Cell-replacement therapy for diabetes: Generating functional insulin-producing tissue from adult human liver cells

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Shortage in tissue availability from cadaver donors and the need for life-long immunosuppression severely restrict the large-scale application of cell-replacement therapy for diabetic patients. This study suggests the potential use of adult human liver as alternate tissue for autologous beta-cell-replacement therapy. By using pancreatic and duodenal homeobox gene 1 (PDX-1) and soluble factors, we induced a comprehensive developmental shift of adult human liver cells into functional insulin-producing cells. PDX-1-treated human liver cells express insulin, store it in defined granules, and secrete the hormone in a glucose-regulated manner. When transplanted under the renal capsule of diabetic, immunodeficient mice, the cells ameliorated hyperglycemia for prolonged periods of time. Inducing developmental redirection of adult liver offers the potential of a cell-replacement therapy for diabetics by allowing the patient to be the donor of his own insulin-producing tissue.

pancreas | transdifferentiation

Large-scale application of islet cell implantations for treating type 1 diabetic patients is hindered by the limited availability of insulin-producing tissues and the subsequent need for life-long immunosuppression (1). It is generally accepted that cell replacement, as a treatment for diabetes, will become widely available only when new sources of pancreatic islets or beta cells are found (2).

Despite the demonstrated self-replication potential in mouse *in vivo* (3), differentiated beta cells cannot be expanded efficiently *in vitro* (4). Therefore, alternative approaches must be explored to generate cell-replacement therapies for diabetics by using pluripotent cells derived from pancreatic (5, 6) or extrapancreatic sources (7, 8). Efficient means to control the development of embryonic (9) and adult stem cells into desired tissues are under extensive study (2, 7, 10). Instructive roles of transcription factors as well as the role of soluble factors (SFs) have been suggested to be important in mediating the process of developmental switching (7, 10–12). Pancreatic and duodenal homeobox gene 1 (PDX-1) has a central role in regulating both pancreas organogenesis, as well as adult beta-cell function. PDX-1 is involved in regulating the expression of multiple beta-cell-specific genes and has a key role in pancreatic morphogenesis in mice and humans (13–15).

The surprising capacity of liver to serve as a potential source of tissue for generating functional endocrine pancreas was demonstrated in mice *in vivo* by using ectopic PDX-1 expression (2, 7, 16). Transient expression of a *PDX-1* transgene in mice induced a comprehensive, irreversible, and functional transdifferentiation process in a subpopulation of cells in the liver (16). Furthermore, the capacity of PDX-1 to convert adult liver into pancreas has been confirmed by using different experimental procedures in *Xenopus* (11) and mice (12, 17).

Here, we demonstrate that freshly isolated adult and fetal human liver cells can be cultured *in vitro* and induced to transdifferentiate along an endocrine pancreatic lineage. Up to 50% of the liver cells that expressed the *PDX-1* transgene activated the otherwise inactive insulin promoter. PDX-1 induced transdifferentiated human liver cells produced the hormone, stored it in granules, and released processed insulin in a glucose-regulated manner. Insulin-producing human liver cells were functional for prolonged periods of time and ameliorated hyperglycemia when implanted under the renal capsule of diabetic immunodeficient mice. The mice reverted to a diabetic state upon removal of the transdifferentiated cells.

This study suggests that adult liver could be considered as a pancreatic progenitor tissue. The potential use of extrapancreatic tissues as a basis for autologous cell-replacement therapy may allow the diabetic patient to be the donor of his own insulin-producing tissue.

Methods

Preparation of Recombinant Adenoviruses. All first-generation recombinant adenoviruses were constructed according to Becker *et al.* (18). Generation of *Ad-RIP-GFP* and the bifunctional recombinant *Ad-RIP-GFP-CMV-PDX-1* adenoviruses is described in *Supporting Methods*, which is published as supporting information on the PNAS web site.

Human Liver Cells. Adult human liver (AHL) tissues were obtained from eight different liver transplantation surgeries from 4- to 10-year-old children and three individuals >40 years old. Fetal human livers were obtained from four deliberate abortions of 20–22 weeks of gestation. Both adult and fetal liver tissues were used with approval from the Committee on Clinical Investigations (Institutional Review Boards of Sheba Medical Center and Rabin Medical Center).

Cell Harvest and Culture Conditions. Isolation of human liver cells was performed as described (19). Briefly, liver samples were cut into thin slices (1- to 2-mm thickness), and digested by 0.03% collagenase type I (Worthington) for 20 min at 37°C. The cells were cultured in DMEM (1 g/liter glucose) supplemented with 10% FCS/100 units/ml penicillin/100 μg/ml streptomycin/250 ng/ml

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Abbreviations: PDX-1, pancreatic and duodenal homeobox gene 1; AHL, adult human liver; TAHL, transdifferentiated AHL; SFs, soluble factors; moi, multiplicity of infection; NOD-SCID, nonobese diabetic severe combined immunodeficient.

See Commentary on page 7781.

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amphotericin B (Biological Industries, Beit Haemek, Israel). Cells were plated on fibronectin-coated (3 μg/cm^2; Biological Industries) plates at 1–2 $\times$ 10^5 cells per ml. The medium was changed daily during the first 3 days to remove nonadherent cells. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Viral Infections and SF Treatment. Liver cells were cultured in DMEM (1g/liter glucose) with or without SFs as indicated separately [SFs, 20 ng/ml EGF (Cytolab, Rehovot, Israel)/10 mM nicotinamide (Sigma)] and infected with recombinant adenoviruses *Ad-RIP-GFP-CMV-PDX-1*, *Ad-RIP-GFP*, *Ad-CMV-PDX-1* (replication-deficient recombinant adenovirus that encodes rat PDX-1 cDNA under the control of the cytomegalovirus promoter), *Ad-CMV-hInsulin* (20), and *Ad-CMV-GFP* (Clontech), at a multiplicity of infection (moi) of 500 for 48 h.

RNA Isolation and Reverse Transcriptase Reactions. Total RNA was isolated and cDNA was prepared as described (16). RNA isolated from human islets was a gift from S. Deng (Department of Surgery, University of Pennsylvania, Philadelphia) and C. Ricordi (Diabetes Research Institute, University of Miami, Miami).

Real-Time RT-PCR. Quantitative real-time RT-PCR was performed by using a Prism 7000 sequence-detection system (Applied Biosystems). The Assay-On-Demand (Applied Biosystems) TaqMan fluorogenic probes that were used in this study are described in *Supporting Methods*.

Amplification conditions included initiation at 50°C for 2 min and denaturation at 95°C for 10 min, followed by 40 cycles; each cycle included denaturation at 95°C for 15 sec and annealing at 60°C for 1 min by using the TaqMan PCR Master mixture (Applied Biosystems). Relative quantitative analysis was performed according to the comparative C_T method by using the arithmetic formula $2^{-(\Delta\Delta Ct)}$. The cDNA levels were normalized to human β-actin cDNA.

Rat *PDX-1* (STF, NM_022852) cDNA was amplified for 30 cycles (94°C for 45 sec, 56°C for 45 sec, and 72°C for 60 sec) by using the following primer pair CCAAAACCGTCGCATGAAGTG (forward) and CAGCTCGCCTGGTGGCTGT (reverse). PCR products were separated by electrophoresis in 2–3.5% agarose gels and visualized by ethidium bromide staining.

Insulin Detection. Insulin secretion was measured in primary cultures of adult liver cells by static incubation 3–5 days after initial exposure to viral and SF treatment. Insulin secretion to the medium was measured by radioimmunoassay by using the Ultra Sensitive Human Insulin radioimmunoassay kit (Linco Research, St. Charles, MO), with only 6% cross-reactivity to human proinsulin. Insulin content was measured as described (16). Insulin content was normalized to total cellular protein measured by the Bio-Rad protein-assay kit.

Immunofluorescence. Liver cells treated by *Ad-CMV-PDX-1* for 5 days were plated on glass cover slides in six-well culture plates. At 48 h later, the cells were fixed in 4% paraformaldehyde, permeablized by using 0.1% Triton X-100, blocked in 1% BSA, and incubated 1.5 h with the primary antibody mouse anti-insulin 1:100 (Sigma) and with rabbit anti-Pdx-1 1:5000 (a gift from C. V. E Wright, Department of Cell Biology, Vanderbilt University, Nashville, TN). Secondary anti-mouse IgG indocarbocyanine (Cy3)-conjugated antibody 1:250 and anti-rabbit IgG FITC-conjugated antibody 1:250 (both from Jackson ImmunoResearch) were used. Last, the cells were stained with DAPI (Sigma). The slides were analyzed by using a fluorescent microscope (Provis, Olympus).

Glucose-Challenge Assay. Adult liver cells were treated with *Ad-CMV-PDX-1* and SFs for 5 days and seeded in six-well dishes at 10^5 cells per well. Cells were preincubated for 2 h in KRB containing 0.1% BSA, rinsed and challenged thereafter with increasing concentrations of glucose (0–30 mM) or 2-deoxyglucose (0–25 mM). Medium aliquots were analyzed for C-peptide (human C-peptide radioimmunoassay kit, Linco Research; <4% cross-reactivity to human proinsulin) and insulin secretion, as described above.

Electron Microscopy. Liver cells were prepared as described (21). The samples were incubated with guinea pig polyclonal antibody against human insulin (7.8 μg/ml, Dako) overnight at 4°C and then incubated with ImmunoGold-conjugated antibody against guinea pig IgG (15-nm gold, 1:40 dilution, British BioCell International) for 1.5 h at room temperature. Last, the sections were stained with 2% uranyl acetate and Reynolds' lead citrate solution and analyzed under a JEOL 100CX electron microscope.

Cell Transplantation. We rendered 7- to 8-week-old nonobese diabetic severe combined immunodeficient (NOD-SCID) male mice (The Weizmann Institute of Science) hyperglycemic by i.p. injection of streptozotocin (Sigma) at 180 mg/kg body weight. When blood glucose levels were ≥300 mg/dl on two consecutive measurements, mice were transplanted under the kidney capsule, with 7 $\times$ 10^6 transdifferentiated AHL (TAHL) cells or with control untreated AHL cells by using a 30-gauge needle. Blood was drawn from the tail for determination of glucose levels (Accutrend GC, Roche Applied Science). Serum was collected from fed mice for human C-peptide, mouse insulin, and amylase (16) levels analyses. The Ultrasensitive Human C-Peptide ELISA kit (Mercodia, Uppsala) with 3% cross reactivity to proinsulin but no cross reactivity to mouse C-peptide and mouse insulin ELISA kit (Mercodia) with no cross-reactivity to human insulin were used according to the manufacturer's instructions. Nephrectomy was performed at the indicated time points, and blood glucose levels were monitored daily until killed. Kidney and pancreas were harvested for immunohistochemical analyses.

Glucose Tolerance Test. Mice fasted for 6 h were injected i.p. with glucose in saline at 1 mg/g body weight. Blood glucose levels were monitored at the indicated time points in samples obtained from the tail vein.

Histology and Staining. Kidney and pancreas were fixed and stained as described above and in refs. 7 and 16.

Statistical Analyses. Statistical analyses were performed by using a two-sample Student's *t* test assuming unequal variances.

Results

PDX-1 Activates the Insulin Promoter in Human Liver Cells. Human liver cells were isolated from both adult and fetal tissues. The cells exhibited a heterogeneous phenotype and proliferated efficiently in culture for up to 20 passages. Only ≈50% of human liver cells in culture were susceptible to recombinant adenovirus infection even at high moi (500 moi of *Ad-CMV-GFP*, Fig. 1 *E* and *F*). We wished to analyze whether human liver cells, without any prior selection, can undergo a process of developmental redirection toward a pancreatic phenotype in response to ectopic PDX-1 expression. To identify liver cells that may have the potential to undergo transdifferentiation, we constructed a bifunctional recombinant adenovirus, *Ad-RIP-GFP-CMV-PDX-1*. This virus encodes *PDX-1* under the control of the heterologous CMV promoter, whereas RIP (the rat insulin-1 promoter) controls *GFP* expression (*Supporting Methods* and Fig. 6, which is published as supporting information on the PNAS web site). Thus, cells capable of activating the ectopic insulin promoter in response to PDX-1 are identified by green fluorescence (Fig. 1 *A–D*). Although non-beta-cell lines did not activate the insulin promoter in response to ectopic *Ad-RIP-GFP-CMV-PDX-1* treatment, 10–25% of AHL cells in culture showed green fluorescence, in response to the viral infection (Fig. 1 *A* and *B*, represen-

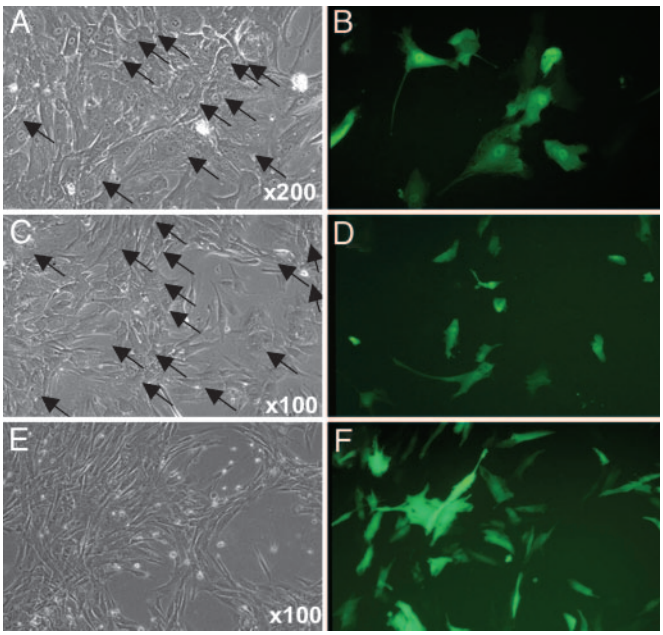


Fig. 1. PDX-1 activates the insulin promoter in human liver cells *in vitro*. Representative phase contrast morphology (*A*, *C*, and *E*), and green fluorescence (*B*, *D*, and *F*) of the same field of adult (*A* and *B*) and fetal (*C* and *D*) human liver cells infected by *Ad-RIP-GFP-CMV-PDX-1*. Arrows indicate the fluorescing cells. *Ad-CMV-GFP*-infected adult liver cells (*E* and *F*) represent the yield of infection at 500 moi used for all viral treatments in this study. Original magnifications are ×200 (*A* and *B*) and ×100 (*C*–*F*).

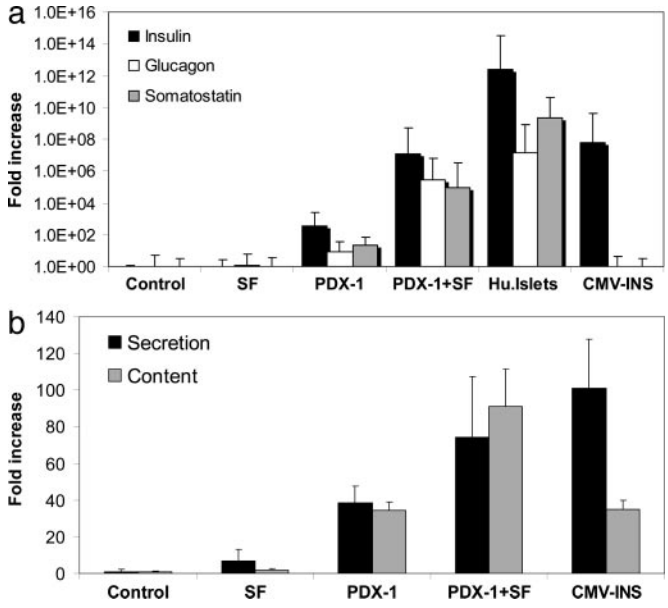


Fig. 2. The promoting effect of SF on pancreatic hormones gene expression, insulin content, and secretion in AHL cells treated by *Ad-CMV-PDX-1*. (*a*) Quantitative real-time RT-PCR analyses of *insulin*, *glucagon*, and *somatostatin* gene expression levels. C_T (threshold cycle) values are normalized to β-actin gene expression within the same cDNA sample ($n \geq 30$ in five different experiments). (*b*) Insulin content ($n \geq 10$) and insulin secretion ($n \geq 25$) by static incubations for 48 h. *Ad-CMV-hIns*-infected cells (500 moi) serve as constitutive control for human (pro)insulin production and secretion. Results are presented as fold of increase of the mean ± SD compared with untreated control liver cells.

tative). The obligatory role of PDX-1 in activating the insulin promoter in liver cells was demonstrated by *Ad-RIP-GFP* treatment that did not result in green fluorescence (data not shown). Considering that only half of cells in culture are susceptible to adenovirus infection (Fig. 1 *E* and *F*), one can suggest that 20–50% of the PDX-1 expressing liver cells may posses the potential of activating the insulin promoter. To determine whether the responsiveness to PDX-1 is limited by the differentiation state of the adult liver cells, we analyzed the activation of the insulin promoter in fetal human liver cells, which are less differentiated and probably contain more pluripotent cells than AHL cells; 10–30% of the fetal human liver cells in culture (isolated from 22 weeks of gestation) responded to ectopic expression of PDX-1 by activation of the insulin promoter (Fig. 1 *C* and *D*, representative), although their susceptibility to *Ad-CMV-GFP* infection was similar to that of AHL cells. This modest increase in number of responding cells suggests that the original differentiation state of the cells plays only a minor role, if any, in the PDX-1-induced developmental-redirection process.

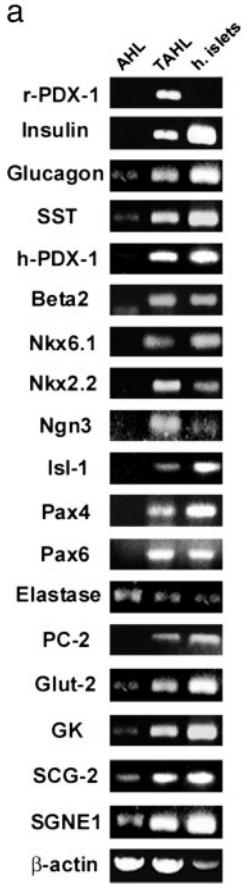
Three observations emerge from the primary culture of human liver cells. First, both adult and fetal liver cells proliferate efficiently *in vitro* for ≈6 months (20 passages) and are capable of activating the insulin promoter in response to PDX-1 treatment. Second, both fetal and adult human hepatic tissues possess similar potential to activate this pancreatic promoter. Third, the capacity to activate the insulin promoter in human liver cells is not restricted to a rare population of cells, because up to one-half of the cells that are capable of being infected by recombinant adenovirus also activated the ectopic insulin promoter in a PDX-1-dependent manner.

PDX-1-Induced Liver-to-Pancreas Transdifferentiation Is Promoted by SFs. A better indication for the extent of the transdifferentiation process is to monitor the expression of the endogenous, otherwise silent, pancreatic genes in PDX-1-treated liver cells. The expression of the three pancreatic hormones genes was induced by ectopic rat PDX-1. The most profound effect was on the activation of the *insulin* gene expression that was increased by two orders of magnitude compared with control untreated liver cells (Fig. 2*a*). Normal pancreatic development depends on the action of various SFs operating in prenatal and postnatal development (22). Nicotinamide and EGF were documented to promote endocrine pancreatic differentiation in several experimental systems (12, 23–25). Therefore, we analyzed their potential to promote the PDX-1 effect on AHL cells. When PDX-1 treatment was supplemented with nicotinamide and EGF (collectively, called SF), pancreatic hormone gene expression was substantially increased. *Insulin* gene expression in primary culture of adult liver cells was increased by seven orders of magnitude compared with that in control untreated liver cells, yet it was still considerably lower than that in freshly isolated human pancreatic islets (Figs. 2*a* and 3). Neither nicotinamide nor EGF alone or in combination increased pancreatic gene expression in the absence of PDX-1 (Fig. 2*a*). Importantly, fetal and AHL cells exhibited similar levels of pancreatic gene expression in response to ectopic PDX-1 expression and SF treatment (data not shown).

The promoting effect of SF is further reflected in increasing the insulin content and secretion of PDX-1-treated cells (Fig. 2*b*). Insulin content in control and SF-treated liver cells was 0.06 ± 0.03 ng per 10^6 cells and 0.26 ± 0.05 ng per 10^6 cells (0.5 mg of protein), respectively. Insulin content in PDX-1-treated liver cells was 2.28 ± 0.3 ng per 10^6 cells and increased to 6.05 ± 1.3 ng per 10^6 cells upon treatment with PDX-1 and SF. Because only ≈10–25% of liver cells in culture are actual insulin producers (Figs. 1 and 4*a*), the calculated amount of processed insulin content is 25–60 ng per 10^6 cells. Insulin content in *in vitro* culture of human islets is 1.8 ng per islet (26).

Our aim in using PDX-1 was not restricted to the induction of insulin production but rather to analyze its capacity of inducing a process of developmental redirection along the pancreatic lineage in liver cells. Indeed, *Ad-CMV-hIns*, a recombinant adenovirus that

a



b

AHL control	TAHL	H. Islets
-	+	-
1	1.2×10^{7}	2.5×10^{12}
1	2.9×10^{5}	1.5×10^{7}
1	8.5×10^{4}	2.1×10^{9}
1	1.2×10^{4}	2.7×10^{7}
1	5.8×10^{4}	1.1×10^{7}
1	6.2×10^{2}	5.1×10^{4}
1	3.5×10^{2}	5.2×10^{2}
1	1.7×10^{4}	1.7×10^{2}
1	1.5×10^{2}	8.7×10^{4}
1	6.2×10^{1}	9.5×10^{3}
1	4.1×10^{1}	8.5×10^{2}
1	0.5×10^{1}	1.1×10^{2}
1	2.7×10^{2}	2.1×10^{6}
1	2.5×10^{1}	1.8×10^{6}
1	3.1×10^{2}	2.8×10^{5}
1	8.5×10^{1}	3.6×10^{2}
1	2.2×10^{1}	1.5×10^{2}

Fig. 3. TAHL express a wide repertoire of pancreatic gene expression. Quantitative RT-PCR analyses of pancreatic genes in THAL cells compared with untreated AHL and human islets demonstrated in ethidium bromide staining of agarose-separated PCR products (*a*) and quantitative analyses levels (*b*). C_T (threshold cycle) values are normalized to β-actin gene expression within the same cDNA sample. Results are presented as fold increase compared with untreated AHL cells, arbitrary set at 1.

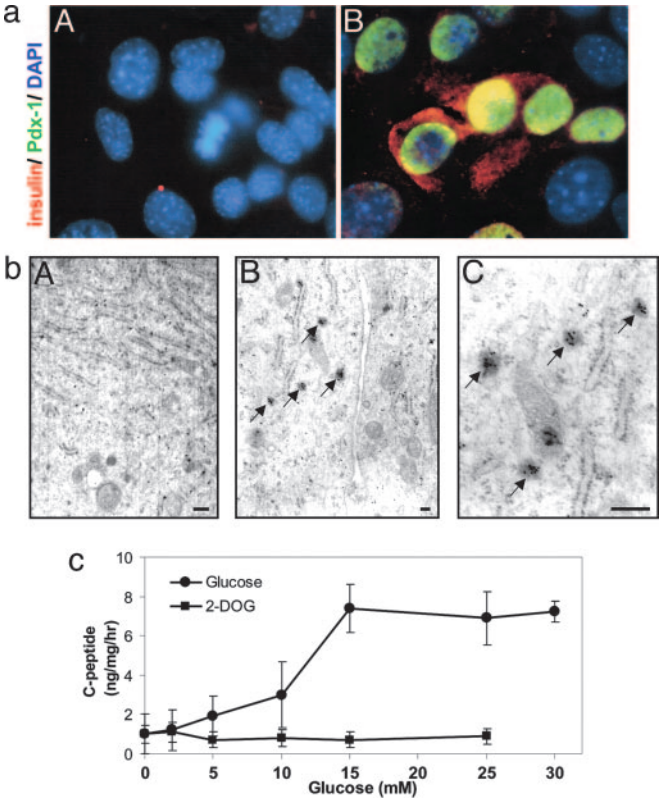


Fig. 4. TAHL cells produce, store, and secrete insulin and C-peptide in a glucose regulated manner. (*a*) Immunofluorescent staining for insulin (cytoplasmatic, red) and Pdx-1 (nuclear, green) in untreated AHL (*A*) and TAHL (*B*) cells. Nuclei are stained in blue (DAPI). Original magnifications are ×600 (*A*) and ×1,000 (*B*). (*b*) Electron microscopy of insulin ImmunoGold histochemistry in untreated AHL (*A*) and in TAHL (*B* and *C*) cells. *C* is a further magnification of an area in *B*. Arrows, ImmunoGold particles concentrated in granules, which appear in TAHL cells. (Scale bar, 0.25 μm.) (*c*) Static incubation of glucose or 2-deoxy-glucose (2-DOG) dose–response (0–30 mM) of C-peptide secretion. Results are presented as the mean ± SD; $n = 30$ in four different experiments.

causes constitutive ectopic expression of human *Proinsulin* cDNA under the control of the CMV promoter, resulted in *insulin* gene expression at levels comparable with those in PDX-1- and SF-treated cells, without inducing *glucagon* and *somatostatin* gene expression (Fig. 2*a*). Moreover, whereas much of the insulin in PDX-1-treated cells was retained within the cells, most of the insulin produced under *Ad-CMV-hIns* treatment was released (Fig. 2*b*).

These data suggest that PDX-1 is necessary for liver to pancreas transdifferentiation, whereas SF, although inactive on their own, possess a synergistic effect on the process. The similar levels of *insulin* transcripts and hormone production in adult and fetal human liver cells induced by PDX-1 treatment motivated us to further analyze the AHL cells capacity to undergo a comprehensive developmental-redirection process in response to PDX-1 and SF, because of their possible advantage for use in autologous cell-replacement therapy.

PDX-1 Induces a Comprehensive Repertoire of Pancreatic Gene Expression in Liver. Along with the induction of the pancreatic hormones, the ectopic rat PDX-1 induced the endogenous human *PDX-1* transcript and many pancreatic transcription factors, such as *Beta-2*, *Nkx6.1*, *Nkx2.2*, *Ngn3*, *Isl-1*, *Pax4* and *Pax6*, in *PDX-1*- and SF-treated TAHL cells (Fig. 3). Although these nuclear factors should be analyzed further by protein detection assays, their induced expression in TAHL cells by ectopic PDX-1, suggests that this factor has a high dominant location in the hierarchy of transcription factors, leading not only to pancreatic development in the embryo (27) but also in the transdifferentiation process of AHL cells. However, the induced expression of *Ngn3* may suggest that some of the developmentally shifted liver cells are at an immature state of endocrine pancreatic differentiation (Fig. 3).

TAHL Cells Produce, Store, and Secrete the Processed (Pro)Insulin in a Glucose-Regulated Manner. Immunofluorescence analysis reveals that TAHL cells produce and store insulin (Fig. 4*a*); ≈50% of the liver cell nuclei are positively stained for the pancreatic nuclear factor, and of these, approximately one-half are positive for insulin staining, demonstrating the heterogeneity of our culture (as shown in Fig. 1).

Further maturation along the endocrine lineage is demonstrated by the increase in the expression of neuroendocrine vesicle markers *SCG-2* (secretogranin 2) and *SGNE1* (secretory granule neuroendocrine 1) and by the induction of *prohormone convertase 2* (*PC-2*) expression in TAHL cells (Fig. 3). Furthermore, electron microscope analysis of ImmunoGold histochemistry by using anti-insulin antibodies revealed that the insulin produced in TAHL cells is stored in granules (Fig. 4*b*). These granules did not contain a characteristic dense core as in intact pancreatic islets *in vivo* but resembled these present in beta cell lines that probably contain a

lower level of stored insulin (21). These data, together with C-peptide secretion from TAHL cells (Fig. 4*c*), suggest the possible induction of the insulin storage and processing machinery in some PDX-1-treated cells. Further HPLC analyses would determine the efficiency of the prohormone processing capacity of TAHL cells.

Glucose-sensing ability and the coupling between glucose sensing and insulin secretion are hallmarks of pancreatic beta-cell function. Exposure of TAHL cells to 25 mM glucose resulted in an immediate and profound increase in C-peptide secretion (from 0.8 to 2.4 ng/mg within 15 min). Glucose dose–response of C-peptide secretion peaked at 15 mM glucose (Fig. 4*c*), resembling that of normal pancreatic islets (26). A nonmetabolizable glucose analog, 2-deoxy-glucose (2-DOG), did not trigger C-peptide secretion in TAHL cells (Fig. 4*c*), suggesting that as in pancreatic beta cells, glucose metabolism is required for the effect on insulin secretion. Although liver cells in culture express *Glut-2* and *glucokinase*, PDX-1 and SF treatment further increased the expression of these genes (Fig. 3). Insulin secretion from TAHL cells is ≈8 ng/mg protein per h, as determined by C-peptide release at 15 mM glucose. Because only ≈10–25% of liver cells in culture are actual insulin producers (Figs. 1 and 4*a*), the calculated mature insulin produced by TAHL cells is 32–80 ng/mg protein per h.

TAHL Cells Ameliorate Diabetes *in Vivo*. To analyze the function of TAHL cells *in-vivo*, we tested their capacity to ameliorate hyperglycemia upon implantation in diabetic immunodeficient mice. Streptozotocin treatment resulted in induction of hyperglycemia, which was associated with a marked decrease in serum insulin levels. Mouse insulin dropped from 1.57 ± 0.14 ng/ml in fed healthy mice to 0.54 ± 0.11 ng/ml upon induction of hyperglycemia. These low levels of mouse insulin persisted throughout the whole duration of the experiment. TAHL cells implantation in diabetic NOD-SCID mice caused a gradual, prolonged, and significant decrease in blood glucose levels that persisted over the 60 days duration of the experiment (Fig. 5*a*). To confirm that the therapeutic effect was attributed to the implanted TAHL cells, we analyzed human C-peptide levels in the immunodeficient mice sera before and at indicated time points after implantation. Human C-peptide levels increased gradually in a manner parallel to the decrease in blood glucose levels (Fig. 5 *a* and *b*), possibly demonstrating further differentiation along the pancreatic lineage or cell proliferation, *in vivo*. From day 12 on, the C-peptide levels remained unchanged and averaged 0.23 ± 0.03 ng/ml, compared with negligible levels in both healthy and diabetic NOD-SCID mice implanted with AHL cells (Fig. 5*b*). Removal of TAHL cells by nephrectomy at 23, 45, and 60 days after implantation resulted in reversal to hyperglycemia that was associated with decreased human C-peptide levels (Fig. 5 *a* and *b*). Immunohistochemical analysis revealed that although mice pancreata were depleted of insulin, TAHL cells implanted under the kidney capsule stained positive for PDX-1 and insulin (Fig. 5*c*). The anabolic effect of the implanted cells was further manifested in an average 10% increase in body weight per month, similar to that of control normoglycemic mice (16). Mice implanted with TAHL cells responded to glucose load with a clearance rate that paralleled that of healthy mice (Fig. 5*d*). AHL-cell-transplanted mice remained hyperglycemic (>400 mg percentage) throughout the assay. Together, these findings indicate that implanted cells were functional *in vivo* and ameliorated hyperglycemia in the NOD-SCID mice. These results establish the potential of PDX-1-treated TAHL cells to replace β cells function *in vivo*.

Discussion

It has long been thought that animal cells, when committed to a specific lineage, generally can no longer change their fate and, thus, become "terminally differentiated" (28). Here, we demonstrate that AHL cells retain substantial plasticity and can be induced to assume new fates and function upon appropriate molecular manipulation.

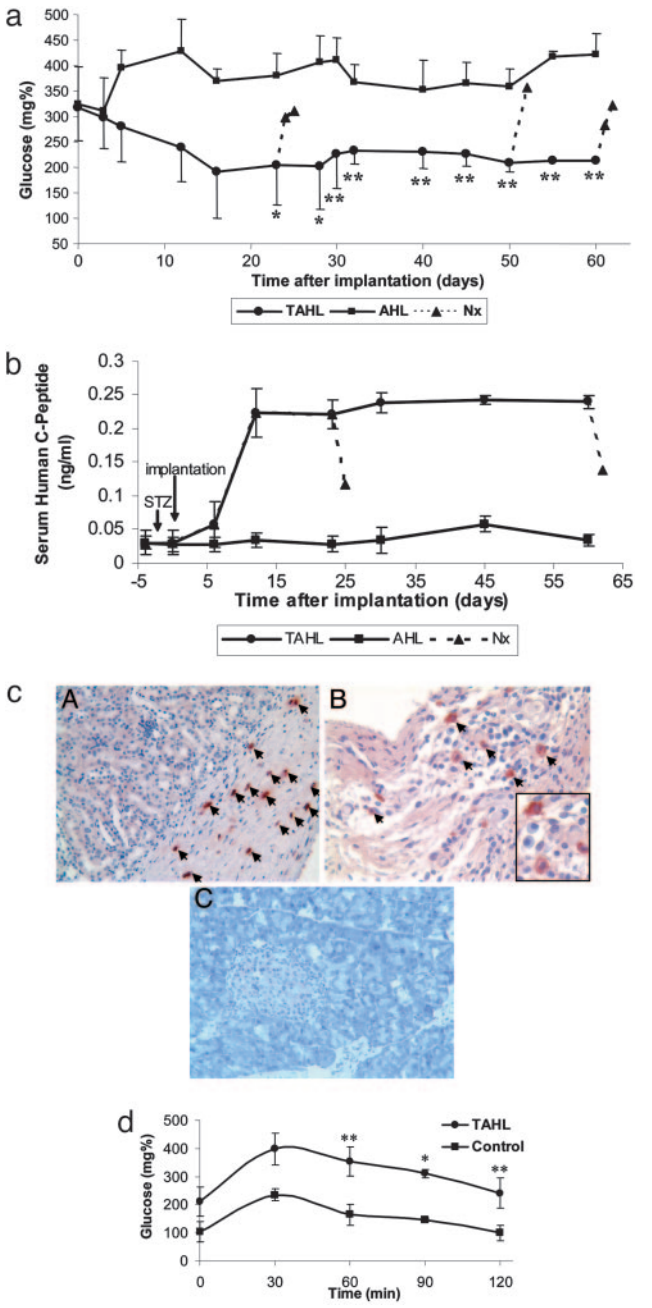


Fig. 5. TAHL cells ameliorate hyperglycemia in NOD-SCID mice. (*a*) Diabetic NOD-SCID mice were implanted under the kidney capsule with 7×10^6 TAHL cells ($n = 15$) or with untreated AHL cells ($n = 9$). Glucose levels at the indicated time points after implantation are given as mean ± SE in mg percentage. Asterisks denote a significant difference (*, $P < 0.05$; **, $P < 0.01$) between the glucose levels of mice implanted by TAHL cells and these implanted by AHL cells. Dashed lines indicate glucose levels measured after nephrectomy (Nx) at the indicated time points. (*b*) Serum human C-peptide levels in mice implanted by TAHL cells ($n = 10$) or with untreated AHL cells ($n = 7$). (*c*) Immunohistochemical analysis of Pdx-1 (*A*) and insulin (*B*) in the kidney capsule sections, 10 days after transplantation of TAHL cells. (*B*) Enlarged magnification of insulin-positive liver cells. (*C*) Insulin staining of the same NOD-SCID mouse pancreas. Arrows indicate positive cell staining. Original magnifications are ×400 (*A* and *B*) and ×200 (*C*). (*d*) Glucose tolerance test in normoglycemic NOD-SCID controls ($n = 3$) and in mice implanted with TAHL cells ($n = 3$), 25–35 days after implantation (*, $P < 0.1$; **, $P < 0.01$).

Our study demonstrates the potential use of AHL as a pancreatic progenitor tissue. It shows that PDX-1 possesses the capacity of instructing differentiation along the endocrine pancreatic lineage

regardless of the hepatic state of differentiation (Fig. 1). Insulin induction by ectopic PDX-1 in AHL cells was determined at the following several levels: the activation of an ectopic insulin promoter (Fig. 1), the induction of endogenous transcripts, hormone production (Fig. 2), secretion, and processing (Fig. 4). The presence of insulin-containing granules (Fig. 4*b*) suggests the possible creation of intracellular compartments characteristic of endocrine tissue. Furthermore, the cells responded to glucose challenge both *in vitro* (Fig. 4*c*) and upon implantation *in vivo* (Fig. 5*d*). The requirement for glucose metabolism for triggering insulin release suggests the possible coupling between the induced insulin storage compartment and the glucose-sensing apparatus in TAHL cells (Fig. 4). Despite the described mature characteristics, the relatively high Ngn3 expression, which in mice does not co-localize with insulin (29), suggests that at least some of the PDX-1-treated TAHL cells are at an immature state of pancreatic endocrine differentiation. Although located at a high position in the transcription factors hierarchy (leading to pancreatic organogenesis), PDX-1 induced neither exocrine gene expression (*Elastase*, Fig. 3) nor function, as amylase secretion upon TAHL cells implantation *in vivo* remained unaltered compared with control mice (data not shown).

Our study reveals a substantial promoting effect of SFs in augmenting the effect of PDX-1 on the developmental-redirection process of liver to pancreas (Fig. 2). SFs could either promote the proliferation of predisposed cells in liver or synergize in a yet-unknown fashion with the pancreatic nuclear factor effect on the process. The mechanism that mediates the SF effect on the transdifferentiation process of liver to pancreas requires further analysis.

The most important "pancreatic-like" function that these cells demonstrate is their capacity to ameliorate hyperglycemia and improve the diabetic state *in vivo* (Fig. 5). Although blood glucose levels of TAHL cells implanted diabetic NOD-SCID mice substantially decreased compared with mice implanted with AHL-control cells, they were higher than in healthy mice (Fig. 5*a*). This outcome could be attributed to the relatively low number of insulin-producing cells, because only 10–25% of the 7×10^6 implanted cells were expected to produce (pro)insulin (Figs. 1 and 4*a*). Separation of TAHL cells according to insulin promoter activation may enrich the insulin-producing population 4- to 10-fold and, therefore, is expected to improve the therapeutic outcome of using liver as functional surrogate beta cells. Nevertheless, the implanted human cells were clearly instrumental in correcting the diabetic state, because mice reverted to diabetes upon removal of TAHL cells by nephrectomy, even at 60 days after implantation.

Several lines of evidence suggest a substantial and persistent therapeutic effect of the implanted liver cells. Despite the episomal nature of the ectopic PDX-1 expression, blood glucose and human C-peptide levels were stable for the whole duration of the experiment (Fig. 5). The expression of the endogenous human *PDX-1* and additional pancreatic transcription factors in preimplanted cells (Fig. 3) are consistent with the continuous functional properties of TAHL cells *in vivo*. The irreversibility of the developmental-redirection process of TAHL cells and the clearance of the ectopic *PDX-1* gene should be analyzed further in longer-term experiments along with molecular analysis of the explants.

The *in vivo* environment may promote TAHL cell differentiation because it allows cell clustering and represents a physiologically balanced environment (30). Therefore, the gradual increase in C-peptide levels upon *in vivo* implantation could result from further maturation of TAHL cells along the pancreatic lineage. It would be interesting to analyze whether factors that are capable of promoting the maturation of dedifferentiated pancreatic derived cells will similarly affect TAHL-cell differentiation along the pancreatic lineage.

The use of AHL cells for generating functional insulin-producing tissue may make autologous implantations possible, thus allowing the diabetic patient to be the donor of his own insulin-producing tissue. This approach may circumvent the shortage in tissue availability, the need for antirejection treatment and the ethical issues associated with the use of fetal or embryonic stem cells for this purpose.

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