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Functional, Persistent, and Extended Liver to Pancreas Transdifferentiation*

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Pancreatic and duodenal homeobox gene-1 (PDX-1) regulates pancreas development during embryogenesis, whereas in the adult it controls β-cell function. Here we analyze whether PDX-1 functions as a pancreatic differentiation factor and a *bona fide* master regulator when ectopically expressed in mature fully differentiated liver *in vivo*. By ectopic and transient *PDX-1* expression in liver *in vivo*, using the first generation recombinant adenoviruses, we demonstrate that PDX-1 induces in liver a wide repertoire of both exocrine and endocrine pancreatic gene expression. Moreover, PDX-1 induces its own expression (auto-induction), which in turn may explain the long lasting nature of the "liver to pancreas" transdifferentiation. Insulin as well glucagon-producing cells are mainly located in the proximity of hepatic central veins, possibly allowing direct hormone release into the bloodstream, without affecting normal hepatic function. Importantly, we demonstrate that hepatic insulin production triggered by *Ad-CMV-PDX-1* recombinant adenovirus administration is functional and prevents streptozotocin-induced hyperglycemia in Balb/c mice even 8 months after the initial treatment. We conclude that PDX-1 plays an important instructive role in pancreas differentiation, not only from primitive gut endoderm but also from mature liver. Transconversion of liver to pancreas may serve as a novel approach for generating endocrine-pancreatic tissue that can replace malfunctioning β-cells in diabetics.

The possible role of "master regulator" transcription factors in directing cell fate, taken together with the documented plasticity of many mature tissues and pluripotent cells, motivated us to analyze whether it is possible to redirect the developmental route of cells in liver toward a pancreatic β-cell phenotype. The strategy used for this purpose is ectopic expression of a specific pancreatic master regulator gene. Our hypothesis was that such a developmental redirection was most likely to occur between tissues that are developmentally related, such as liver and pancreas (1–4). Transdifferentiation of pancreas to liver has been described in both experimental models and human pathology (5–10).

Pancreatic and duodenal homeobox gene-1 (PDX-1,[1] also known as IDX-1, IPF-1, STF-1, or IUF-1) possesses a dual role in the pancreas; it is essential for normal pancreas development during embryogenesis and for maintenance of β-cell function in the adult (11, 12). During organogenesis *PDX-1* is expressed in all cells differentiating toward the exocrine and endocrine components of the pancreas (11–13). In the adult, its expression is restricted to β-cells and to 20% of somatostatin producing δ-cells. *PDX-1* is neither expressed in glucagon producing α-cells nor in mature exocrine pancreas (11, 12, 14).

We demonstrated previously that transdifferentiation of liver to pancreas could be induced by ectopic expression of *PDX-1*; expression of *PDX-1* in liver induced expression of the otherwise silent endogenous insulin genes and was sufficient to direct the production and secretion of mature, biologically active insulin from a restricted population of cells in the liver *in vivo*. Furthermore, insulin secreted from liver of PDX-1-treated mice ameliorated streptozotocin (STZ)-induced diabetes (1).

In the present study *PDX-1* cDNA is delivered *in vivo* by systemic administration of recombinant adenoviruses that carry the rat *PDX-1* gene under the cytomegalovirus promoter (*Ad-CMV-PDX-1*). This gene delivery system results in efficient transgene expression, mainly in the liver (15). Because genes delivered *in vivo* by recombinant adenoviruses do not integrate into the host genome, ectopic expression is transient, lasting from days to a few weeks (15–17). Therefore, our previous study monitored the effects of the ectopic *PDX-1* over a short period (up to 2 weeks), to parallel the predicted time frame of the *PDX-1* transgene expression (1).

In order to determine whether PDX-1 fulfills the role of a differentiation factor in mature hepatic tissues, we sought to determine whether it induces, in addition to insulin, a broad repertoire of pancreatic markers, especially these that are not considered its immediate targets. Moreover, if indeed PDX-1 acts as a differentiation factor also in mature liver and not only in the primitive gut endoderm, then it may trigger a cascade of events that do not require continuous expression of the transgene. Therefore, we sought to determine whether the transient ectopic PDX-1 induces a long lasting process of liver to pancreas transconversion. Moreover, *bona fide* master regulators such as MyoD have the capacity to activate their own expres-

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[1] The abbreviations used are: PDX-1, pancreatic and duodenal homeobox gene-1; *Ad-CMV-PDX-1,* replication-deficient recombinant adenovirus that carries expression of PDX-1 cDNA under the control of the cytomegalovirus promoter; STZ, streptozotocin; RT, reverse transcriptase; RIA, radioimmunoassay.

TABLE I
Primer sequences and PCR conditions
Denaturation was at 94 °C for 1 min; annealing was as presented in the table and extension was at 72 °C for 1 min.

Gene	Primer sequences	CDNA	Product	Annealing		Cycles
				°C	s	
		μl	*bp*			
β-Actin	F, ATGGATGACGATATCGCT R, ATGAGGTAGTCTGTCAGGT	1	570	56	45	35
Rat PDX-1[a]	F, CCAAAACCGTCGCATGAAGTG R, CAGCTCGCCTGGTGGCTGT	1	628	60	60	35
Mouse PDX-1[b]	F, CCTTCGGGCCTTAGCGTGTC R, CGCCTGCTGGTCCGTATTG	3	396	59	90	38
Insulin I	F, TTGCCCTCTGGGAGCCCAAA R, CAGATGCTGGTGCAGCACTG	1	253	62.6	60	38
Insulin II	F, TCTTCCTCTGGGAGTCCCAC R, CAGATGCTGGTGCAGCACTG	1	259	62.6	60	36
Somatostatin	F, CAGACTCCGTCAGTTTCTGC R, ACAGGATGTGAAAGTCTTCCA	3	262	54	90	38
Glucagon	F, ATCATTCCCAGCTTCCCAGA R, CGGTTCCTCTTGGTGTTCAT	2	161	52	60	38
IAPP	F, CCACTTGAGAGCTACACCTG R, GGATTCCCTATTTGGATCC	2	205	54	60	37
Ad-CMV-PDX-1	F, CTCAATGGGAGTTTGTTTTGG R, GGGGGATTAGCACTGAACTCT	1 (DNA)	569	58	60	26
Elastase	F, GGGCACAAACAGACCATCAC R, GGGATGGGTAAGAAGGTGGT	2	298	55	60	38
p48	F, GAAGGTTATCATCTGCCATCG R, GGGTGGTTCGTTCTCTATGTT	3	211	54	60	38

[a] Specific primer to rat PDX-1, no recognition of mouse PDX-1.
[b] Specific primer to mouse PDX-1, no recognition of rat PDX-1.

sion (18). This has been proven also for *PDX-1* but only in pancreatic β-cells (19, 20). Because additional transcription factors such as hepatic nuclear factor 1α and 3β that are known to control *PDX-1* expression in pancreatic β-cells are also expressed in liver and could cooperate with the *PDX-1* transgene, we sought to analyze whether the endogenous otherwise silent homologue of *PDX-1* is induced also in liver in response to the transgene expression, thus in turn directing a long lasting process of liver to pancreas transdifferentiation, also when the transgene is no longer expressed.

The present study analyzes PDX-1 capacity to function as a pancreatic differentiation factor in mature liver and investigates part of the mechanism of liver to pancreas transconversion. Moreover, it examines whether the PDX-1-induced liver to pancreas transdifferentiation process carries a therapeutic benefit long after the initial ectopic *PDX-1* gene expression decays.

MATERIALS AND METHODS

Recombinant Adenoviruses—Ad-CMV-PDX-1 was constructed as described previously (1, 21). It contains the STF-1 cDNA, the rat homologue of *PDX-1* ligated into *Bam*HI site of *pACCMVpLpA* vector. *Ad-CMV-β-galactosidase* (generously contributed by C. B. Newgard) serves as a control for nonspecific effect of adenovirus-mediated gene delivery.

Cell Culture—The mouse pancreatic derived cell lines β-TC-1 and α-TC-1 and the rat pancreatic cell line RIN1046-38 were cultured according to conditions published previously (21, 22).

Animals and Recombinant Adenoviruses—Mice were housed in an air-conditioned environment, under a 12-h light/dark cycle, and handled according to institutional animal welfare regulations. 8–9-week-old (18–19 g) BALB/c mice were treated by 1–5 × 10^{10} plaque-forming units of the indicated recombinant adenoviruses by systemic injection into the tail vein (in a volume of 200–300 μl of physiological saline). Blood was drawn from the tail, for determination of glucose concentration (Accutrend® GC, Roche Applied Science). Liver was harvested for immunohistochemical staining (fixed in 4% formaldehyde and embedded in paraffin), for analysis of gene expression (total RNA), and for determination of pancreatic hormone content in liver. For the last two analyses, hepatic specimens were immediately frozen in liquid nitrogen and stored at −70 °C.

RNA Isolation and RT-PCR Analysis—Total RNA was isolated from frozen tissues using Tri-Reagent (Molecular Research Center). RNA samples were treated by 10 units of RQ1 RNase-free DNase I (Promega) for 60 min. cDNA was prepared by reverse transcription (native avian myeloblastosis virus reverse transcriptase, Chimerx) by using 4 μg of DNA-free total RNA and 0.5 μg of oligo$(dT)_{15}$. PCR was performed using T3 Thermocycler (Biometra, Göttingen, Germany), and products were separated on 1.8% agarose gels and visualized with ethidium bromide. The sequence of the primers used for PCR and reactions conditions were as listed in Table I. Note that in order to discriminate between expression of the endogenous mouse *PDX-1* and the ectopic rat homologue, two sets of specific oligonucleotide primers were designed (see Table I). RNA isolated from the β-TC-1 cell line was used as a control for insulin and somatostatin genes expression, and the α-TC-1 cell line was used for glucagon.

Quantitative Analysis for Pancreatic Gene Expression Using Real Time PCR—RT-PCR was performed on the LightCycler (Roche Applied Science) using SYBR-Green I dye.

Amplification conditions included initial denaturation at 95 °C for 10 min, followed by 55 cycles for both mouse and rat *PDX-1*, or 30 cycles for β-actin. For both *PDX-1* homologues, each cycle included denaturation at 95 °C for 15 s, annealing at 59 °C, and extension at 72 °C for 15 s. Annealing for *β-actin* was performed at 56 °C for 10 s. The fluorescent signal was monitored 5 s after each cycle at 90 °C for *β-actin*, 87 °C for mouse *PDX-1*, and 88 °C for rat *PDX-1*. Melting curve program was carried out at 68 °C for 40 s to analyze the specificity of the generated products.

The RT-PCR for mouse *PDX-1* was performed 3 times and for rat PDX-1 2 times. Both rat and mouse *PDX-1* levels were normalized to the respective β-actin mRNA levels in the same samples.

Pancreatic Hormones Analysis by Immunohistochemistry—Slides of 4-μm paraffin-embedded sections were deparaffinized and incubated in 3% H_2O_2 and in blocking solution (for both insulin and glucagon detection), using the commercially available Histomouse™-SP Kit (Zymed Laboratories Inc., South San Francisco, CA). Sections were then incubated for 1 h at 37 °C with monoclonal antibodies against human insulin and against human glucagon (Sigma), both at a dilution of 1:200. Because both insulin and glucagon are detected within the same tissue, at the same time using the same dilution of distinct monoclonal antibodies, they are considered extraneous to each other. Slides were exposed to the secondary biotinylated IgG for 30 min at room temperature and then incubated in streptavidin-peroxidase followed by a chromogen peroxide solution. A control using only secondary antibody without primary antibodies followed by streptavidin-peroxidase and a chromogen peroxide solution was performed to rule out possible background of the system.

Radioimmunoassay (RIA) of Pancreatic Hormones—Frozen tissues

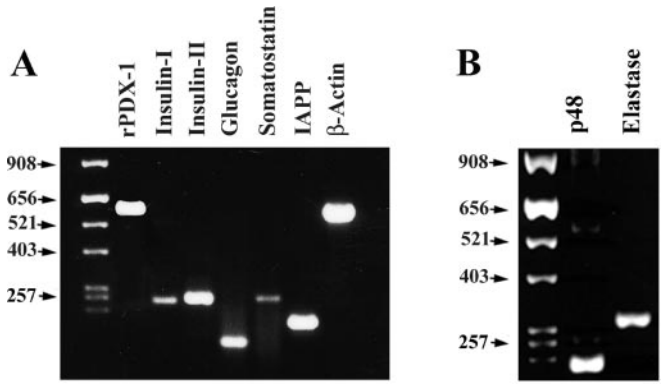

FIG. 1. **Ectopic *PDX-1* expression induces both endocrine (*A*) and exocrine (*B*) repertoire of pancreatic gene expression in mature liver *in vivo*.** Total RNA was extracted from livers of mice treated by *AdCMV-PDX-1*. The RNA was reverse-transcribed, and RT-PCR was performed to detect the expression of genes characteristic to endocrine (*A*) and exocrine (*B*) pancreas. The exact conditions for each analyzed gene expression are detailed in Table I. A representative analysis for each gene expression is demonstrated, with similar results in $n = 35–40$ for endocrine (*A*) and $n = 20$ for exocrine (*B*) in PDX-1-treated mice, and $n = 25–30$ in control treated mice (data not presented for controls that were mostly negative for the analyzed genes, see text). *IAPP,* islet amyloid polypeptide.

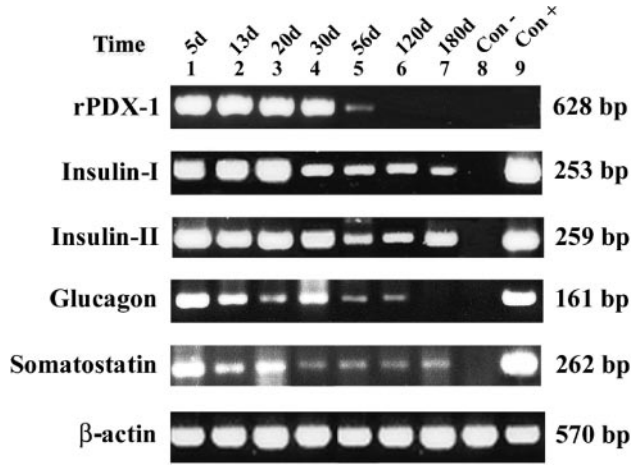

FIG. 2. **PDX-1 induces long lasting expression of insulin, glucagon, and somatostatin genes in liver.** RT-PCR analysis of pancreatic gene expression in PDX-1-treated livers. Pancreatic gene expression was analyzed at different time points after *Ad-CMV-PDX-1* recombinant adenovirus administration (*lanes 1–7*). RT-PCR was performed to detect *rPDX-1* (rat, ectopic *PDX-1*), *insulin I*, *insulin II*, glucagon, somatostatin, and β-actin gene expression. The mouse insulinoma cell lines β-TC-1 and α-TC-1 cDNAs were used as a positive control for insulin and glucagon, respectively (*lane 9*) (*rat PDX-1* is not detected in β-TC-1 cells as the primers used for the reaction identify the rat but not the mouse homologue), and *lane 8* is *Ad-CMV-β-g*alactosidase-treated liver (a representative control of $n = 30$, at various time points analyzed). *Right margin*, molecular size in bp. Number of mice analyzed at each indicated time point following systemic *Ad-CMV-PDX-1* administration: 5 days, $n = 6$; 13 days, $n = 4$; 20 days, $n = 4$; 30 days, $n = 5$; 56 days, $n = 3$; 120 days, $n = 7$; 180 days, $n = 4$.

were homogenized in 0.18 N HCl, 35% ethanol. The homogenates were extracted overnight at 4 °C with continuous stirring, and the supernatants were lyophilized. Samples were dissolved in 0.8 ml of RIA Assay Buffer, supplemented by a mixture of protease inhibitors (Sigma). Hepatic insulin and glucagon levels were determined using rat radioimmunoassay (RIA, catalog numbers SRI-13K and GL-32K, Linco, MO, and Coat-A-Count, Diagnostic Products Corp.). Somatostatin concentrations were determined by RIA (Euro-Diagnostica, Sweden). Hepatic content of pancreatic hormones was normalized to the wet weight of extracted tissues.

Analyses of Hepatic Function—Serum biochemistry profile consisting of albumin, aspartate aminotransferase, alanine aminotransferase, and total bilirubin and amylase levels was determined using Olympus AU 2700 Apparatus (Olympus, Germany) in serum samples.

Statistical Analysis—Statistical analysis was preformed using Two-sample Assuming Unequal Variances *t* test.

RESULTS

Both Pancreatic Endocrine and Exocrine Markers Are Induced in Liver by Ectopic PDX-1 Expression—Fig. 1 shows that ectopic *PDX-1* expression in mature liver *in vivo* activates a wide repertoire of pancreatic genes. Both endocrine and exocrine markers including the exocrine pancreas transcription factor p48 were uniquely expressed in response to ectopic *PDX-1* expression in liver (Fig. 1). Control treated mice were mostly negative to pancreatic gene expression. Although insulin gene expression was induced in close to 100% of mice treated by ectopic *PDX-1*, it was expressed at very low levels that were not translated into protein also in 20–30% of the control treated mice.

In the developing pancreas PDX-1 serves as an early molecular marker that temporally correlates with pancreatic commitment (13, 23, 24). Our data suggest that PDX-1 recapitulates to some extent its role in pancreas organogenesis, also in a mature fully differentiated tissue, such as liver.

PDX-1 Triggers a Long Lasting Process of Liver to Pancreas Developmental Shift—In order to explore the mechanism underlying the PDX-1-induced developmental shift, and to specifically determine whether cells in liver that ectopically express *PDX-1* exhibit several pancreatic developmental options that persist for long periods of time, we carefully followed insulin, *glucagon*, and *somatostatin* gene expression and protein production for 6 months after the initial, single adenovirus-mediated *PDX-1* administration to mice livers *in vivo*.

8–9-Week-old mice were treated by systemic injection of *Ad-CMV-PDX-1*, a recombinant adenovirus that carries the rat *PDX-1* gene (STF-1) under the control of the CMV promoter (1). Pancreatic gene expression in liver was analyzed in comparison to age-matched control mice (treated by either *Ad-CMV-β-galactosidase* or untreated).

Despite the expected transient *PDX-1* expression achieved by adenovirus-mediated delivery of the gene to liver, expression of insulin and somatostatin persisted for 6 months, at both the mRNA (Fig. 2) and protein levels (Fig. 4). Glucagon gene expression was evident during the first 4 months (Figs. 2 and 3). Importantly, insulin I and insulin II genes expression was evident in 80–100% of PDX-1-treated mice even 6–8 months after the initial PDX-1 treatment. The increase in *insulin* and *glucagon* expression in PDX-1 *versus* control treated mice livers was further confirmed using real time PCR (data not presented).

The temporal differences between insulin and glucagon gene expression could reflect a unique phenomenon that characterizes pancreas organogenesis in mature liver, and may suggest a more stable transconversion toward the β- and δ-cell phenotype. Glucagon gene is not a direct *PDX-1* target gene, and its persistent expression in liver suggests that PDX-1 is functioning as a differentiation factor in this organ.

Quantitative Analysis of Insulin, Glucagon, and Somatostatin Hormones Production in PDX-1-treated Livers as a Function of Time after Initial Ad-CMV-PDX-1 Administration—Our data demonstrate that the pancreatic genes expressed in liver as a consequence of ectopic *PDX-1* expression are successfully translated into the respective proteins.

Immunohistochemical analysis (Fig. 3) localizes the insulin-producing cells mainly in the proximity of central veins even 4–6 months after *PDX-1* ectopic gene delivery (Fig. 3, *A* and *C*). Although glucagon-positive cells are also localized in the proximity of central veins (Fig. 3*B*), immunohistochemical analysis of these two hormones performed on sequential slides suggest

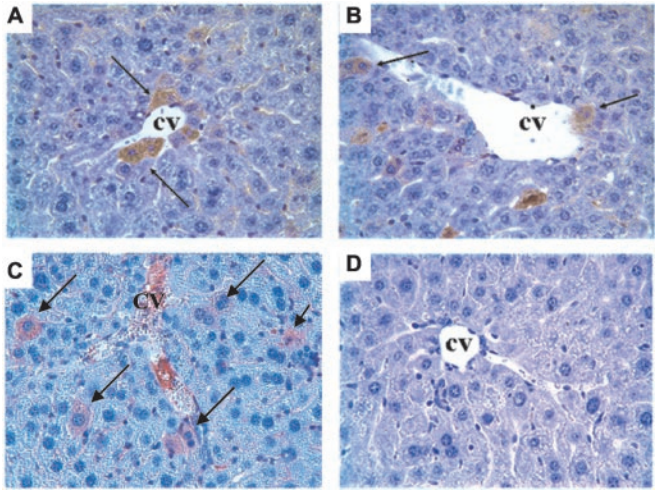

FIG. 3. **Insulin- and glucagon-positive cells are located in the proximity of central veins (*cv*) 4–6 months after treatment.** Immunohistochemical analysis of insulin (*A*), glucagon (*B*) 120 days, and insulin-positive cells (*C*) 180 days after *Ad-CMV-PDX-1* administration compared with control (*D*) using only secondary without primary antibodies followed by streptavidin-peroxidase. A chromogen peroxide solution was performed to rule out possible background of the system (*A* and *B* are sequential sections *upside down*). *Arrows* indicate positive cells, mostly located at the proximity of central veins (*cv*). Original magnification ×400.

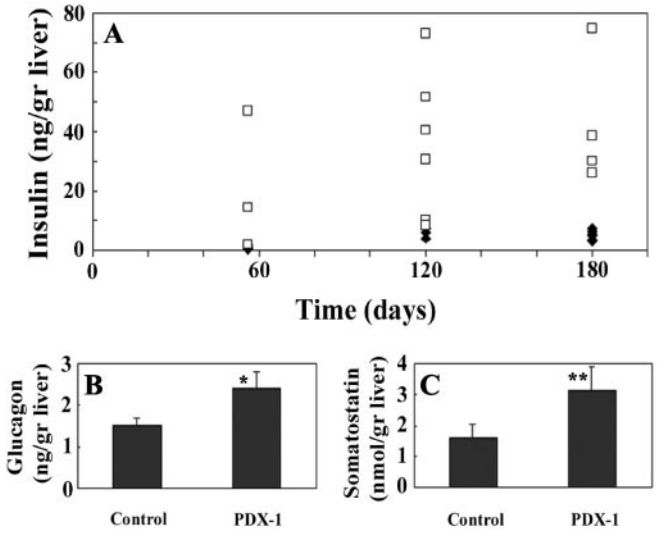

FIG. 4. **Quantitative analysis of hepatic content of pancreatic hormones in PDX-1-treated mice.** *A*, hepatic insulin content in individual mice as a function of time after systemic *Ad-CMV-PDX-1* administration: at 56 days (PDX-1-treated, $n = 3$, control, $n = 6$), 120 days (PDX-1-treated, $n = 7$, control, $n = 3$), and 180 days (PDX-1-treated, $n = 4$, control, $n = 5$). Hepatic immunoreactive insulin content in PDX-1-treated (□) or control (♦) mice are presented separately for each individual mouse. Hepatic glucagon content (PDX-1-treated, $n = 10$; control, $n = 10$) (*B*) and hepatic somatostatin (PDX-1-treated, $n = 9$; control, $n = 7$) content (*C*) are presented as mean ± S.E. for mice 2–4 months after recombinant adenovirus administration. Hormonal levels are determined by radioimmunoassay and normalized to the weight of the homogenized tissue (*, $p < 0.05$; **, $p < 0.06$).

that these hormones do not co-localize within the same cell. Liver cells present in areas close to the central vein in liver are known to correspond to mature cells (25).

Quantitative analysis of hepatic insulin stored in the liver of PDX-1-treated mice indicates that even 4–6 months after treatment, hepatic insulin content is about 30–75 ng/g tissue, compared with 1–9 ng/g tissue in age-matched control livers (Fig. 4*A*). Although this represents a substantial increase compared with control liver, it is about 1.3–3% of pancreatic content (51280 ng/g tissue), even after we take in consideration that liver is about 20-fold larger than pancreas. A significant 2-fold increase in hepatic pro/glucagon and somatostatin content was observed up to at least 4 months after initial *Ad-CMV-PDX-1* administration (Fig. 4, *B* and *C*).

Despite hepatic insulin production, serum insulin and glucose levels in PDX-1-treated mice bearing normal pancreatic function were normal throughout the duration of the experiment (insulin, 1.0 ± 0.5 *versus* 0.9 ± 0.4 ng/ml, and glucagon, 0.16 ± 0.08 *versus* 0.12 ± 0.05 ng/ml in PDX-1-treated compared with controls, respectively).

The persistent production of pancreatic hormones in liver suggests that ectopic PDX-1 triggers a cascade of events that may not require the continuous presence of the *PDX-1* transgene.

Ectopic PDX-1 Triggers the Expression of the Endogenous, Otherwise Silent PDX-1 Gene in Liver; a Possible Mechanism of Sustained Liver to Pancreas Transdifferentiation—In order to explain the sustained developmental shift in liver, triggered by the transient ectopic *PDX-1* expression, we analyzed whether the transgene induces the expression of otherwise silent pancreatic transcription factors, which in turn dictate the continuous expression of pancreatic genes. An obvious candidate is the endogenous *PDX-1* gene itself.

To analyze the induction of the endogenous and otherwise silent *PDX-1* gene in liver by the ectopic gene, we treated mice by systemic delivery of recombinant adenovirus that directs expression of the rat *PDX-1* homologue, and we used specific oligonucleotide primers to distinguish between the ectopic *PDX-1* transgene (rat) mRNA (cDNA) and the endogenous mouse mRNA, by RT-PCR.

PCR analysis of DNA samples isolated from liver of *Ad-CMV-PDX-1*-treated mice confirms that the virally encoded transgene disappears between 30 and 56 days after adenovirus injection (Fig. 5*A*).

Fig. 5*B* demonstrates that the ectopic rat *PDX-1* expression parallels the observed presence of delivered viral DNA in liver and also extinguishes after 1 month (Fig. 5*A*). The only homologue of *PDX-1* expressed in treated livers for the whole duration of the experiment is the endogenous and otherwise silent mouse homologue (Fig. 5*B*). Endogenous *PDX-1* expression was exclusive to mice that received the rat *PDX-1* transgene, was evident in 75% of ectopic PDX-1-treated mice (21 of 28 mice), and in none of the 25 control treated livers analyzed. By using real time PCR, we further analyzed the identity and quantities of the relative levels of mouse *versus* rat *PDX-1* gene expression in liver as a function of time after the initial treatment, using identical conditions (but different primers), and we normalized it to β-actin within the same samples.

As demonstrated in Fig. 5*C*, the mRNA encoding the ectopic rat *PDX-1* is maximal at 5 days, drops by 85% at day 30, and disappears thereafter. By contrast, the endogenous mouse *PDX-1* is expressed at substantial levels for the whole duration of the experiment. Unfortunately, due to the high level of homology between the two nuclear proteins, no specific antibody could distinguish between the rat and the mouse homologues of PDX-1, although it seems highly unlikely that the nuclear protein detected only in mouse livers 2–6 months after the initial adenovirus delivery corresponds to the ectopic transiently expressed transgene (data not presented). These data taken together suggest an auto-induction of the endogenous and otherwise silent *PDX-1* in liver, which in turn may suggest a mechanistic explanation for the long lasting mode of liver to pancreas transconversion process.

Insulin Produced in Liver in Response to PDX-1 Transgene Expression Is Functional and Prevents STZ-induced Hyperglycemia—In order to determine whether *PDX-1* gene delivery

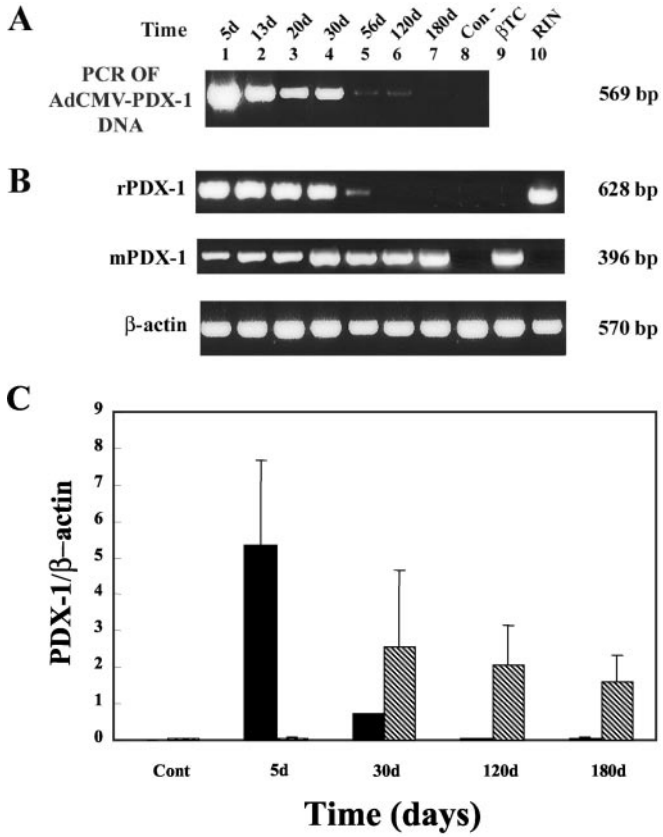

FIG. 5. **Ectopic rat PDX-1 delivered by *Ad-CMV-PDX-1* induces expression of the endogenous mouse *PDX-1* gene in liver.** *A*, PCR analysis of *Ad-CMV-PDX-1* DNA. DNA was extracted from mice livers at indicated time points and analyzed for the systemically delivered recombinant adenovirus infection, using specific primers (see Table I). Primers used for the PCR analysis were designed to amplify a DNA sequence that spans part of the CMV promoter and part of the PDX-1 cDNA and is therefore unique to the ectopically delivered transgene and the specific recombinant adenovirus, *Ad-CMV-PDX-1*. *B*, RT-PCR analysis of rat PDX-1 (*rPDX-1*), mouse PDX-1 (*mPDX-1*), and β-actin gene expression. Total RNA was extracted from livers of mice treated by *Ad-CMV-PDX-1* (*lanes 1–7*) or untreated (*lane 8*, representative sample of 25 controls analyzed and all tested negative to endogenous PDX-1). *β-TC* (*lane 9*) and RIN-38 (*lane 10*) are controls for mouse and rat PDX-1, respectively. *Right margin*, molecular sizes in bp. Both *A* and *B* demonstrate representative results out of at least four mice livers at each indicated time point. *C*, quantitation of ectopic (rat) *versus* endogenous (mouse) *PDX-1* expression as a function of time after initial *Ad-CMV-PDX-1* treatment using real time PCR. Mouse *PDX-1* (▨) and rat *PDX-1* (■) expression was analyzed at different time points by LightCycler. The calculation is performed relative to β-actin expression in each individual sample. Data presented are mean values ± S.E. of samples presented in *B*, each analyzed at least twice on LightCycler PCR.

induces long lasting production of biologically active insulin, we analyzed whether it provides protection against STZ-induced diabetes, even 8 months after the initial *Ad-CMV-PDX-1* administration. Mice were treated by 220 mg/kg STZ, and the incidences of hyperglycemia were compared with those in age-matched controls. Sixty percent of the control BALB/c mice developed hyperglycemia (6 of 10) within 3–5 days of STZ injection. By contrast, only 1 of 5 PDX-1-treated mice developed hyperglycemia in response to STZ treatment (20%). In order to analyze whether hepatic insulin production contributed to such a protection, we analyzed the hepatic and the pancreatic insulin content in PDX-1-treated and control mice.

Immunohistochemical studies and insulin content analyses revealed that in response to STZ treatment, pancreatic β-cells were mostly destroyed, and pancreatic insulin content in both control diabetic mice and in PDX-1-treated mice (that remained normoglycemic) dropped by 95 ± 1%. By contrast, hepatic insulin content in PDX-1-treated mice liver remained unaffected by the STZ treatment and was 40-fold increased compared with PDX-1-untreated mice. Fig. 6 demonstrates the ratio between hepatic and pancreatic insulin content upon STZ treatment in control *versus* PDX-1-treated mice. Whereas in both groups pancreatic insulin content was similar, and corresponded to 5% of normal pancreas, only mice treated by PDX-1 remained normoglycemic. Moreover, because hepatic insulin content remained unaffected by STZ, the relative contribution of hepatic insulin production increased to 25.6% that of STZ-treated pancreata (instead of 1.3–3% when compared with normal pancreas). Although these results demonstrate that the PDX-1-induced developmental shift is both long lasting and functional, they may also suggest that developmentally shifted cells in liver resist β-cell-specific toxins.

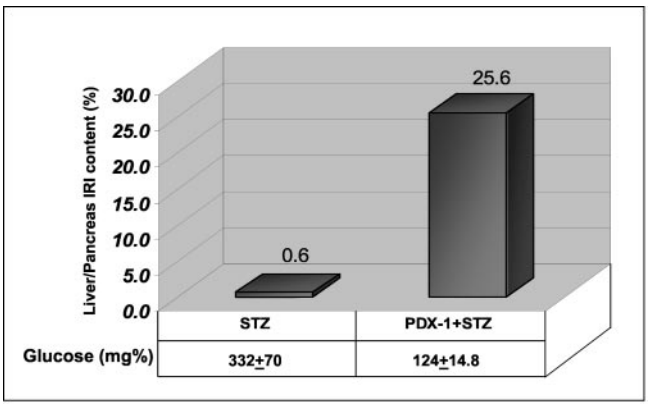

FIG. 6. **Hepatic insulin production protects mice from STZ-induced hyperglycemia 8 months after the initial *Ad-CMV-PDX-1* treatment.** Eight months after *Ad-CMV-PDX*-1 administration, BALB/c mice and age-matched control group were treated by 220 mg/kg STZ. In both groups pancreatic immunoreactive insulin (*IRI*) content dropped to 95 ± 1% that in normal pancreas. Four of the five PDX-1-treated mice remained normoglycemic (n = 4; 124 ± 14.8 mg % despite pancreas destruction) and 6 of 10 age-matched control mice developed hyperglycemia (n = 6; 332 ± 70 mg %). The figure demonstrates the ratio of hepatic to pancreatic insulin content as a function of PDX-1 treatment.

The profound biological activity in face of a relatively modest hepatic insulin production may suggest an apparent preferential efficacy of insulin produced in liver, which remains to be further investigated.

Importantly, despite the ongoing local insulin production in liver even 6–8 months after initial viral infection, hepatic functions were not perturbed and bilirubin levels remained unaltered (Table II). Transient alterations in hepatic functions occurred in response to adenovirus administration; however, hepatic function returned back to normal levels within 1–2 months. Moreover, serum amylase levels were not affected by ectopic *PDX-1* expression in liver, suggesting that the pancreatic exocrine function in liver was not induced (Table III). PDX-1-treated mice consistently gained weight with a slightly increased rate compared with that of age-matched controls (Fig. 7).

DISCUSSION

Our data demonstrate that PDX-1 recapitulates its role in pancreas organogenesis when ectopically expressed in liver. Thus, we show that *PDX-1* expression in liver induces a wide array of endocrine and exocrine pancreatic genes. PDX-1 induces its own expression in liver (auto-induction), which in turn explains the prolonged nature of the "liver to pancreas" transdifferentiation process embodied by pancreatic hormone production in liver of adenovirus-treated mice that persists

TABLE II
Hepatic function in Ad-CMV-PDX-1 treated mice

Blood biochemistry measurements (mean ± S.E.) of mice after PDX administration. ALB, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T.bil, total bilirubin.

	ALB	AST	ALT	T. bil
	gr/dl	*IU/liter*	*IU/liter*	*mg/dl*
Control (10)	2.5 ± 0.05	82 ± 5.75	36.6 ± 3.2	0.1
PDX 5 days (5)	2.5[a]	121[a]	102[a]	0.1[a]
PDX 20 days (5)	2.5 ± 0.05	111 ± 21	73 ± 10	0.1
PDX 60 days (12)	2.5 ± 0.05	101 ± 31	53.6 ± 13.6	0.1
PDX 120 days (3)	2.5	81.6 ± 29	29 ± 2	0.1
PDX 180 days (3)	2.5 ± 0.05	61 ± 7	21 ± 2	0.1

[a] Pooled samples, numbers of mice analysed are given in parentheses.

TABLE III
Serum amylase levels in PDX-1 treated mice

Serum amylase levels were determined in pooled samples of serum in both groups; numbers of mice in each group are indicated in parentheses.

Time after adenoviruses administration	Amylase	
	Control	PDX-1
	IU/liter	
5 days	1850 (3)	1709 (2)
60 days	1909 (2)	1876 (2)
120 days	2240 (3)	1744 (7)
180 days	1978 (4)	2477 (4)
240 days	2298 (6)	2343 (4)
280 days	2634 (3)	2570 (4)

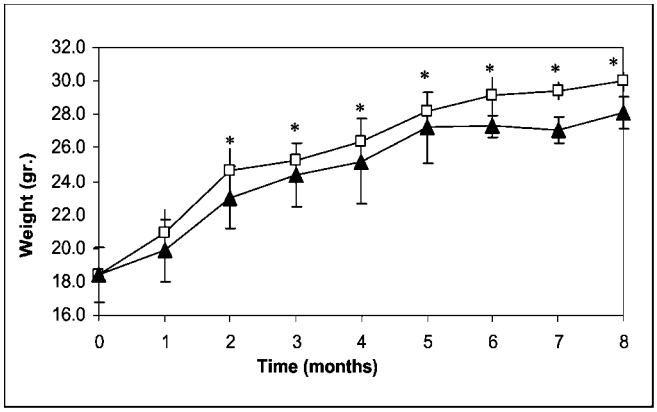

FIG. 7. **Body weight of in PDX-1-treated *versus* control treated mice.** 8-week-old mice were systemically injected by *Ad-CMV-PDX-1* (□) as well as by control virus (▲) as described under "Materials and Methods." Body weight was monitored once a month after 4 h of fasting. $n = 6$ at every time point analyzed; * indicates statistical significant increase in body weight upon PDX-1 treatment; $p < 0.05$.

long after the ectopic gene expression extinguishes. Insulin as well as glucagon-producing cells are mainly located in the proximity of central veins. Importantly, the *PDX-1*-induced insulin production in liver proves functional and is sufficient to protect mice from STZ-induced diabetes even 8 months after the initial *Ad-CMV-PDX-1* administration. Moreover, these data suggest that the developmentally shifted cells in liver may have maintained their original resistance to β-cell-specific toxins.

Although the PDX-1 role in pancreas organogenesis is mainly studied during embryogenesis, our data demonstrate that PDX-1 possesses an instructive role in pancreas differentiation also when ectopically expressed in mature and fully differentiated tissue such as liver. PDX-1 triggers the expression of pancreatic genes that are not its known immediate targets, such as glucagon, p48, and elastase (Fig. 1). *PDX-1* is not expressed in mature pancreatic α-cells, and several lines of evidence suggest that glucagon gene expression is indirectly repressed by PDX-1 (26). The fact that ectopic *PDX-1* expression leads to activation of glucagon as well as acinar gene expression in liver suggests that PDX-1 acts in this tissue as a differentiation factor, mediating its effect via downstream induced or pre-existing transcription factors in liver (26, 27). The development of distinct cell types characteristic of endocrine pancreas in mature liver could also be explained by the formation of a gradient of PDX-1 protein levels that may differentially affect the endocrine pancreatic lineage identity in liver (21, 28). For example, transient *PDX-1* expression followed by a decrease in PDX-1 levels could promote the activation of glucagon gene expression, as suggested for INS-1 cells (26).

The capacity of PDX-1 to induce its own expression in an extra-pancreatic tissue, such as liver, strengthens the view that it functions as a *bona fide* master regulator (18). Several transcription factors including PDX-1 itself (20, 29) have been suggested as regulators of *PDX-1* gene expression in pancreatic β-cells, most also present in liver (19, 20, 30). Indeed, liver may possess a unique predisposition to activate endogenous *PDX-1* gene expression due to pre-existing hepatocyte nuclear factor 3β and hepatocyte nuclear factor 1α in this tissue.

Our data are consistent with the notion that only a subpopulation of cells in the liver are predisposed to undergo a long lasting and comprehensive PDX-1-induced developmental shift. Despite the initial and relatively abundant ectopic *PDX-1* expression in liver, uniformly spread among 30–50% of the cells, insulin- and glucagon-positive cells represent less than 1% of the total cells in liver. Immunohistochemical analysis localizes pancreatic hormone-producing cells mainly to the proximity of central veins (Fig. 3) (1). This localization is not attributable to blood circulation because the blood supply that originally brings the recombinant adenoviruses into the liver enters via the hepatic artery and drains through the central veins (31). Therefore, we suggest that liver cells located in the proximity of central veins may possess a certain predisposition that allows them to undergo PDX-1-induced developmental shift. Hepatocytes located in the proximity of central veins are definitely mature and were reported to express high levels of glutamine synthetase (25). A metabolic zonation in liver was suggested by many researchers, and oxygen pressure was suggested to develop and maintain gradients of gene expression in liver (25, 32–35). Therefore, the different populations of cells in liver may consist of slightly distinct levels of certain hepatic transcription factors expression that in turn may affect the ability of these cells to undergo a PDX-1-induced developmental shift (33). An additional possible reason for preferred central vein localization of PDX-1-induced developmentally shifted cells could be the proximity to signals released from endothelial cells around blood vessels. These were documented to induce essential steps in organ formation such as morphogenesis and cell differentiation of both liver and pancreas (36, 37). These cells may harbor signals that instruct *PDX-1*-expressing cells in liver epithelium to adapt pancreatic cell fate (36, 37). However, we do not know whether signals released from endothelial cells that surround central veins are different from these released from endothelial cells that surround other blood vessels. Additional, yet unknown factors may be involved in the process of liver to pancreas developmental shift that occurs in response to ectopic *PDX-1* expression. The location of insulin-producing cells in the proximity of central veins may have a substantial functional advantage in allowing direct release of the produced hormones into the bloodstream, thus preventing intra-hepatic accumulation of pancreatic hormones that could adversely affect carbohydrate metabolism and glycogen storage.

Whereas *PDX-1* expression alone in our study is sufficient to induce pancreatic hormones in mice liver, studies performed in transgenic *Xenopus* tadpoles suggest that PDX-1 should be further activated by VP-16 to exert profound effects and convert most of the liver into pancreas (38). This study confirms the pivotal role of PDX-1 in controlling the process of liver to pancreas fate redirection; however, the functional consequences of converting most of the liver into pancreas are in question and are possibly demonstrated in the study by Kojima *et al*. (39).

Additional pancreatic transcription factors (40–42) may possess the capacity to induce pancreatic repertoire of gene expression in liver. Recently Kojima *et al*. (39) demonstrated the capacity of NeuroD to induce pancreatic markers. Interestingly, ectopic NeuroD combined with ectopic betacellulin expression in liver induced both downstream and upstream transcription factors that are part of the pancreatic transcriptional network including the *PDX-1*, and corrected hyperglycemia in STZ diabetic mice (39). In addition, the same study suggests that ectopic *PDX-1* expression is toxic to liver due to the induction of the pancreatic acinar function. Hepatic function analyses performed in our model do not demonstrate similar effects; serum amylase levels in PDX-1-treated mice were normal, and mice consistently gained weight (Tables II and III and Fig. 7). The discrepancy between our approach and the approach by Kojima and co-workers is likely to be caused by the distinct levels and the longer time frame of the ectopic *PDX-1* expression. The use of helper-dependent adenovirus combined with the potent mammalian elongation factor-1α promoter used in the study by Kojima *et al.* (39) may result in substantial increase in ectopic *PDX-1* expression in liver that may span several orders of magnitude, in close to 100% of cells in liver, in an almost irreversible mode (43–45).

Recombinant adenoviruses seem to be an optimal tool used for gene delivery *in vivo* for the purpose of inducing a transdifferentiation process. The ectopic PDX-1 serves only as a transient trigger for the persistent transdifferentiation process, leaving the host genome with no modification or insertions of foreign DNA (Fig. 5) and with normal hepatic function as demonstrated in our model (Table II).

Importantly, our study demonstrates that despite the short term expression of the ectopic *PDX-1* transgene, the triggered developmental shift remains functional for the whole duration of the experiment, which is at least 8 months, and possibly forever.

Moreover, our data suggest that the developmentally shifted cells in liver may resist the deleterious effects of the β-cell-specific toxins. The distinct STZ effects on liver *versus* pancreatic β-cells may stem from the fact that β-cells unlike liver cells possess a restricted capacity to metabolize hydroxyl radicals and hydrogen peroxide. By contrast, liver cells are equipped with high enzymatic activities of peroxidase, catalase, and superoxide dismutase, which together prevent accumulation of free radicals, thus rendering the cells resistant to many toxins and cytokines (46, 47). The exact mechanism of the therapeutic effect and the possible resistance of developmentally shifted liver cells to β-cell toxins should be further analyzed.

Additional studies are needed to fully understand the mechanism of PDX-1 and possibly additional pancreatic transcription and growth factors, mediated liver to pancreas transdifferentiation process. Markers of predisposed cells should be identified, and transdifferentiated cells should be better characterized in order to determine to what extent they mimic normal pancreatic β-cell functions.

Islet cell implantation as a treatment for diabetic patients will be widely available only when new sources of islets or β-cells are found. Our study suggests that liver could serve as an important source of tissue for generating functional insulin-producing cells to be used in replacing malfunctioning β-cells in patients with diabetes.

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