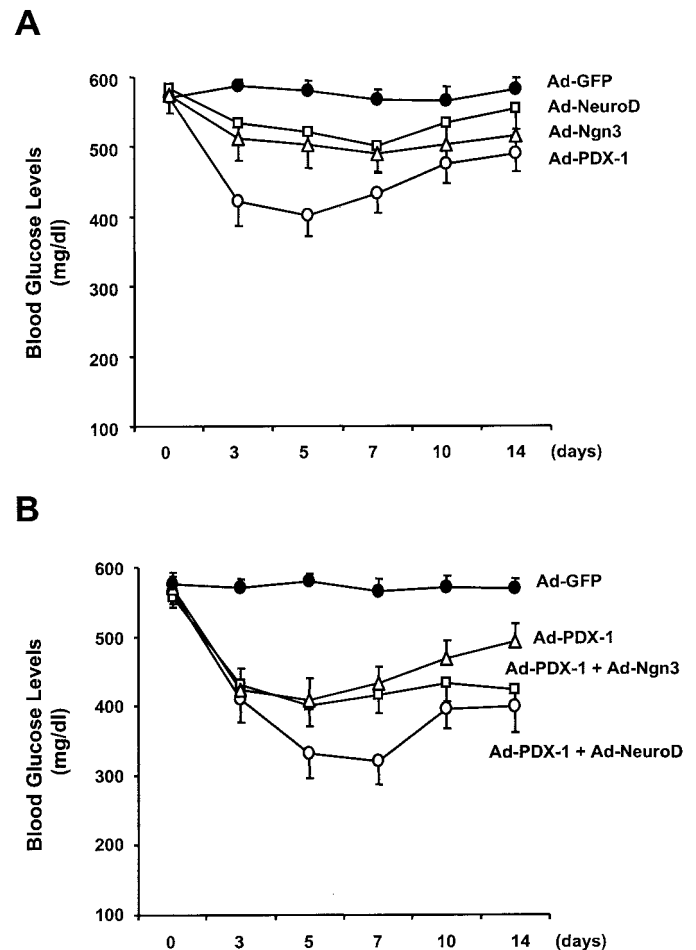


FIG. 3. Adenoviral PDX-1 expression in the liver, together with NeuroD or Ngn3, decreases blood glucose levels in diabetic animals. Male C57BL/6 mice were made diabetic with STZ and 1 week later infected with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (**A**) and Ad-PDX-1 plus Ad-NeuroD or Ad-Ngn3 (**B**) (1×10^{10} PFU/ml). Nonfasting blood glucose levels were measured with a portable glucose meter after tail snipping. Data are means \pm SE ($n = 6$).

PDX-1 plus Ad-NeuroD (*lane 5*) or Ad-Ngn3 (*lane 6*), insulin mRNA was also detected by Northern blot analysis, although insulin mRNA was not detected by Ad-PDX-1, Ad-NeuroD, or Ad-Ngn3 alone (*lanes 2-4*)

To examine whether insulin protein was synthesized and stored in the liver, we examined insulin protein expression in the liver after treatment with the adenoviruses. As shown in Fig. 7A (*upper panel*), immunostaining for insulin 3 days after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD revealed several insulin-positive cells in cytoplasm (*arrows; red cells*). Insulin was not detected at all in the control liver (*middle panel*) or by immunostaining without primary antibody (*lower panel*). Similarly, several insulin-positive cells were observed after treatment with Ad-PDX-1/VP16 plus Ad-Ngn3 (data not shown).

To examine insulin processing, we performed immunostaining for C-peptide. As shown in Fig. 7B, C-peptide was clearly detected in the liver after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD (*arrows; brown cells*). Similarly, C-peptide-positive cells were observed in the liver after treatment with Ad-PDX-1/VP16 plus Ad-Ngn3 but were not detected at all in the control liver or by immunostaining

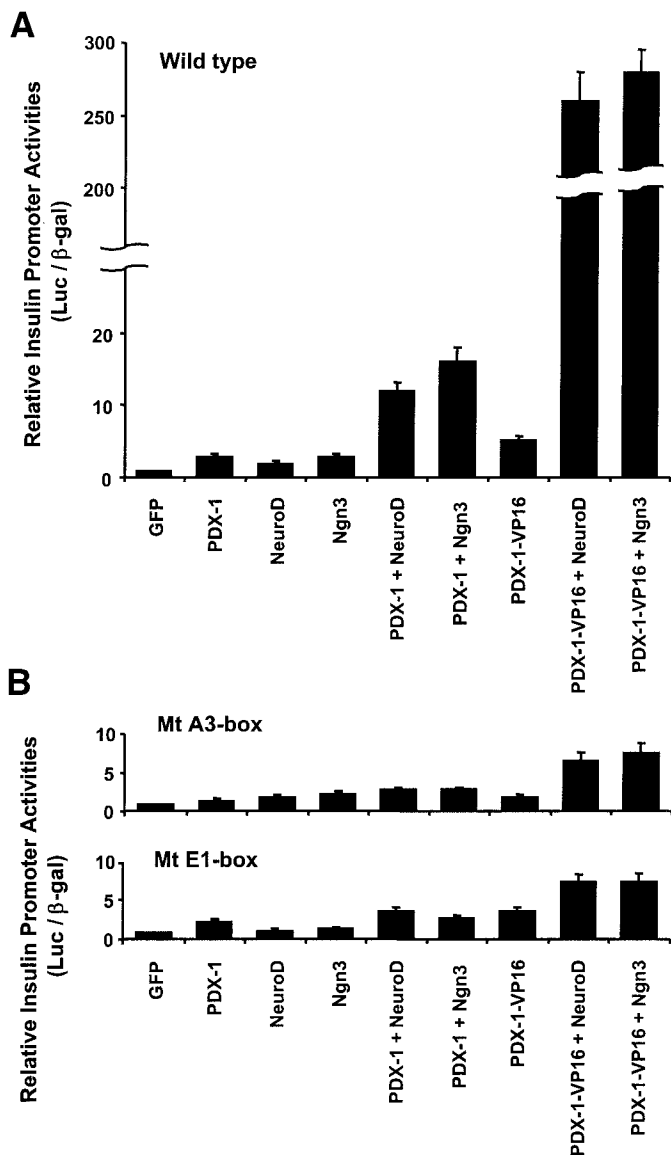


FIG. 4. PDX-1/VP16 expression, together with NeuroD or Ngn3, markedly increases insulin gene promoter activity in HepG2 cells. **A:** The rat insulin promoter-reporter (luciferase; Luc) plasmid and pSV- β -galactosidase control vector (β -gal) were cotransfected into HepG2 cells with the PDX-1, PDX-1/VP16, NeuroD, and/or Ngn3 expression plasmids (or empty vector). After 48 h, luciferase and β -galactosidase assays were performed. **B:** The A3-box- or E1-box-mutated rat insulin promoter-reporter plasmid and the pSV- β -galactosidase control vector were cotransfected into HepG2 cells with the PDX-1, PDX-1/VP16, NeuroD, and/or Ngn3 expression plasmids (or empty vector). After 48 h, luciferase and β -galactosidase assays were performed. The luciferase results were normalized with respect to the transfection efficiency assessed from the results of the β -galactosidase assays. Data are means \pm SE, with the basal insulin promoter activity being arbitrarily set at 1 ($n = 4$).

without primary antibody (data not shown). Although many cells in the liver were infected with the adenovirus, all cells did not necessarily express substantial amounts of insulin; ~ 3 of 100 cells in the liver started expressing substantial amounts of insulin in the liver. To further evaluate insulin biosynthesis in the liver, we performed electron microscopy. As shown in Fig. 7C, several insulin secretory granule-like granules (arrows in left upper and lower panels) were observed in the liver after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD, although these

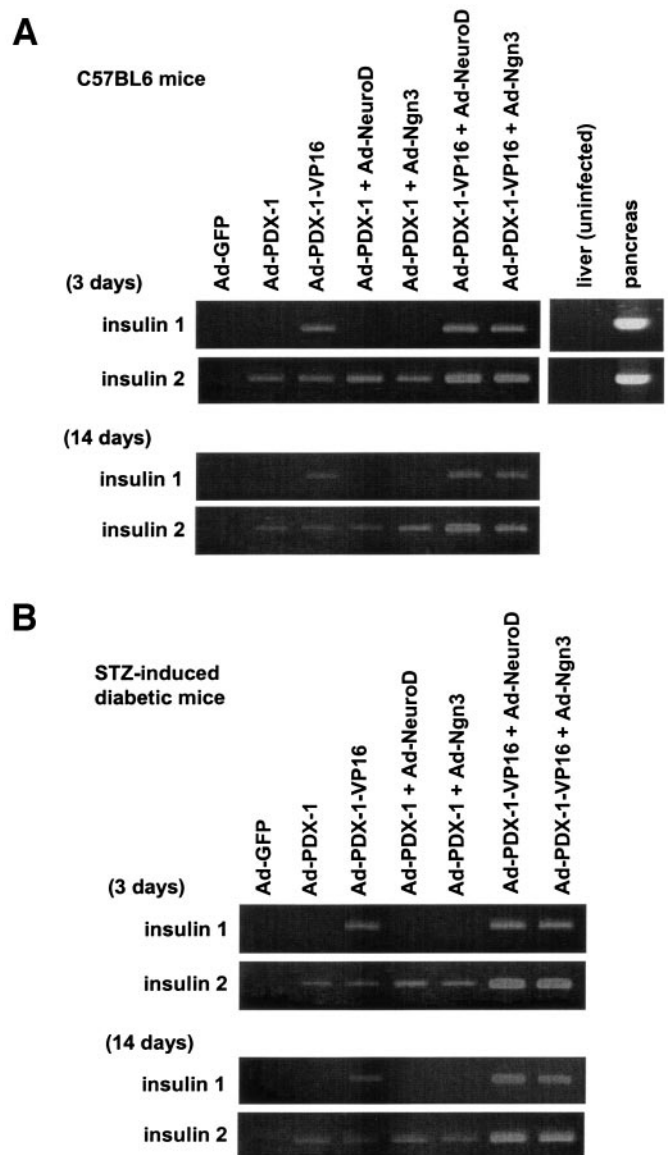


FIG. 5. Adenoviral expression of PDX-1/VP16 in the liver, together with NeuroD or Ngn3, induces insulin gene expression. **A:** Male C57BL/6 mice were injected with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml) into the cervical vein. Then, 3 (upper panel) and 14 (lower panel) days after the injection we examined insulin 1 and 2 mRNA expression by RT-PCR. Insulin mRNA expression in the liver and pancreas without the adenovirus treatment is also shown (right panel). **B:** Male C57BL/6 mice were made diabetic with STZ and 1 week later were injected with Ad-PDX-1/VP16, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml). Then 3 (upper panel) and 14 (lower panel) days after the injection, we examined insulin 1 and 2 mRNA expression by RT-PCR. Similar results were obtained in three independent experiments.

were not observed in the control liver (right panel). These granules had the typical crystal-like structure inside (left upper panel), which is characteristic of insulin secretory granules. In addition, it is known that insulin secretory granules are often withdrawn into their vesicles. As shown in Fig. 7C (left lower panel), several granules were withdrawn into their vesicles. Peroxisome was observed even in control liver (arrow heads in right panel) but was different from insulin secretory granules. In this study, we did not examine the secretory granules in detail, and thus the morphology of the granules is not conclusive. How-

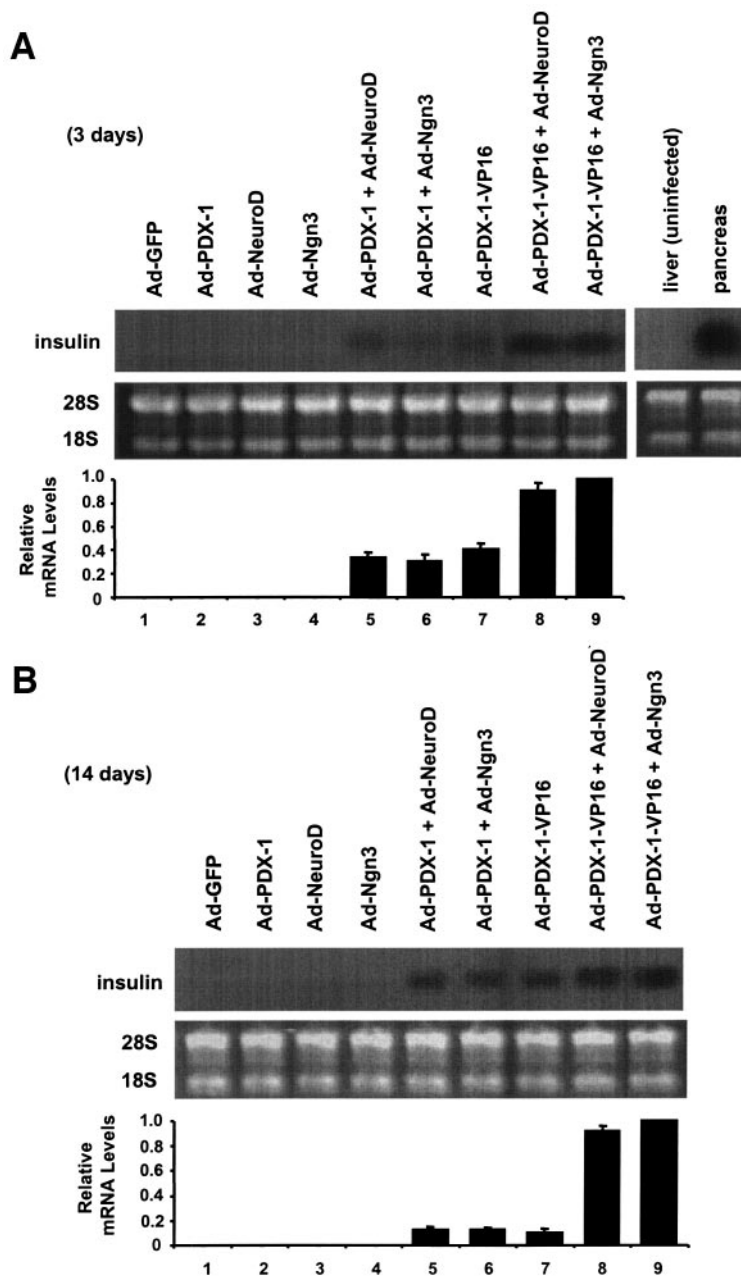


FIG. 6. Quantitative analysis of insulin gene expression induced by adenoviral expression of PDX-1/VP16 in the liver, together with NeuroD or Ngn3. Male C57BL/6 mice were injected with Ad-PDX-1, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml) into the cervical vein. We then examined insulin mRNA expression levels by Northern blot analysis 3 (A) and 14 (B) days after the injection. Data are means \pm SE, with the insulin mRNA levels after treatment with Ad-PDX-1/VP16 plus Ad-Ngn3 being arbitrarily set at 1.0 ($n = 3$). Also shown is insulin mRNA expression in the liver and pancreas without the adenovirus treatment (right panel).

ever, based on various results of insulin expression in this study, it is likely that insulin secretory granules were induced by PDX-1/VP16 overexpression together with NeuroD. Similar secretory granules were observed in the liver after treatment with Ad-PDX-1/VP16 plus Ad-Ngn3 (data not shown). In all the observed cells containing insulin secretory granules, there were several characteristics of hepatocytes, such as big Golgi complex and a large number of mitochondria, indicating that the insulin-producing cells in the liver were hepatocytes, although we cannot deny the possibility that some other cells in the liver also have insulin secretory granules. These results also suggest that hepatocyte-type characteristics are preserved even after insulin expression is induced by the adenovirus infection. Thus PDX-1/VP16 expression together with NeuroD or Ngn3 indeed induces insulin biosynthesis in addition to inducing insulin promoter activity (Fig. 4) and its gene expression (Figs. 5 and 6). Further-

more, to determine whether the insulin produced was secreted into the blood stream, we measured serum immunoreactive insulin levels after a 6-h fast. As shown in Fig. 8, serum insulin levels were significantly increased after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3, although a moderate increase was observed after treatment of Ad-PDX-1 plus Ad-NeuroD, Ad-PDX-1 plus Ad-Ngn3, or Ad-PDX-1/VP16 alone. For reference, we show serum insulin levels after a 6-h fast in untreated nondiabetic mice in Fig. 8. Severe hyperglycemia was not observed after a 6-h fast even in Ad-GFP-treated STZ-induced diabetic mice, and the difference in blood glucose levels after a 6-h fast among the groups was much smaller than the difference in nonfasting blood glucose levels. In particular, blood glucose levels after a 6-h fast in the mice treated with Ad-PDX-1/VP16 plus Ad-NeuroD (or Ad-Ngn3) were similar to those in untreated control mice. However, we cannot exclude the possibility that the

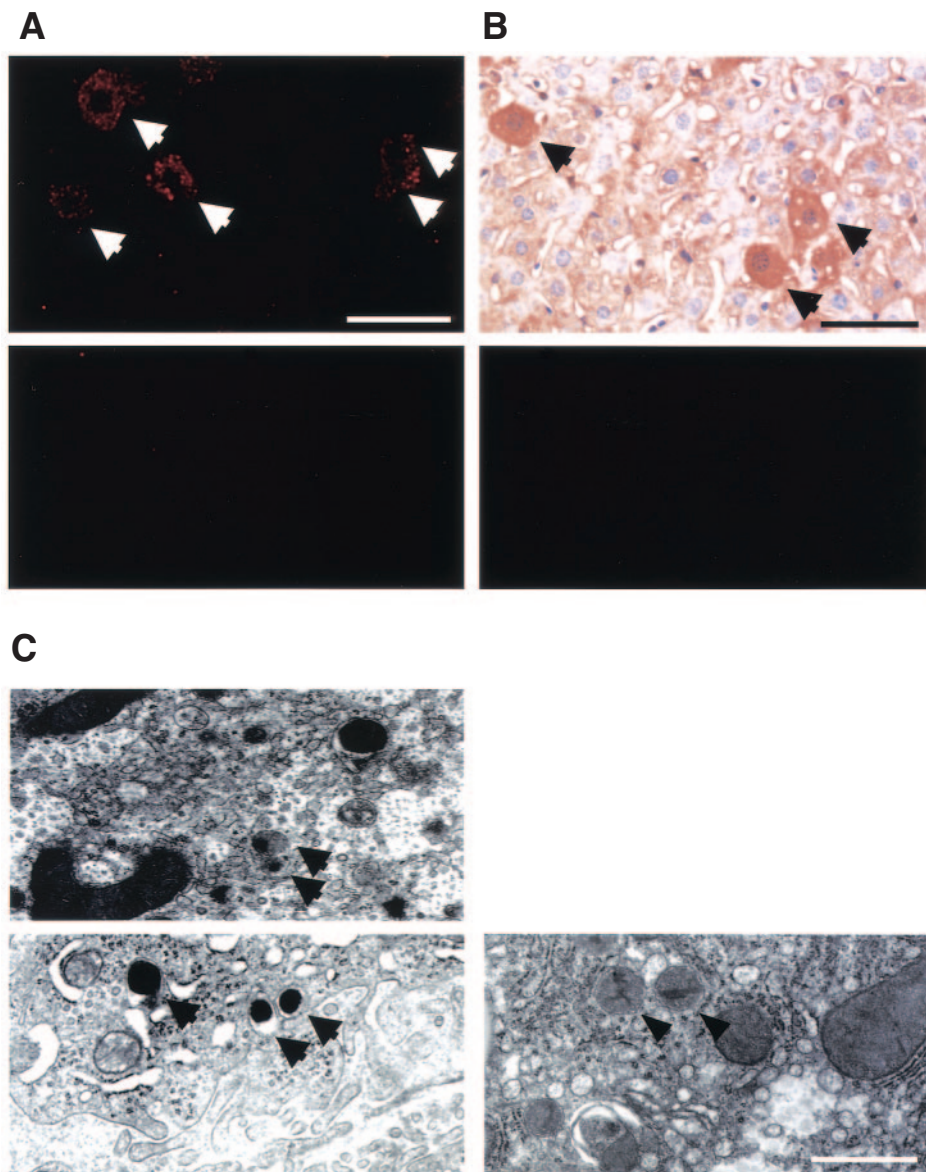


FIG. 7. Adenoviral PDX-1/VP16 expression in the liver, together with NeuroD or Ngn3, induces insulin protein. **A:** To examine insulin protein expression in the liver, we performed immunostaining for insulin after the adenovirus injection. Insulin immunostaining 3 days after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD is shown (upper panel). Several insulin-positive cells (arrows; red cells) were clearly observed in cytoplasm. In contrast, insulin was not detected at all in the control liver (left lower panel) or by immunostaining without primary antibody (right lower panel). Bar, 20 μ m. **B:** To examine insulin processing in the liver, we performed immunostaining for C-peptide after the adenovirus injection. The panel shows C-peptide immunostaining 3 days after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD. Several C-peptide-positive cells (arrows; brown cells) were clearly observed. Bar, 20 μ m. **C:** To further evaluate insulin biosynthesis in the liver, we performed electron microscopy. The panel shows an electron microscopy in the liver 3 days after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD. Several insulin secretory granules (arrows in left upper and lower panels) were observed. Peroxisome was observed even in control liver (arrowheads in right panel) but was different from insulin secretory granules.

difference in insulin levels among the groups is at least in part due to differences in blood glucose levels.

To examine pancreas-related gene expression caused by ectopic expression of PDX-1, PDX-1/VP16, NeuroD, and Ngn3, we evaluated mRNA expression of various pancreas-related genes by RT-PCR analysis. First, we examined the expression of other β -cell-related genes: islet-type glucokinase, SUR1, and Kir6.2. As shown in Fig. 9A, islet-type glucokinase, which is different from hepatic-type glucokinase in the first exon, was detected in the liver after treatment with Ad-PDX-1, Ad-PDX-1 plus Ad-NeuroD or Ad-Ngn3, or Ad-PDX-1/VP16; larger amounts of glucokinase expression were observed after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3. Similarly, large amounts of SUR1 and Kir6.2 mRNA expression were observed after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3, although some expression was detected without these combinations. Also, as shown in Fig. 9B, endocrine hormones such as glucagon, somatostatin, and pancreatic polypeptide were detected in the liver after treatment with Ad-PDX-1, and larger amounts of endo-

crine hormone expression were observed after treatment with Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3. These results suggest that the liver after PDX-1/VP16 overexpression, together with NeuroD or Ngn3, expresses various pancreas-related factors.

To examine whether various pancreatic markers are expressed in the same cells (in insulin-producing cells), we performed immunostaining for various pancreas markers such as glucagon, somatostatin, and pancreatic polypeptide. Although their mRNAs were detected by RT-PCR (Fig. 9B), we failed to detect their protein expression by immunostaining, probably because of low (or diffuse) expression of such markers. These results imply that the cells generated by PDX-1/VP16 overexpression, together with NeuroD or Ngn3, are still quite different from real pancreatic endocrine cells. If protein expression levels of such endocrine cell markers were similar to those in real endocrine cells, they should be readily detectable as they are in real endocrine cells. In addition, to examine exocrine cell differentiation caused by ectopic expression of PDX-1, PDX-1/VP16, NeuroD, and Ngn3, we evaluated

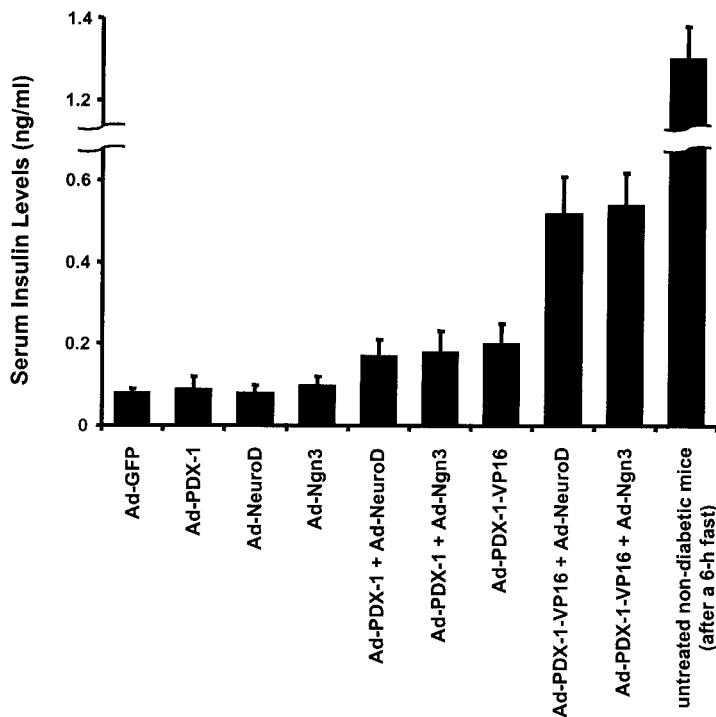


FIG. 8. Adenoviral PDX-1/VP16 expression in the liver, together with NeuroD or Ngn3, substantially increases serum insulin levels. Male C57BL/6 mice were made diabetic with STZ and 1 week later were infected with Ad-PDX-1, Ad-PDX-1/VP16, Ad-NeuroD, Ad-Ngn3, or Ad-GFP. After the adenovirus infection, we determined serum insulin levels. For reference, we show serum insulin levels after a 6-h fast in untreated nondiabetic mice. Data are means \pm SE ($n = 4$).

mRNA expression of an exocrine hormone elastase 1. As shown in Fig. 9B, elastase 1 mRNA expression was detected after Ad-PDX-1 or Ad-PDX-1/VP16 treatment but was not further increased by the presence of NeuroD or Ngn3. These results suggest that PDX-1 is important for exocrine cell differentiation but that the presence of NeuroD and Ngn3 does not facilitate exocrine cell differentiation.

To examine whether the adenovirus infection and consequent local insulin production influences liver function, 14 days after the adenovirus injection we measured levels of aspartic acid aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin levels. AST and ALT levels after treatment with Ad-GFP were 40 ± 3 and 29 ± 3 IU/l, respectively, with Ad-PDX-1/VP16 plus Ad-NeuroD were 43 ± 1 and 30 ± 1 IU/l, and with Ad-PDX-1/VP16 plus Ad-Ngn3 were 42 ± 8 and 26 ± 2 IU/l. In addition, 14 days after treatment with Ad-GFP or Ad-PDX-1/VP16 plus Ad-NeuroD or Ngn3, total bilirubin levels in the three groups were within the normal range (<0.1 mg/gl). These results suggest that liver physiology is not influenced by ectopic pancreatic gene expression.

Adenoviral PDX-1/VP16 expression, together with NeuroD or Ngn3, ameliorates glucose tolerance in diabetic model animals more effectively compared with wild-type PDX-1. To examine whether hepatic insulin production induced by PDX-1/VP16 plus NeuroD or Ngn3 is capable of controlling blood glucose levels in diabetic mice, we injected 220 mg/kg STZ into C57BL/6 mice and 1 week later treated the mice with Ad-PDX-1/VP16 with and without Ad-NeuroD or Ad-Ngn3. As shown in Fig. 10A, nonfasting blood glucose levels were decreased by PDX-1/VP16 alone, which was a more pronounced change compared with the effects of wild-type PDX-1. As shown in Fig. 10B, nonfasting blood glucose levels were further decreased by overexpression of PDX-1/VP16 plus NeuroD or Ngn3. The effects of PDX-1/VP16 plus NeuroD or Ngn3 were more pronounced and persis-

tent compared with those of PDX-1 plus NeuroD or Ngn3. We assume that the capacity of hepatic insulin induced by such transcription factors to lower blood glucose levels in mice with STZ-induced diabetes was due to the induced production and secretion of insulin. Thus, PDX-1/VP16 expression together with NeuroD or Ngn3 markedly induced insulin gene transcription and ameliorated glucose tolerance in diabetic model animals, implying that this combination is useful for replacing the reduced β -cell function found in diabetes.

Furthermore, 3 and 14 days after the adenovirus injection, we performed an intraperitoneal glucose tolerance test. As shown in Fig. 11A (3 days) and B (14 days), there was a marked difference in glucose tolerance between untreated mice and mice treated with Ad-PDX-1/VP16 plus Ad-NeuroD or Ngn3. In addition, as shown in Fig. 11C (3 days) and D (14 days), serum insulin levels before glucose challenge in the mice treated with Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3 were much higher than those in mice treated with Ad-GFP. We think that this difference in insulin secretion was due to unregulated production of insulin by Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3. However, there was an additional increase in serum insulin levels in response to a glucose challenge in mice treated with Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3, whereas no such additional increase was observed in mice treated with Ad-GFP. These results suggest that some glucose-mediated regulation of insulin secretion is achieved by Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3.

DISCUSSION

In this study, we examined the effects of adenovirus-mediated expression of various pancreas-related transcription factors on insulin gene expression and glucose tolerance and found that PDX-1/VP16 overexpression, together with NeuroD or Ngn3, markedly induces insulin

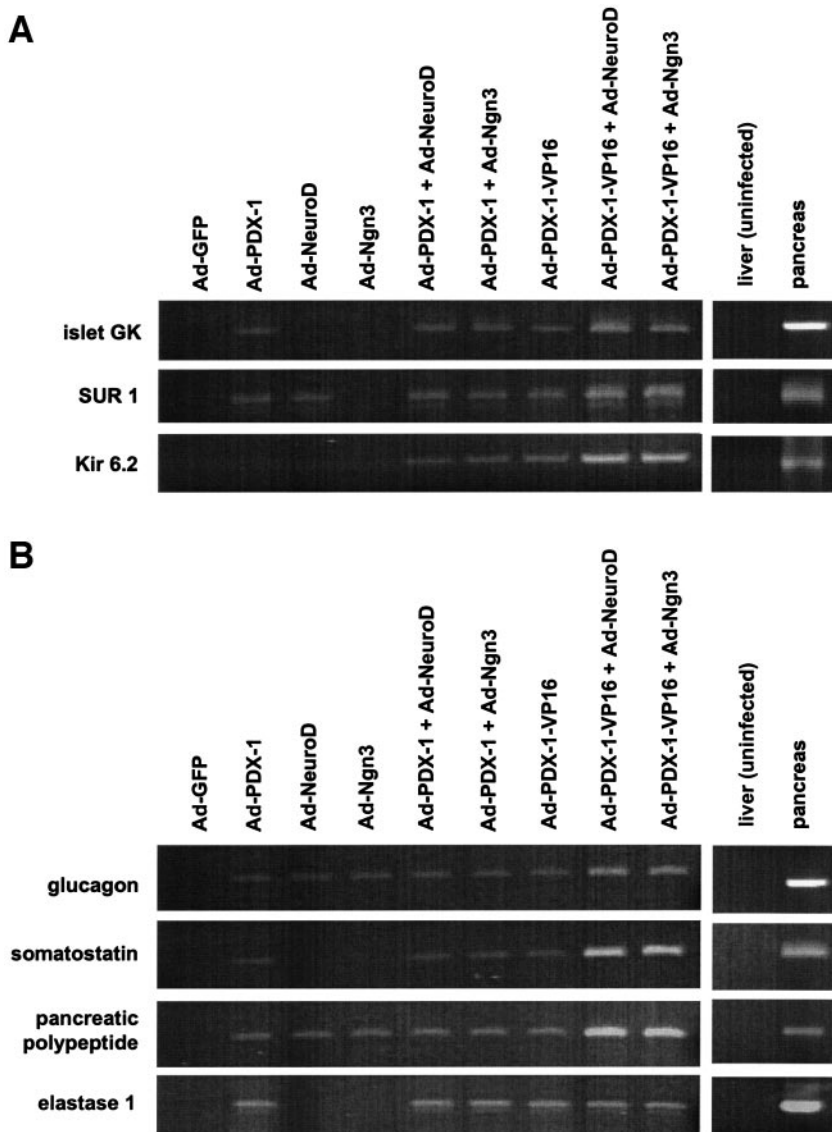


FIG. 9. Adenoviral expression of PDX-1, PDX-1/VP16, NeuroD, and Ngn3 in the liver induces various pancreas-related genes. Male C57BL/6 mice were injected with Ad-PDX-1, Ad-PDX-1/VP16, Ad-NeuroD, Ad-Ngn3, or Ad-GFP (1×10^{10} PFU/ml) into the cervical vein. Then, 14 days after the injection, we examined mRNA expression levels of the β -cell-related genes islet-type glucokinase (GK), sulfonylurea receptor 1 (SUR 1), and Kir6.2 (*A*) and the endocrine hormones glucagon, somatostatin, and pancreatic polypeptide (*B*). Also shown is insulin mRNA expression in the liver and pancreas without the adenovirus treatment (*right panel*). Similar results were obtained in three independent experiments.

production in the liver and ameliorates glucose tolerance in diabetic animal models. PDX-1 (1–3) plays a crucial role in pancreas development (4–14), β -cell differentiation (15–26), and the maintenance of normal β -cell function by regulating several β -cell-related genes (14,27–37). Previously it has been shown that PDX-1 functions in concert with other transcription factors (e.g., NeuroD) in regulating the expression of insulin and several other islet-specific genes (39–43) and that VP16 enhances PDX-1 function as a transdifferentiation factor from the liver to the pancreas (72,73). In this study, we examined the effects of PDX-1/VP16 expression on insulin gene expression and glucose tolerance in the absence and presence of other transcription factors such as NeuroD and Ngn3. The marked effects of PDX-1/VP16 expression together with NeuroD or Ngn3 on insulin production and glucose tolerance indicate that the combinations are useful and efficient for replacing the reduced insulin biosynthesis found in diabetes and that PDX-1 requires the recruitment of coordinately functioning transcription factors or cofactors to fully exert its function. PDX-1, NeuroD, and Ngn3 are important molecules for determining the lineage to pan-

creatic endocrine cells, and thus we thought the combination of such transcription factors would be a useful tool for inducing insulin-producing cells. We would like to emphasize that the combinations of PDX-1/VP16 plus Ngn3 and PDX-1/VP16 plus NeuroD are useful as means to induce insulin-producing cells in non- β -cells and to replace the impaired β -cell function found in diabetes. In our study, many cells in the liver were infected with the adenovirus (Fig. 1A), but not all cells expressed substantial amounts of insulin; in fact, ~ 3 of 100 cells in the liver started expressing substantial amounts of insulin in the liver (Fig. 7A and B). We assume that because the liver is a very large tissue, the total amounts of insulin biosynthesis in the whole liver were enough to decrease blood glucose levels.

PDX-1/VP16 overexpression, together with NeuroD or Ngn3, substantially induced insulin biosynthesis in the liver (Figs. 5–8) and the various pancreas-related factors that are necessary for β -cell function (Fig. 9). Therefore, we assume that the liver cells started secreting insulin after the adenovirus injection, thus leading to a substantial increase in serum insulin levels (Fig. 8) and marked

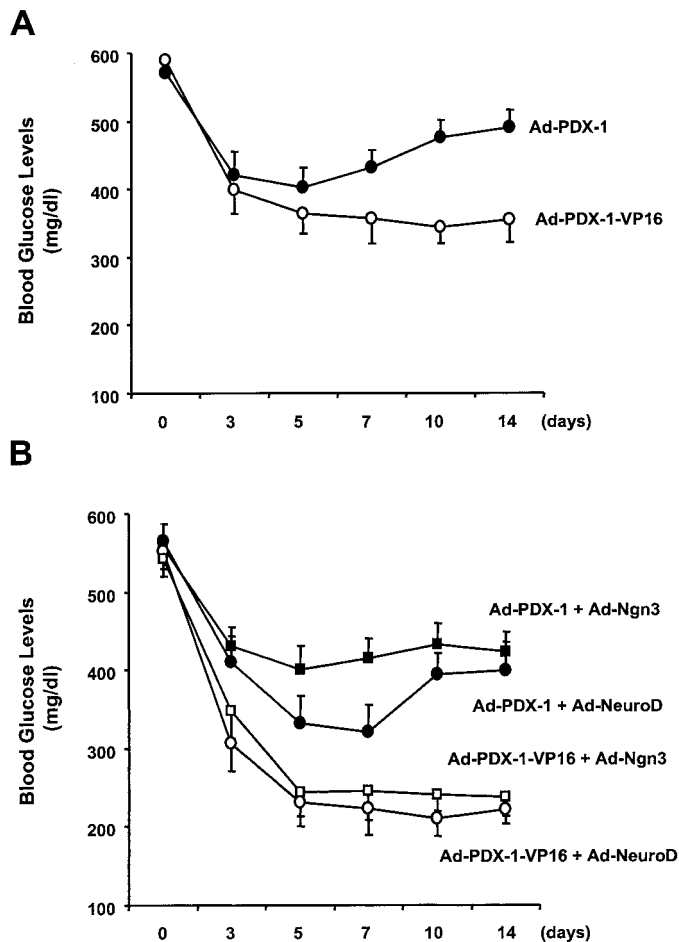


FIG. 10. Adenoviral PDX-1/VP16 expression in the liver, together with NeuroD or Ngn3, markedly decreases blood glucose levels in diabetic animals. Male C57BL/6 mice were made diabetic with STZ and 1 week later were infected with Ad-PDX-1 or Ad-PDX-1/VP16 (A) and Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3 (B) (1×10^{10} PFU/ml). Nonfasting blood glucose levels were measured with a portable glucose meter after tail snipping. Data are means \pm SE ($n = 6$).

improvement of glycemic control (Figs. 10 and 11). In addition, as shown in Fig. 1B, the adenovirus is infected only in the liver, when the virus is injected into the cervical vein, suggesting that the improved glycemic control was initiated by a direct effect of adenoviral overexpression of such transcription factors in the liver. However, once the vicious circle of the glucose toxicity is prevented by achieving good glycemic control by some means, the circle then starts moving in the opposite direction: the toxic effects of high glucose are reduced, which results in further improvement of glycemic control. Because the adenoviral overexpression of such transcription factors reduced blood glucose levels (Figs. 10 and 11), it must have reduced glucose toxicity and contributed in part to further improvement of glycemic control.

It has been reported that insulin mRNA expression was detected after Ad-PDX-1 administration alone (16). In that study, the investigators detected insulin mRNA expression by RT-PCR, but not by Northern blot analysis. We also detected insulin mRNA expression by RT-PCR after treatment with Ad-PDX-1 alone (Fig. 2A and B). As shown in Fig. 6A and B, however, insulin mRNA was not detected by Northern blot analysis after treatment with Ad-PDX-1

alone (lane 2), when film was exposed overnight with an intensifying screen at -80°C . When exposure time was increased up to 3 days, small amounts of insulin mRNA expression were detected by Ad-PDX-1 alone. Because RT-PCR is much more sensitive than Northern blot analysis, we think it is reasonable to hypothesize that insulin mRNA is detected by RT-PCR but not easily detected by Northern blot analysis. In addition, in one study (16), the adenovirus was infected 3 days after STZ administration, whereas we injected the adenovirus 2 weeks after STZ injection. We think that this difference might be important in explaining the difference of the effect of PDX-1 alone on the induction of insulin gene expression. Although not examined in detail in our study, it is likely that 3 days after STZ administration, pancreatic β -cells are not yet completely destroyed. Indeed, in our study, blood glucose levels continued to increase until 1 week after STZ administration. In contrast, it is likely that 2 weeks after STZ administration, β -cells are almost completely destroyed. In addition, it has been reported that although ectopic PDX-1 expression alone is insufficient to induce insulin-producing cells in the liver, hepatic regeneration stimulates the transdifferentiation of the liver into insulin-producing cells; in STZ-induced diabetic mice, adenoviral PDX-1 overexpression plus partial hepatectomy induces substantial amounts of insulin biosynthesis in the liver (26). Those researchers found a significant difference in insulin biosynthesis in the liver with and without partial hepatectomy. Therefore, we assume that in some conditions, PDX-1 alone (without other transcription factors such as NeuroD or Ngn3) is enough to induce some amounts of insulin gene expression in the liver and ameliorate glucose tolerance and that even after β -cells are completely destroyed, the combination of PDX-1/VP16 and NeuroD or Ngn3 can induce substantial amounts of insulin in the liver and markedly ameliorate glucose tolerance.

As shown in Fig. 4, PDX-1 or NeuroD expression induced insulin promoter activity, and cotransfection of these two expression plasmids exerted synergistic effects. Furthermore, the expression of PDX-1/VP16 together with NeuroD or Ngn3 much more markedly induced insulin promoter activity. In addition, as shown in Figs. 5 and 6, insulin gene expression was much more markedly induced by PDX-1/VP16 in the presence of NeuroD or Ngn3. There are several possible explanations as to why the combination of PDX-1/VP16 and NeuroD or Ngn3 is much more active compared with wild-type PDX-1. One possibility is that the transcription partners required for PDX-1 to induce sufficient insulin production are absent in the liver, but that their expression is induced by the combination of PDX-1/VP16 and NeuroD or Ngn3 expression. Indeed, PDX-1 has been shown to require interactions with other proteins such as PBX (76–80), and thus overexpression of unmodified PDX-1 without these protein partners may not be sufficient to induce the production of large amounts of insulin.

In summary, PDX-1/VP16 expression together with NeuroD or Ngn3 markedly induces insulin gene transcription and ameliorates glucose tolerance. This is an approach that warrants further investigation and may turn out to have utility in the treatment of diabetes.

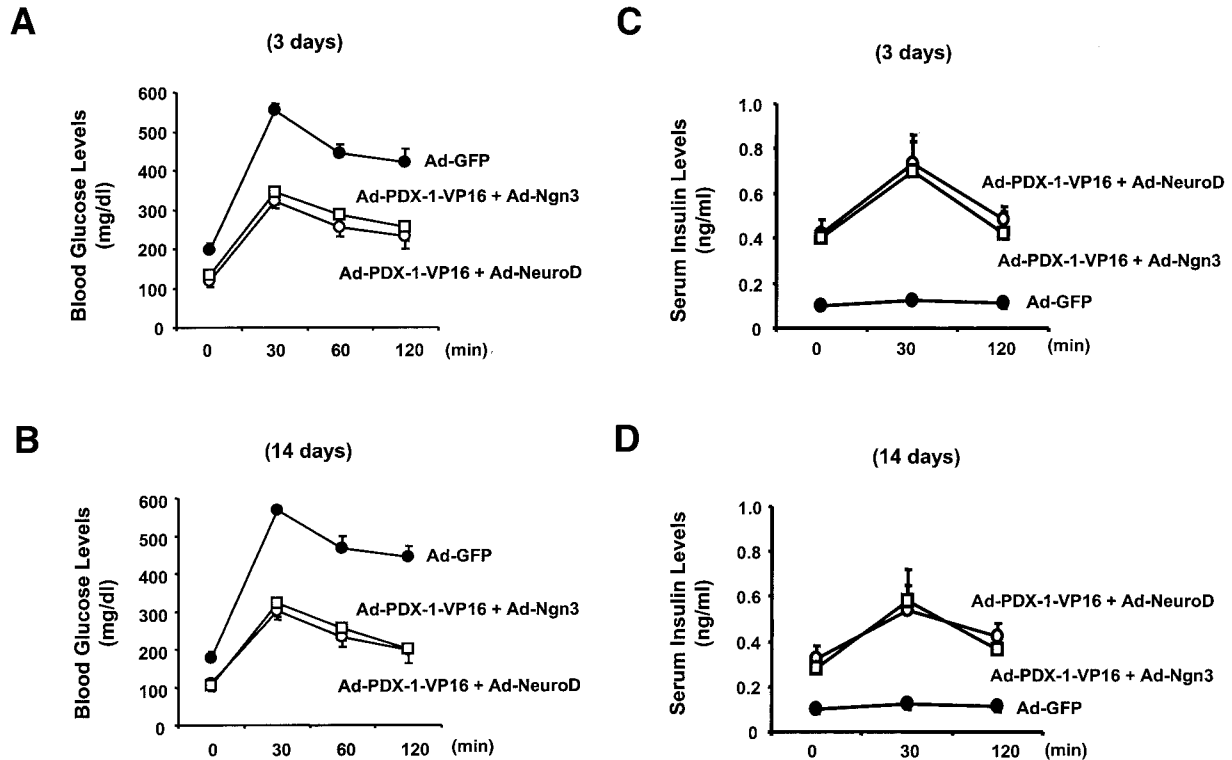


FIG. 11. Adenoviral PDX-1/VP16 expression in the liver, together with NeuroD or Ngn3, markedly ameliorates glucose tolerance in diabetic animals. Male C57BL/6 mice were made diabetic with STZ and 1 week later were infected with Ad-GFP or Ad-PDX-1/VP16 plus Ad-NeuroD or Ad-Ngn3 (1×10^{10} PFU/ml). An intraperitoneal glucose tolerance test was performed 3 and 14 days after the adenovirus infection. Blood glucose levels (A and B) and serum insulin levels (C and D) were measured 3 and 14 days after the adenovirus infection. ●, Ad-GFP; ○, Ad-PDX-1/VP16 plus Ad-NeuroD; □, Ad-PDX-1/VP16 plus Ad-Ngn3. Data are means \pm SE ($n = 4$).

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