# Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia

SARAH FERBER[1], AMIR HALKIN[2], HOFIT COHEN[1], IDIT BER[1,3], YULIA EINAV[4], IRIS GOLDBERG[5], IRIS BARSHACK[5], RHONA SEIJFFERS[1,3], JURI KOPOLOVIC[3,5], NURIT KAISER[6] & AVRAHAM KARASIK[1,3]

[1]*Endocrine Institute and* [5]*Pathology Institute, Sheba Medical Center, Tel-Hashomer 52621, Israel*
[2]*Deptartment of Cardiology Sourasky Medical Center, Tel-Aviv 64239, Israel*
[3]*Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel*
[4]*Life Science Dep. Tel-Aviv University, Ramat-Aviv 69978, Israel*
[6]*Department of Endocrinology and Metabolism, The Hebrew University-Hadassah Medical Center, Jerusalem 91120, Israel*
*Correspondence should be addressed to S.F.; email: berezin@post.tau.ac.il*



**Insulin gene expression is restricted to islet β cells of the mammalian pancreas through specific control mechanisms mediated in part by specific transcription factors[1,2]. The protein encoded by the pancreatic and duodenal homeobox gene 1 (PDX-1) is central in regulating pancreatic development and islet cell function[3]. PDX-1 regulates insulin gene expression and is involved in islet cell-specific expression of various genes[4–7]. Involvement of PDX-1 in islet-cell differentiation and function has been demonstrated mainly by 'loss-of-function' studies[8–11]. We used a 'gain-of-function' approach to test whether PDX-1 could endow a non-islet tissue with pancreatic β-cell characteristics *in vivo*. Recombinant-adenovirus-mediated gene transfer of PDX-1 to the livers of BALB/C and C57BL/6 mice activated expression of the endogenous, otherwise silent, genes for mouse insulin 1 and 2 and prohormone convertase 1/3 (PC 1/3). Expression of PDX-1 resulted in a substantial increase in hepatic immunoreactive insulin content and an increase of 300% in plasma immunoreactive insulin levels, compared with that in mice treated with control adenovirus. Hepatic immunoreactive insulin induced by PDX-1 was processed to mature mouse insulin 1 and 2 and was biologically active; it ameliorated hyperglycemia in diabetic mice treated with streptozotocin. These data indicate the capacity of PDX-1 to reprogram extrapancreatic tissue towards a β-cell phenotype, may provide a valuable approach for generating 'self' surrogate β cells, suitable for replacing impaired islet-cell function in diabetics.**

To assess the effect of ectopic expression of pancreatic and duodenal homeobox gene 1 (PDX-1) in liver, we delivered *AdCMV–PDX-1* recombinant adenovirus[12] (encoding the rat homolog of PDX-1) to 11–14 week-old male BALB/c and C57BL/6 mice. RT–PCR analysis of total RNA showed that administration of *AdCMV–PDX-1* resulted in expression of PDX-1 mainly in the liver. Spleen, pancreas and kidney from the same mice lacked the rat homolog of PDX-1, as determined by RT–PCR, except in two mice that expressed the message in the spleen (data not presented).

PDX-1 induced expression of the genes for endogenous mouse insulin 1 (mI-1) and mI-2 in liver. By RT–PCR, 75% (25 of 34) of the mice that had the ectopic rat PDX-1 message expressed the gene for mI-2 in this organ, but only 10 mice expressed the gene for mI-1 (Fig. 1). The mouse insulin genes were not expressed in the two mice that had ectopic PDX-1 in the spleen.

Rodents have two non-allelic insulin genes, and in mice they are located on chromosomes 6 and 7 (ref. 13). Expression of endogenous mI-1 could be more stringently protected than that of mI-2 by chromatin structure and/or DNA methylation[14,15]. Alternatively, mI-1 promoter activation could be affected differently by the identity or the levels of transcription factors present in liver cells expressing PDX-1. To distinguish between these two possibilities, we co-delivered the rat insulin 1 promoter (RIP-1), which is closely related to the mI-1 promoter[13], with PDX-1 using *AdRIP–1hIns*, a recombinant adenovirus containing human insulin cDNA (hIns, reporter) under the control of RIP-1. In livers in which PDX-1 induced only the expression of the gene for endogenous mI-2 but not mI-1, it activated RIP-1 (Fig. 1). This indicates that different levels of DNA methylation or distinct chromatin structures could be the cause of the low efficiency of activation of expression of endogenous mI-1 by expression of PDX-1 in liver. The expression of mouse or human

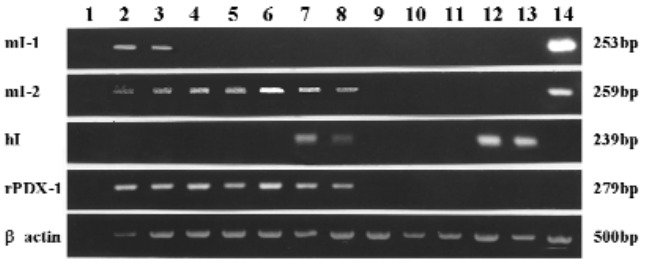


**Fig. 1** RT–PCR analysis of mRNA for mI-1, mI-2, human insulin, PDX-1 and β-actin. BALB/c mice were treated with adenovirus and 1 week later were killed and analyzed for gene expression in livers from mice treated with: lanes 2–6, *AdCMV–PDX-1* ($n = 35$; mI-2 positive in 25 and mI-1 positive in 10); lanes 7 and 8, *AdCMV–PDX-1 + AdRIP–1-hIns* ($n = 4$; human insulin and mI-2 positive in 3); lanes 9–11,*AdCMV–β-gal + AdRIP-1–hIns* (control; $n = 20$); lanes 12 and 13, *AdCMV–hIns* ($n = 20$; human insulin positive in all). Lane 1, no DNA (negative control for PCR); lane 14: normal mouse pancreas (PDX-1 is not detected in pancreas as the primers used for the reaction identify the rat but not the mouse homolog). hI, human insulin; rPDX-1, ectopic rat homolog of PDX-1 (STF-1). Right margin, molecular sizes in base pairs (bp).

**Table 1** Blood glucose and immunoreactive insulin levels in serum and liver extracts

| | Mice treated with control virus | mice treated with *AdCMV–PDX-1* |
|---|---|---|
| Blood glucose (mg/dl) | 228 ± 15.74 ($n$ = 18) | 197 ± 11.2 ($n$ = 40) |
| Serum IRI (μU/ml) | 118.2 ± 23.7 ($n$ = 14) | 323 ± 48.4 ($n$ = 26) |
| Liver extracts IRI (μU/mg protein) | 0.78 ± 0.25 ($n$ = 10) | 20.7 ± 3.9 ($n$ = 12) |
| Pancreas extracts IRI (μU/mg protein) | 2627 ± 24 ($n$ = 6) | |

IRI, immunoreactive insulin. Statistical analysis used Sigma-Stat software, using two-way ANOVA and the Mann-Witney rank sum test. Blood glucose was significantly lower in mice treated with PDX-1 than in mice treated with control ($P$ = 0.0098). Serum immunoreactive insulin was significantly higher in mice treated with PDX than in mice treated with control ($P$ = 0.0023). The immunoreactive insulin content of mice treated with PDX-1 was significantly higher than that of mice treated with control ($P$ < 0.05; Kruskal-Wallis one way analysis of variance on ranks, Dunn's method).

insulin genes was not affected by treatment with the same concentration of the control recombinant adenoviruses *AdCMV–β-gal* or *AdCMV–β-gal* and *AdRIP–1hIns* ($n$ = 20). These results show that expression of PDX-1 is essential and sufficient to induce expression of the endogenous insulin genes and to activate RIP-1 in tissue outside of the pancreas.

To determine whether hepatic insulin mRNA was effectively translated into protein, we measured by radioimmunoassay the content of immunoreactive insulin in extracts derived from hepatic tissue. Livers from mice treated with PDX-1 that showed expression of the insulin gene by RT–PCR (Fig. 1) contained about 2,500% more immunoreactive insulin than did livers of mice treated with a control virus (Table 1). The mean immunoreactive insulin level in extracts from livers of mice treated with PDX-1 was 20.7 ± 3.97 μU/mg protein, whereas in control livers (from mice treated with the same number of *AdCMV–β-gal* recombinant adenoviruses), the level of immunoreactive insulin was only 0.78 ± 0.25 μU/mg protein. Although immunoreactive insulin detected in liver extracts from mice treated with PDX-1 was less than 1% of that in pancreatic extracts (Table 1), serum immunoreactive insulin levels in mice treated with PDX were almost 300% higher than in control mice (323 ± 48.4 μU /ml and 118.2 ± 23.7 μU /ml, respectively; Table 1), indicating that insulin was being synthesized and that a large portion of it was secreted into the blood stream. Mice treated with *AdCMV–PDX-1* were not hypoglycemic, but their blood glucose level was slightly lower than that of mice treated with *AdCMV–β-gal* (Table 1). As we used mice with intact pancreatic islets (Table 1), we did not expect extreme alterations in blood glucose levels. However, the normoglycemia associated with a substantial increase of serum immunoreactive insulin levels indicates that the hormone produced and secreted from the liver may be partially unprocessed.

We next determined whether the liver was able to retain the insulin induced by PDX-1. Although treating mice with *AdCMV–hIns* adenovirus (human proinsulin cDNA driven by the CMV promoter) resulted in a substantial increase in plasma levels of immunoreactive insulin, it did not result in positive immunostaining for insulin in liver (H.C. and S.F., data not shown). Immunohistochemical analysis of liver sections from mice treated with PDX-1 showed expression of the homeoprotein in 30–60% of hepatocyte nuclei (depending on the efficiency of systemic viral administration; Fig. 2*a*), with 0.1–1% of the liver cells staining positive for insulin or proinsulin (Fig. 2*b*). Control livers from mice treated with *AdCMV–β-gal* did not stain positive for insulin or proinsulin (Fig. 2*d*), although β-galactosidase activity was evident in 50% of the nuclei (the *AdCMV–β-gal* construct has a nuclear localization signal for β-galactosidase; Fig. 2*e*). The fact that the ectopic expression of PDX-1 but not of insulin resulted in positive immunostaining for insulin or proinsulin may indicate the induction of a cellular modification that supports insulin retention in a small subpopulation of liver cells, which may have shifted towards a β-cell phenotype.

High-performance liquid chromatography analysis of mice treated with PDX-1 showed that the immunoreactive insulin content of the liver was 59 ± 7% ($n$ = 3) fully processed mI-1 and mI-2. In comparison, pancreatic extracts contained 85 ± 5% ($n$ = 3) mature insulin (Fig. 3*a*). Ectopic expression of human insulin (*AdCMV–hIns*) did not result in retention of immunoreactive insulin in the liver cells except for one mouse liver in which most of the extracted immunoreactive insulin was immature insulin. This is in agreement with observations in insulin-gene-transfected FAO hepatoma cell line in which the proinsulin gene product was secreted by the constitutive secretory pathway[16]. Moreover, only livers from mice treated with PDX-1 showed induction of expression of prohormone convertase 1/3 (PC1/3), a Kexin family protease whose expression is restricted to endocrine and neuroendocrine cells with regulated secretory pathway[16,17] (Fig. 3*b*).

To determine whether hepatic insulin production induced by PDX-1 was capable of controlling blood glucose levels in diabetic mice, we injected 220 mg/kg streptozotocin into C57BL/6 mice, which produced hyperglycemia (more than 600 mg/dl glucose) and ketonurea 24 hours later. We treated mice systemically, 24–48 hours after the injection of streptozotocin, with either *AdCMV–PDX-1* or *AdCMV–β-gal* (control) recombinant adenoviruses. In control mice treated with *AdCMV–β-gal*, hyper-

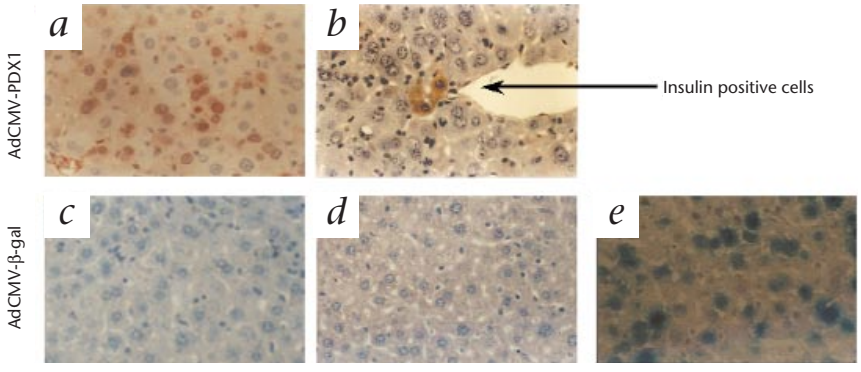


**Fig. 2** Immunohistochemical staining of livers. Mice were treated *in vivo* with *AdCMV–PDX-1* (top row) or *AdCMV–β-gal* (control containing a nuclear localization signal; bottom row), and protein was detected in liver sections by immunostaining for PDX-1 (***a*** and ***c***) or human insulin (***b*** and ***d***). Arrow (*b*), a cluster of three cells positive for insulin in the cytoplasm. ***e***, β-galactosidase activity in the cryopreserved liver of a control mouse treated with *AdCMV–β-gal*.

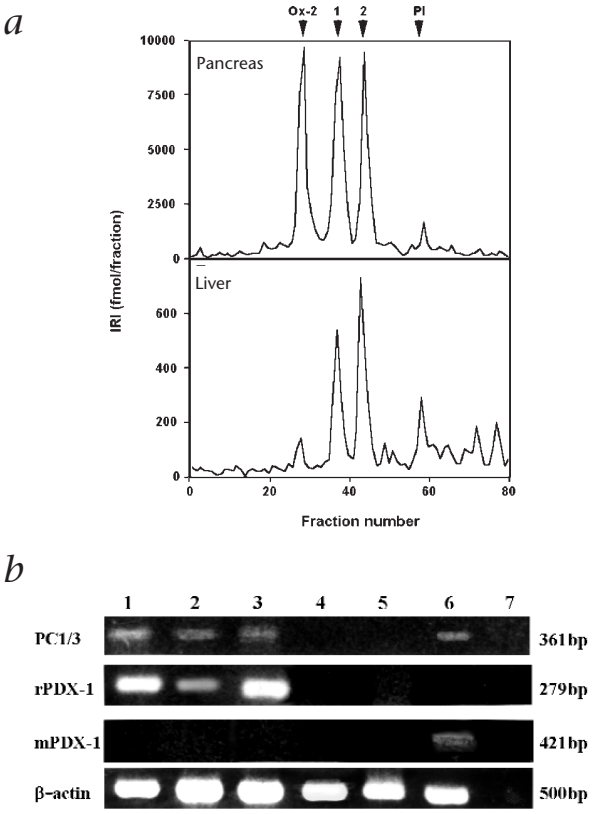


**Fig. 3** Liver from PDX-1 treated mice contain processed insulin and express the prohormone convertase PC1/3. ***a***, High-performance liquid chromatography elution profiles of insulin-related peptides extracted from mouse pancreas (top) and from the liver of a mouse treated with PDX-1 (bottom). Downward arrows, positions of insulin-related peptides: PI, mouse proinsulins; 1, mouse insulin 1; 2, mouse insulin 2; Ox-2, the oxidized form of mouse insulin 2. Proinsulin conversion intermediates, identified by pulse–chase experiments[25], are eluted at fractions 46–51 and 69–76. ***b***, RT–PCR analysis of mRNA for PDX-1, proinsulin convertase (PC1/3) and β-actin. Total RNA was extracted from mice treated with PDX-1 and control mice, and was reverse-transcribed using a PC1/3-specific primer. Lanes 1–3, mice treated with *AdCMV–PDX-1*; lanes 4 and 5, mice treated with *AdCMV–β-gal*; lane 6, pancreas; lane 7, no cDNA (control for PCR). The primers designed to detect the ectopic rat homolog of PDX-1 (rPDX-1 or STF-1) do not detect the mouse homolog (mPDX-1 or IPF-1). Right margin, molecular sizes in base pairs (bp).

glycemia persisted and was associated with ketonuria, resulting in an increased rate of mortality. Of 22 control treated diabetic mice, 12 died 2–3 days after receiving *AdCMV–β-gal*, and none survived beyond 8 days after receiving streptozotocin. In contrast, all mice treated with *AdCMV–PDX-1* survived for the duration of the experiment. Moreover, they showed a gradual decrease in blood glucose levels starting 2 days after treatment with recombinant adenovirus, from 600 mg/dl to about 200 mg/dl 1 week after receiving virus (Fig. 4).

Our data have shown that expression of PDX-1 is sufficient to induce mature, biologically active insulin production in liver. Although the insulin content in livers of mice treated with PDX-1 was much lower than that of pancreata, there was an increase of 300% in serum immunoreactive insulin levels. The capacity of hepatic insulin induced by PDX-1 to lower blood glucose levels in mice with streptozotocin-induced diabetes could have resulted from combined effects: In addition to the induction of production and secretion of insulin, ectopic expression of PDX-1 in liver could also increase hepatic glucose clearance by increasing expression and activity of glucokinase, directly or by the locally produced insulin[18].

In pancreatic islets, PDX-1 functions in concert with additional transcription factors in regulating expression of insulin and additional islet-specific genes[1,4–6]. Naturally occurring transcription factors in the liver, ubiquitous as well as tissue-specific, may act in concert with the ectopic PDX-1 to induce and regulate insulin gene expression in this organ. Indeed, pancreatic and liver tissues share common expression of several transcription factors such as HNF1α, CEPB/β and E47 (refs. 19,20). Alternatively, PDX-1 may promote expression of additional β cell-specific transcription factors in liver, similar to the induction of muscle-specific transcription factors by ectopic expression of MyoD in non-muscle tissue[21], although this remains to be demonstrated.

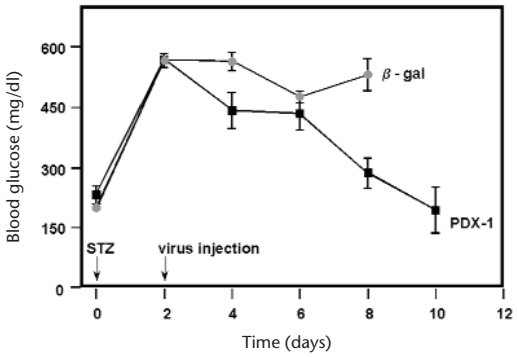


**Fig. 4** Ectopic expression of PDX-1 in mice livers ameliorates streptozotocin-induced hyperglycemia. Male C57BL/6 mice were treated with 220 mg/kg streptozotocin (STZ) and 36–48 h later were injected with *AdCMV–PDX-1* ($n$ = 15 mice) or *AdCMV–β-gal* (control; $n$ = 22) Glucose levels were determined in blood samples drawn from the ocular vein.

It is unknown at present which sub-population of liver cells supports the developmental shift induced by ectopic expression of PDX-1. *In vitro* transduction of primary culture of hepatocytes (which consists of 98% mature hepatocytes) did not result in induction of the endogenous insulin genes (Y.E., S. Brill and S.F., unpublished data). This may indicate that the transdifferentiation process induced by ectopic expression of PDX-1 could have occurred in a pluripotent population of progenitor liver cells present *in vivo* but not in *in vitro* culture of mature hepatocytes. Ectopic expression of PDX-1 in the intestines of transgenic mice leads to cecal agenesis but does not activate the endogenous insulin gene expression[22]. Liver, in contrast to intestine, has a high regenerative capacity and a relatively high percentage of functional progenitor cells that can be further activated by cellular injury. These cells are pluripotent and retain the ability to differentiate into hepatocytes or biliary, intestinal or pancreatic acinar epithelium[23,24]. Recombinant adenovirus infection could be recognized by the liver (*in vivo*) as a 'cellular assault', leading to an increase in the number of progenitor cells[24]. In turn, these cells could be more 'permissive' than mature hepatocytes or cells of the digestive tract to a developmental shift mediated by PDX-1. However, viral infection itself could not be responsible for the developmental modulation, as insulin gene expression was not apparent after treatment with control *AdCMV–β-gal* or *AdCMV–hIns*.

We can rule out the possibility that PDX-1 induced expression of insulin in progenitor ductal cells in the pancreas, or in any of the endocrine cell populations (β, α or δ) in pancreatic islets. We analyzed pancreas, kidney and spleen for ectopic expression of rat PDX-1 (our rat homolog primers do not amplify the mouse homolog of PDX-1; Figs. 1 and 3). Although there was ectopic expression of PDX-1 in spleen in two mice, it was low and was not accompanied by expression of endogenous mouse insulin genes. Rat PDX-1 systemically delivered by recombinant adenoviruses was never detected in RNA extracts from mouse pancreatic tissue. Moreover, even direct injection of recombinant adenovirus into pancreatic tissue *in vivo* (*AdCMV–β-gal*) resulted only in massive β-gal activity in acinar but not endocrine tissue (D. Castel, R.S. and S.F., unpublished results).

β-cell destruction and/or insufficient insulin production are the hallmarks of diabetes mellitus. Pancreas transplantation or islet-cell implantation efficiently restores normoglycemia but require life-long immunosuppressive therapy and are limited by tissue supply. Our study presents a new approach for extending the β-cell phenotype to additional 'self' tissues, which may compensate for the inadequate β-cell function, and thereby circumvent the need for transplantation and immunosuppression.

## Methods

**Recombinant adenoviruses.** *AdCMV–PDX-1* was constructed as described before[12]. It contains STF-1 cDNA, the rat homolog of PDX-1, ligated into the *Bam*H1 site of pACCMVpLpA vector. *AdCMV–β-gal* (contributed by C.B. Newgard) contains a nuclear localization signal for β-galactosidase. *AdCMV–hIns* contains human insulin cDNA under the control of a heterologous cytomegalovirus promoter. *AdRIP-1-hIns* contains human insulin cDNA under the control of RIP-1 (410 bases of the 5' flanking DNA region of the rat insulin-1 gene; a gift from L.J. Moss and C.B. Newgard).

**Animals and recombinant adenoviruses.** Mice 25–28 g in body weight were housed in an air-conditioned environment, with a 12-hour light–dark cycle, and were fed a regular unrestricted diet. Mice were treated with $2 \times 10^9$ plaque-forming units of recombinant adenovirus by systemic injection into the tail vein and were killed 1 week later. Blood was drawn from the inferior vena cava for the determination of glucose concentration (Accutrend® GC; Boehringer) and insulin levels by radioimmunoassay (Coat-a-count; DPC, Los Angeles, California) using rat insulin standards (Linco, St. Charles, Missouri); the antibody against insulin has only 60% cross-reactivity with human proinsulin. Liver and additional tissues for RNA isolation and determination of gene expression by RT–PCR were immediately frozen in liquid nitrogen and stored at –70 °C. Additional samples from the same tissues were fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical staining. Two-thirds of the liver and pancreatic tissues were homogenized in 70% ethanol and 0.18 N HCl, lyophilized, and resuspended either in phosphate-buffered saline for radioimmunoassay determination of immunoreactive insulin content, or in 0.1 M HCl and 0.1% BSA for high-performance liquid chromatography analysis.

**Induction of hyperglycemia by streptozotocin and treatment with recominant adenovirus**. C57BL/6 male mice 12–13 weeks old were treated with 220 mg/kg streptozotocin in citrate buffer. Then, 36–48 h later, mice were injected with *AdCMV–PDX-1* ($n$ = 15) or *AdCMV–β-gal* (control; $n$ = 22). None of the hyperglycemic mice treated with *AdCMV–β-gal* (control) survived longer than 8 d after treatment with streptozotocin. No mortality occurred in the PDX-1 treated group of hyperglycemic mice and they were killed 10 d after treatement with streptozotocin. Glucose levels were determined in blood samples drawn from the ocular vein.

**High-performance liquid chromatography analysis of insulin-related peptides.** Insulin-related peptides from liver and pancreatic extracts were resolved by reverse-phase high-performance liquid chromatography using a LiChrospher™ 100 RP-18 column (Merck, Darmstadt, Germany) and elution conditions as described[25]. Fractions 1 ml in volume were collected into tubes containing 0.1 ml 0.1% BSA in water, dried in a Speed-Vac apparatus and reconstituted in 1 ml radioimmunoassay buffer (0.1% BSA in phosphate-buffered saline) for peptide determination by radioimmunoassay. Guinea pig antibodies against porcine insulin (Linco, St. Charles, Missouri), with either rat or human insulin standards, were used for determination of mouse or human immunoreactive insulin, respectively[25].

**RT–PCR analysis for determination of gene expression induced by PDX-1.** Total RNA was isolated from frozen tissues using RNAzol B (Tel-Test, Friendswood, Texas). RNA samples were treated with 10 U DNase I (Promega). cDNA was prepared by reverse transcription, using 1 μg DNA-free total RNA and 0.5μg oligo$(dT)_{15}$. Prohormone convertase 1/3 (PC1/3) cDNA was reverse-transcribed using a gene-specific oligonucleotide (5'–TCCAGGTGCCTACAGGATTCTCT–3') instead of oligo $(dT)_{15}$. Of the total RT reaction volume, 1.5 μl was amplified using primers and PCR conditions as described: All samples were denatured at 94 °C for 60 s; primers were annealed for rat PDX-1 (STF-1) and the insulin cDNAs at 62 °C for 60 s and for mouse β-actin, prohormone convertase 1/3 (PC1/3) and PDX-1 (IPF-1) at 56, 55 and 58 °C, respectively, for 45 s; and 31–38 cycles were used for amplification. Primer sequences for all cDNAs analyzed are available on request. PCR used a GeneAmp PCR system 2400 (Cetus–Perkin Elmer, Foster City, California), and products were separated by 1.7% agarose gel electrophporesis.

A separate PCR reaction was done for each RNA sample without reverse transcriptase, to ensure that the amplified product was not due to DNA contamination. The primers designed to detect the ectopic rat PDX-1 (STF-1) expression do not amplify the mouse homolog, and the primers for mI-2 amplification are located on different exons.

**PDX-1 and insulin immunohistochemistry in mice treated with *AdCMV–PDX-1* or *AdCMV–β-gal*.** Sections of paraffin-embedded tissues 5 μm in thickness were deparaffinized, incubated in 3% $H_2O_2$, and then were either microwaved in citrate buffer for antigen retrieval before being incubated in blocking solution (PDX-1 detection) or immediately exposed to the blocking solution (insulin detection). The reagents are commercially available (Histomouse™-SP Kit; Zymed laboratories, South San Francisco, California). For detection of PDX-1, sections were incubated overnight at 4 °C with antiserum raised against the N-terminal portion of frog PDX-1 (a gift from C.V.E. Wright). For detection of insulin, sections were incubated for 1 h at 37 °C with a monoclonal antibody against human insulin (Sigma). Slides were exposed to the secondary biotinylated IgG for 30 min and then incubated in streptavidin–peroxidase followed by a chromogen peroxide solution.

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