CELLULAR REPROGRAMMING
Volume 12, Number 6, 2010

DOI: 10.1089/cell.2010.0030

NKX6.1 Promotes PDX-1-Induced Liver to Pancreatic β-Cells Reprogramming

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Abstract

Reprogramming adult mammalian cells is an attractive approach for generating cell-based therapies for degenerative diseases, such as diabetes. Adult human liver cells exhibit a high level of developmental plasticity and have been suggested as a potential source of pancreatic progenitor tissue. An instructive role for dominant pancreatic transcription factors in altering the hepatic developmental fate along the pancreatic lineage and function has been demonstrated. Here we analyze whether transcription factors expressed in mature pancreatic β-cells preferentially activate β-cell lineage differentiation in liver. NKX6.1 is a transcription factor uniquely expressed in β-cells of the adult pancreas, its potential role in reprogramming liver cells to pancreatic lineages has never been analyzed. Our results suggest that NKX6.1 activates immature pancreatic markers such as NGN-3 and ISL-1 but not pancreatic hormones gene expression in human liver cells. We hypothesized that its restricted capacity to activate a wide pancreatic repertoire in liver could be related to its incapacity to activate endogenous PDX-1 expression in liver cells. Indeed, the complementation of NKX6.1 by ectopic PDX-1 expression substantially and specifically promoted insulin expression and glucose regulated processed hormone secretion to a higher extent than that of PDX-1 alone, without increasing the reprogrammed cells. This may suggest a potential role for NKX6.1 in promoting PDX-1 reprogrammed cells maturation along the β-cell-like lineage. By contrast, NKX6.1 repressed PDX-1 induced proglucagon gene expression. The individual and concerted effects of pancreatic transcription factors in adult extra-pancreatic cells, is expected to facilitate developing regenerative medicine approaches for cell replacement therapy in diabetics.

Introduction

Cells in adult mammalian tissues have long been thought to be terminally and irreversibly differentiated. In the last few years, a role has been demonstrated for specific transcription factors in the diversion of the original developmental fate of cells in adult tissues to either pluripotency or alternate committed lineages (Lewitzky et al., 2007; Yamanaka, 2008). The first demonstration of the activation of a functional pancreatic lineage was in adult mouse liver cells *in vivo* by ectopic pancreatic and duodenal homeobox gene-1 (PDX-1) expression (Ber et al., 2003; Ferber et al., 2000; Shternhall-Ron et al., 2007). Insulin producing cells have been generated by a similar approach in adult human liver cells *in vitro*. Reprogrammed human liver cells produce and process the hormone, secrete it in a glucose-regulated manner (Aviv et al., 2009; Meivar-Levy and Ferber, 2006; Meivar-Levy et al., 2007; Sapir et al., 2005), and ameliorate hyperglycemia upon implantation *in vivo* (Sapir et al., 2005). During the last decade, the potential for converting liver into pancreas has been demonstrated by many groups (Meivar-Levy and Ferber, 2006; Zaret, 2008), both *in vivo* and *in vitro*, in *Xenopus* (Horb et al., 2003), rodents (Ber et al., 2003; Ferber et al., 2000; Kaneto et al., 2005a; Kaneto et al., 2005b; Koizumi et al., 2004; Nakajima-Nagata et al., 2004; Tang et al., 2006b), and in human tissues (Sapir et al., 2005; Zalzman et al., 2003,

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2005). The efficiency of the reprogramming process is significantly improved by coexpressing PDX-1 with additional pancreatic transcription factors, such as NEUROD-1, MAF-A, PAX-4, and NGN3 (Kaneto et al., 2005b; Kojima et al., 2003; Tang et al., 2006a; Wang et al., 2007). Using a similar approach, the activation of the endocrine pancreatic lineage has been demonstrated in acinar and pancreatic duct cells (Baeyens et al., 2005; Bonner-Weir et al., 2008; Zhou et al., 2008). Whether the sequence of developmental events that control embryonic pancreas organogenesis is recapitulated in reprogramming adult extra-pancreatic tissues along the pancreatic lineage is unclear. However, recent data demonstrating adult liver cell plasticity in culture make these cells a convenient experimental system for analyzing the individual or concerted roles of pancreatic transcription factors using a gain-of-function approach.

Ectopic PDX-1 expression in the liver induces the expression of markers characteristic of pancreatic endocrine cells, such as insulin, glucagon, and somatostatin, as well as well as numerous pancreatic transcription factors expression (Ber et al., 2003; Sapir et al., 2005). Moreover, ectopic NEUROD-1 expression activates the expression of pancreatic transcription factors considered to be higher in the hierarchy of transcription factors that control pancreatic organogenesis (Kojima et al., 2003).

In type 1 autoimmune diabetes patients, only the β-cells in the pancreas are selectively destroyed; we sought to analyze whether transcription factors that are specifically expressed in pancreatic β-cells direct liver cell reprogramming preferentially along the pancreatic β-cell lineage.

NKX6.1 is a transcription factor expressed in pancreas and central nervous system (CNS) development (Habener et al., 2005; Nelson et al., 2005; Sander and German, 1997), and is broadly expressed in the early developing pancreatic bud. Its expression becomes restricted to β-cells in the adult pancreas (Iype et al., 2004; Sander et al., 2000), and its function is critical to the formation of functional insulin-producing cells, as NKX6.1-null mice have markedly fewer insulin-producing cells (Sander et al., 2000). NKX6.1 functions as both a transcriptional repressor and activator of gene expression via its N- and C-terminus domains, respectively (Iype et al., 2004). NKX6.1 possesses the capacity to activate insulin gene expression (Jorgensen et al., 1999) but suppresses glucagon gene expression in β-cells by potentially binding to the endogenous glucagon promoter (Schisler et al., 2005), and it was demonstrated to maintain the mature β-cell phenotype in a β-cell line, in part through the suppression of glucagon gene expression (Schisler et al., 2005).

The present study demonstrates that ectopic NKX6.1expression in adult human liver cells is insufficient for inducing a functional endocrine pancreatic lineage, possibly due to its restricted capacity for activating endogenous pancreatic transcription factor expression in liver cells. However, NKX6.1 promotes the PDX-1-induced reprogramming process and specifically diverts it along the β-cell lineage by both increasing insulin production and repressing glucagon expression.

Using liver as a pancreatic progenitor tissue may serve as an experimental system for unraveling fundamental, yet unknown, roles of distinct transcription factors in the network leading to pancreas development using a gain-of-function approach.

Materials and Methods

Human liver cells

Adult human liver tissues were obtained from 20 different liver specimens taken from individuals 4–40 years old. Liver tissues were used with approval from the Committee on Clinical Investigations (institutional review board). The isolation of human liver cells was performed as described (Meivar-Levy et al., 2007; Sapir et al., 2005). The cells were cultured in Dulbecco's minimal essential medium (1 g/L of glucose) supplemented with 10% fetal calf serum, 100 units/mL penicillin; 100 ng/mL streptomycin; 250 ng/mL amphotericin B (Biological Industries, Beit Haemek, Israel), EGF (20 ng/mL; Cytolab, Ltd., Israel) and nicotinamide (10 mM; Sigma, St. Louis, MO, USA) and kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Viral infection

Liver cells were infected with recombinant adenoviruses at 1000 multiplicity of infection (moi) for 5 days. The adenoviruses used in this study were as follows: *Ad-CMV-PDX-1*, *Ad-RIP-GFP* (Meivar-Levy et al., 2007; Sapir et al., 2005) *Ad-CMV-GFP* (Clontech, BD Biosciences, San Jose, CA, USA), *Ad-CMV-β-Gal*, *Ad-CMV-NKX6.1*(generous gift from Newgard, C.B., Duke University). *Ad-GLUP-Luciferae* was prepared by insertion of 350 nucleotides of the 5′-flanking region of the rat glucagon promoter (generous gift from A. Manin) driving the expression of *Firefly Luciferase* (Promega, Madison, WI, USA) into pShuttle plasmid vector of the AdEasy™ system (Stratagene, La Jolla, CA, USA). Recombinant adenoviruses were generated from the constructed pShuttle transfer vectors using the AdEasy vector system (Stratagene), according to the manufacturer's instructions. The viral particles were generated by standard protocol (He et al., 1998).

Flow cytometry

Five days after *Ad-RIP-GFP* infection, cells (5×10^5) were collected, washed twice in phosphate-buffered saline (PBS) (5 min, $1000\times g$), and resuspended in 400 μL of PBS. Flow cytometry was performed (FACSCalibur, Becton Dickinson, Heidelberg, Germany) using the CellQuest program.

Luciferase assay

Liver cells were coinfected with *Ad-GLUP-Luciferase* and *Ad-CMV-PDX1*, *Ad-CMV -NKX6.1* or *Ad-CMV-β-Gal* for 48 h. Lucifease activity was measured using Lucifersase assay System (Promega) and was normalized was normalized to total cellular protein measured by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA).

RNA isolation, RT, and RT-PCR reactions

Total RNA was isolated, cDNA was prepared and amplified as described previously (Ber et al., 2003; Sapir et al., 2005). Quantitative real-time RT-PCR was performed using ABI Prism 7000 sequence Detection system (Applied Biosystems, Foster City, CA, USA) as described previously (Aviv et al., 2009; Meivar-Levy et al., 2007; Sapir et al., 2005).

The primer sets used in this study are; ectopic rat-PDX-1, F: CCAGTT TGCAGGC TCGCTGG; R: GCTGCGTATGCA CCTCCTGC; ectopic hamster NKX6.1, F: AG AGCACGCTTG GCC TAT TC; R: GGA ACCAGACCT TGACCTGACT ARX, F:GCTGGGCCTGAGCACTTTC; R: AAAAGAGCCTGCCG AATGC; BRAIN-4, F: GGGAGTCCTTTCCGCAACC; R: GT GATGGATGATCGCACCCA; CGC, F: CCAAGATTTTGTG CAGTGGT; R: GGTAAAGGTCCCTTCAGCAT; GK, F: CAT CTCTGAGTGCATCTCCGACT; R: TCGCAGTGATGGTCTT CGT AGTA; GLUT-2, F: TCCAGCTACCGACAGCCTATT; R: CCAGCCGTCTGAAAAATGCT; INS, F: GCAGCCTTTGT GAACCAACA; R: CGGGTCTTGGGTGTGT AGAAGAAG; MAF-A, F: AGCAGCGGCACATTCTGG; R: TTGTACAGG TCC CGCTCTTTG; MAF-B, F: CGCCTCCTAGACTCGAGC AG, R: GAGTCTCCA GATGGCCTTGGT; MYT-1, F: TGAAG AATG AAGGACCGACC, R: TTTCCAGCAAAGGTTGCT CT, NEUROD-1, F:ATGACCAAATCGTACAGCGAG; R: GT TCATGGCTTCGAGGTCGT; NGN-3, F: ACCCCATTCTC TCTTCTTTTCTC CT; R: GAG GCGTCATCCTTTCTACCG; PAX-4, F: CAGAGGCACTGGAGAA AGAGTTC; R: GGGCT TGAGAC AGGCTTTAGG; PAX-6, F: CGAATTCTGCA GGT GTCCAA; R: ACAGACCCCCTCGGACAGTAAT, PC1/3, F: CTCTGGCTG CTGGCATCT; R: CTGCATATCTCGCCAG GTG; PC2, F: GAGAAGACGCAGCCTACACC; R: CTGCAA AGCCATCTTTACCC; PDX-1, F: CCATGGATGAAGTCTA CCA; R: GTGCGCGTCCGCTTGTTCTC; SCG2, F; GGAG GAATATGCTGTGGAGCTC; R: CAGCCCCAGAGATGA GGAAA; SGNE-1, F: GACCGGGTCTCAGAAGCAGATA; R: AGTCAACTCTGCCACGATGTT; SST, F: ATGATGCCCTG GAACCTGAAG; R: GCCGGGTTTGAGTTAGCAGAT.

C-peptide detection

C-peptide secretion were measured by static incubations of primary cultures of adult liver cells 3–5 days after the initial exposure to the viral treatment as described (Aviv et al., 2009; Meivar-Levy et al., 2007; Sapir et al., 2005). The glucose regulated C-peptide secretion were measured at 17.5 mM glucose, which was determined by dose-dependent analyses to maximally induce insulin secretion from transdifferentiated liver cells, without having adverse effects on treated cells function (Aviv et al., 2009; Meivar-Levy et al., 2007; Sapir et al., 2005). C-peptide secretion was detected by radioimmunoassay using human C-peptide radioimmunoassay kit (Linco Research, St. Charles, MO; <4% crossreactivity to human proinsulin). The secretion was normalized to the total cellular protein measured by a Bio-Rad protein assay kit.

Immunofluorescence

Human liver cells treated with *Ad-CMV-PDX-1*, *Ad-CMV-NKX6.1*, for 5 days were plated on glass cover slides in six-well culture plates. Three to 4 days later, the cells were fixed and stained as described (Aviv et al., 2009; Meivar-Levy et al., 2007; Sapir et al., 2005). The antibodies used in this study were: anti human C-peptide (1:200 Biodesign, ME, USA), antimouse NKX6.1 (1:6000, a generous gift from C.B. Newgard, Duke University), antirabbit PDX-1, antigoat PDX-1 (both 1:10000 a generous gift from C.V.E. Wright). Anti-secondary antirabbit IgG Cyanine (cy2) conjugated antibody 1:200, antirabbit IgG indocarbocyanine (cy3) conjugated antibody 1:200, antigoat IgG Cyanine (cy2) conjugated

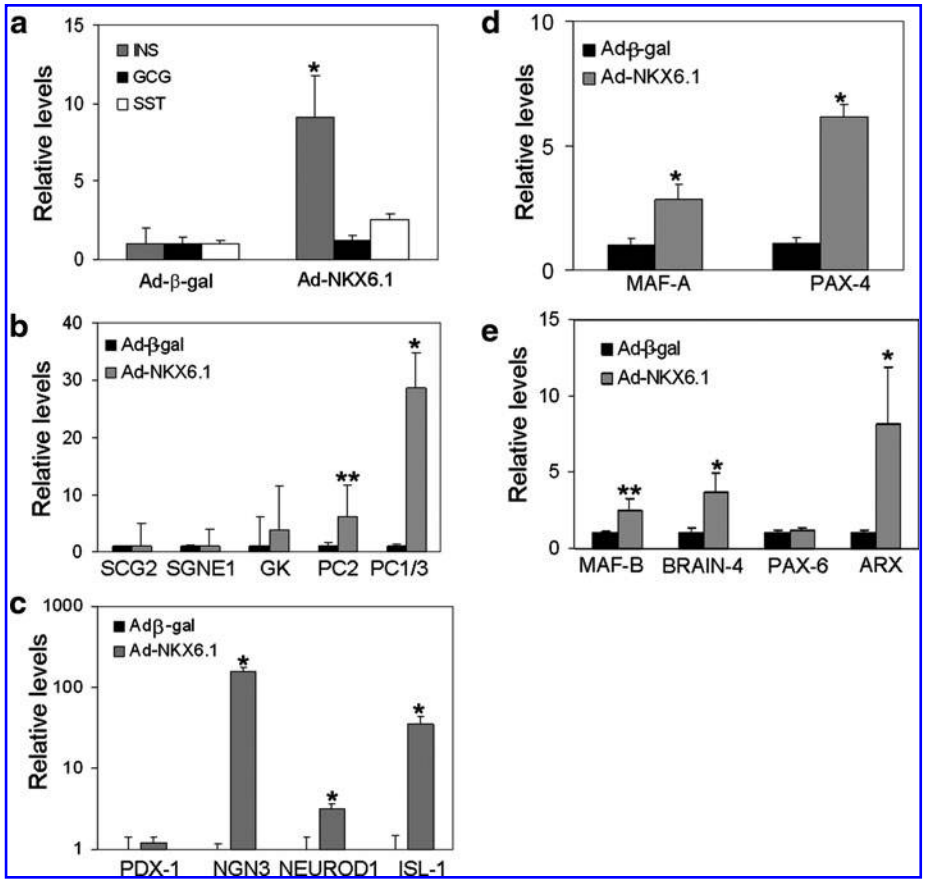

FIG. 1. Ectopic NKX6.1 expression activates a restricted pancreatic phenotype in adult human liver cells *in vitro*. Cultured adult human liver cells were infected with *Ad-CMV-NKX6* (1000 moi) or control virus (*Ad-CMV-β-gal*, 1000 moi) and analyzed by quantitative RT-PCR 5 days later: **(a)** activation of pancreatic hormone genes, **(b)** *β*-cell-specific markers, **(c)** pancreatic-specific transcription factor genes, **(d)** *β*-cell-specific transcription factor genes, and **(e)** α-cell-specific transcription factor genes. The results were normalized to *β*-actin gene expression within the same cDNA sample and are presented as the mean ± SD of the relative expression versus cells infected with the control virus *Ad-CMV-β-gal*. $n \geq 9$ in three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

antibody 1:200, antigoat IgG indocarbocyanine (cy3) conjugated antibody 1:200, and antimouse IgG indocarbocyanine (cy3) conjugated antibody 1:200 (all from Jackson Immuno-Research, West Grove, PA). Finally, the cells were stained with 4′, 6-diamidino-2-phenyl-indole (DAPI; Sigma). The slides were analyzed using a fluorescent (Provis, Olympus), or laser scanning confocal microscopy (Bio-Rad; LSM-1024). Differential interference contrast (DIC) images were analyzed using AxioImager Z1 microscope (Zeiss, Thornwood, NY, USA) with ApoTom.

Statistical analyses

Statistical analyses were performed with a two-sample Student *t*-test assuming unequal variances.

Results

NKX6.1 activates the txpression of pancreatic markers in adult human liver cells

NKX6.1 expression by recombinant adenoviruses resulted in high expression of the ectopic gene (Fig. 2a) and the nuclear protein in 79% of the liver cells in culture (*Ad-CMV-NKX6.1*) (Fig. 2c).

Molecular analyses revealed that NKX6.1 modestly activates insulin gene expression to levels that are 10-fold increased over the control *Ad-β-gal* treatment. However,the levels represent only about 0.0001% of that expressed in intact pancreatic islets (Russ et al., 2008) (Fig. 1a). None of the other pancreatic hormones were induced, and acinar marker gene expression was not observed (data not shown). NKX6.1 is sufficient to activate the prohormone convertases 1/3 (PC1/3), but many of the markers that are characteristic to β-cells, such as genes involved in granule assembly, were not activated (Fig. 1b).

This gain-of-function experiment suggested a restricted individual capacity of NKX6.1 in the activation of pancreatic markers in the liver.

NKX6.1 activates several pancreatic transcription factors in the liver, but not that of PDX-1

The concerted action of numerous transcription factors is needed for efficient activation of pancreatic specific markers.

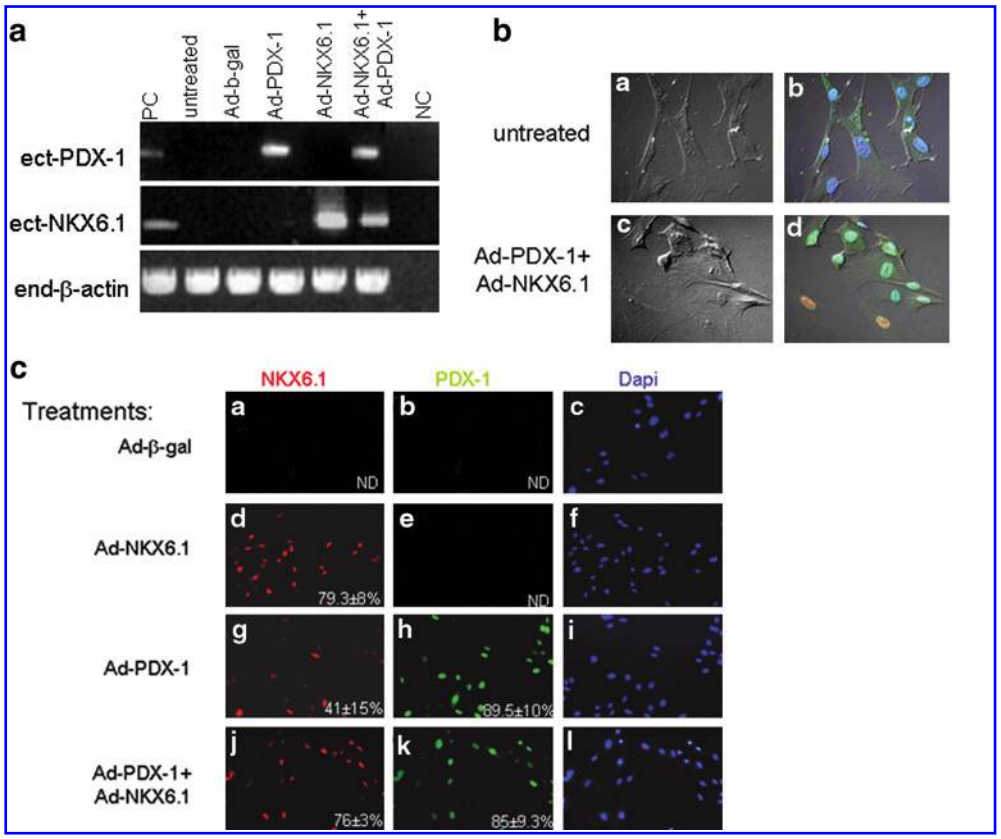

FIG. 2. Ectopic NKX6.1 expression does not induce PDX-1 expression in human liver cells. Adult human liver cells infected with *Ad-CMV-β-gal, Ad-CMV-NKX6,* or *Ad-CMV-PDX*-1, or coinfected with *Ad-CMV-NKX6* and *Ad-CMV-PDX*-1, all at 1000 moi. RNA was extracted and the levels of the ectopic genes expression (rat-PDX-1 or hamster-NKX6.1) were analyzed by RT-PCR. **(a)** Representative results of Ethidium Bromide staining of agarose separated PCR products. PC; positive control, C; negative control. **(b)** Differential Interference Contrast images **(a, c)** and IMF images **(b, d)** using anti-PDX-1 (red) + anti-NKX6.1 (green) of untreated liver cells **(a, b)** or cells coinfected with *Ad-CMV-NKX6.1* and *Ad-CMV-PDX*-1 **(c, d)**. **(c)** Double Immunofluorescence analyses for NKX6.1 (red**; a, d, g, j**) and PDX-1 (green; **b, e, h, k**) in adult human liver cells infected with *Ad-CMV-β-gal,* **(d–f)** *Ad-CMV-NKX6,* or **(g–i)** *Ad-CMV-PDX*-1, or **(j–l)** coinfected with *Ad-CMV-NKX6* and *Ad-CMV-PDX*-1. The nuclei were stained using DAPI (blue) at original magnification×200 **(a–l)**. The numbers in the right lower corner represent the percentile of positive cells, as average ± SD, $n > 3$. ND not detected.

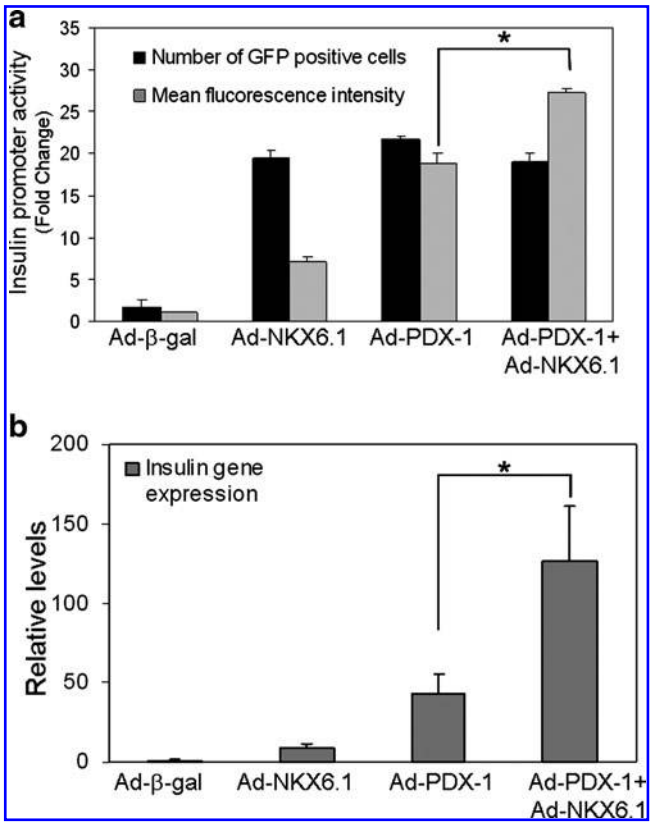

FIG. 3. Ectopic coexpression of NKX6.1 and PDX-1 in human liver cells *in vitro* promotes the activation of insulin expression. Cultured adult human liver cells were infected with *Ad-CMV-PDX-1* and/or *Ad-CMV-NKX6.1* supplemented with adenoviruses carrying a *GFP* reporter gene under the control of the insulin promoter: (*Ad-RIP-GFP*). **(a)** GFP fluorescence of the infected liver cells. Results are expressed as the ratio GFP fluorescence observed in each treatment compared to control *Ad-RIP-GFP*-treated cells. Data are presented as the mean ± SD; $n \geq 6$ in two different experiments. **(b)** Quantitative RT-PCR analysis of insulin gene expression. The results were normalized to β-actin gene expression within the same cDNA sample and are presented as the mean ± SD of the relative expression versus cells infected with the control virus *Ad-CMV-β-gal*. $n \geq 9$ in three independent experiments. $^*p < 0.05$.

To explain the restricted capacity of NKX6.1 to activate pancreatic markers in liver cells, we analyzed the profile of pancreatic transcription factors activated by its ectopic expression in liver. NKX6.1 activated the expression of several transcription factor genes, some of which may precede its expression during embryonic pancreas organogenesis, such as ISl-1, NGN-3, and to a lower extent NEUROD-1, and PAX-4 (Fig. 1c and d). Moreover, NKX6.1 also modestly activated the expression of MAF-B, BRAIN- 4, and ARX, which is restricted to pancreatic β-cells in the adult pancreas (Fig. 1e). However, NKX6.1 did not activate endogenous PDX-1 expression in the liver cells (Fig. 1c and 2c).

These data suggest that NKX6.1 plays instructive roles in activating several otherwise silent pancreatic transcription factors in liver cells, even in the absence, or at undetectable levels, of PDX-1. However, the incapacity of NKX6.1 to activate PDX-1 and profound expression of β-cell-like markers is not related to its potential low expression levels. Ectopic, individual NKX6.1 expression is higher than its levels upon coexpression with PDX-1 or of that expressed in β-cell lines (Fig. 2a). Immunofluorescence analyses demonstrate that although expressed in 79.3% of the cells, ectopic NKX6.1 (Fig. 2c(d–f)) does not activate PDX-1 (Fig. 2c(e)), whereas ectopic PDX-1 expression (Fig. 2c(g–i)) activates the endogenous NKX6.1 nuclear factor in 41% of the liver cells (Fig. 2c(g)).

Other factors, such as NGN-3 and NEUROD-1 were documented to activate the endogenous PDX-1 expression in extra-pancreatic experimental systems (Kojima et al. 2003; Yechoor et al. 2009).

Complementing NKX6.1 treatment with ectopic PDX-1 expression enhances β-cell lineage differentiation and function in the liver

Because PDX-1 plays a crucial role in pancreatic organogenesis, β-cell function (Marshak et al., 2001; Melloul, 2004; Offield et al., 1996), and reprogramming extra-β-cells to be insulin-producing cells in numerous experimental models (Cao et al., 2004; Fodor et al., 2007; Horb et al., 2003; Imai et al., 2005; Jin et al., 2007; Kaneto et al., 2005a, 2005b; Kojima et al., 2003; Li et al., 2005; Miyatsuka et al., 2003; Muniappan et al., 2007; Song et al., 2007; Tang et al., 2006a, 2006b; Wang et al., 2007; Yamada et al., 2006; Yatoh et al., 2006; Zalzman et al., 2003, 2005), we analyzed the molecular and functional consequences of PDX-1 ectopic complementation in NKX6.1-treated cells.

Coexpression of NKX6.1 and PDX-1 did not alter the cells morphology (nor did each of the factors alone, data not presented) (Fig. 2b), but promoted liver reprogramming along the pancreatic lineage. This combination increased the intensity of insulin promoter activity (Fig. 3a, mean fluorescence intensity), without increasing the number of liver cells capable of activating this promoter (Fig. 3a) (number of GFP-positive cells), compared to cells treated by either PDX-1 or NKX6.1 alone. Insulin gene expression was substantially 15-fold increased in adult human liver cells coinfected with the two recombinant adenoviruses compared to cells treated with NKX6.1 and threefold increased compared to PDX-1 alone (Fig. 3b). However, the levels of insulin gene expression in reprogrammed cells constitutes only about 0.1% of that in freshly isolated islets (Ouziel-Yahalom et al. 2006; Sapir et al. 2005). Importantly, the insulin gene expression in reprogrammed liver cells is higher than that of a monolayer culture of isolated human islets at similar passages (Ouziel-Yahalom et al. 2006; Russ et al. 2008), which may serve as a better control. Taken together, these data suggest a potential obligatory role of PDX-1 in the induction of an active β-cell-like lineage in liver cells, and a potential promoting role for NKX6.1 in this process. Because NKX6.1 promoted both insulin promoter activity and gene expression without increasing the number of PDX-1 reprogrammed liver cells, it is suggested that NKX 6.1 may increase the maturation of PDX-1 induced transdifferentiated liver cells along the β-cell lineage.

NKX6.1 preferentially promotes the PDX-1-induced β-cell phenotype and function in adult human liver cells

The higher state of β-cell differentiation in cells treated with both PDX-1 and NKX6.1 was manifested in a 60%

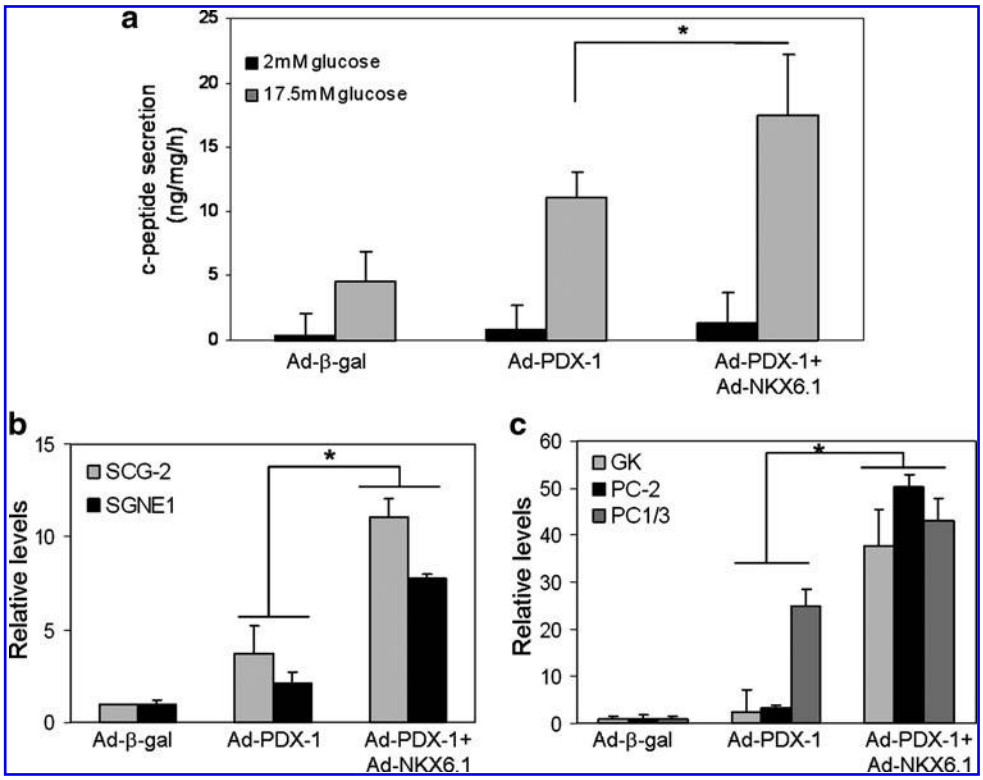

FIG. 4. Coexpression of *PDX-1* and *NKX6.1* improves glucose-regulated C-peptide secretion and increases the expression of *β*-cell-related genes in human liver cells. **(a)** C-peptide secretion at 2 and 17.5 mM glucose in response to *Ad-CMV-PDX-1* alone or in combination with *Ad-CMV-NKX6.1*; $n \geq 7$ in four different experiments. **(b)** Quantitative RT-PCR analyses of SGNE1 and SCG2 or **(c)** GK, PC2, and PC1/3 gene expression. CT (threshold cycle) values are normalized to *β*-actin gene expression within the same cDNA sample ($n \geq 10$ in three independent experiments). Results are presented as the fold increase in the mean ± SD compared to untreated control liver cells. *$p < 0.01$.

increase in C-peptide secretion and improved glucose sensing compared to PDX-1-treated liver cells (Fig. 4a).

To explain the contribution of NKX6.1 to PDX-1-induced *β*-cell-like function, we analyzed its effect on promoting the expression of *β*-cell-specific markers. Along with the profound increase in glucose-regulated C-peptide secretion, was an increase in the expression of *β*-cell specific genes that participate in granule assembly (SGNE1 and SCG-2; Fig. 4b), insulin processing (PC2 and PC1/3; Fig. 4c), and glucose sensing (GK; Fig. 4c).

Moreover, we examined the profile and extent of pancreatic transcription factors in cells treated with both PDX-1 and NKX6.1 compared to PDX-1 treatment alone. As previously reported, PDX-1 alone induced the expression of most of the pancreatic transcription factor genes, including NGN3, NEUROD1, ISL-1, PAX4, and MAF-A (Aviv et al., 2009; Meivar-Levy et al., 2007; Sapir et al., 2005) (Fig. 5a). However, coexpression of PDX-1 and NKX6.1 in liver cells significantly augmented the activation of all of these pancreatic transcription factor genes (Fig. 5a), whereas NKX6.1 alone activated only a few of these transcription factors (Fig. 1c and d).

Notably, the expression of MYT1 was induced only by the ectopic expression of both transcription factors (Fig. 5b). MYT1 is a possible downstream target of NKX6.1 (Sander et al., 2000) and has been suggested to function as a cofactor of NGN3 in promoting pancreas development in the embryo (Wang et al., 2008). These data taken together suggest a reciprocal promoting effect of PDX-1 and NKX6.1 in activating pancreatic transcription factors expression, which may have collectively contributed to the increased pancreatic marker expression and improved *β*-cell-like function.

Coexpression of NKX6.1 and PDX-1 activates the β-cell lineage in liver cells while decreasing PDX-1-induced glucagon expression

Both PDX-1 and NKX6.1 have been suggested to be a detriment to the expression of the glucagon gene in *β*-cells by either protein–protein interactions between PDX-1 and PAX-6 or competitive binding of NKX6.1 to the PAX-6 binding site on the G1 element of the glucagon promoter (Gauthier et al., 2007). Forced expression of NKX6.1 in insulinoma cell line subclones represses the leaky glucagon gene expression and improves the glucose sensing for insulin secretion from these subclones (Schisler et al., 2005). In liver reprogramming, PDX-1 not only induces the *β*-cell lineage, as demonstrated by increased *β*-cell specific transcription factor expression, but also activates glucagon gene expression (Aviv et al., 2009; Meivar-Levy et al., 2007; Sapir et al., 2005). Thus, we analyzed whether NKX6.1 also turns from being a glucagon repressor in the pancreatic milieu into an activator of glucagon gene expression when ectopically expressed in adult liver cells. In PDX-1-treated liver cells, NKX6.1 both

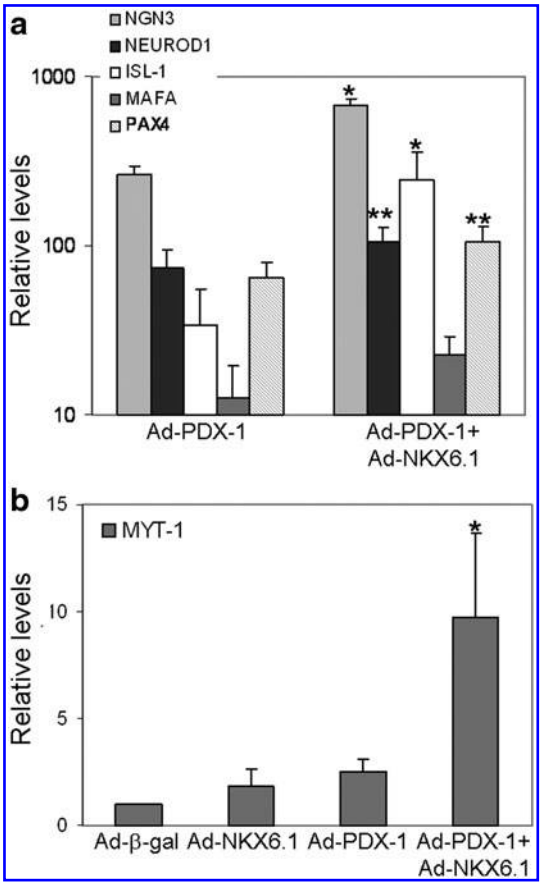

FIG. 5. Ectopic coexpression of PDX-1 and NKX6.1 in liver cells activates the expression of pancreatic transcription factor genes. **(a)** Quantitative RT-PCR analyses of NGN3, NEUROD-1, PAX-4, MAF-A, ISL-1, and **(b)** MYT-1 expression. CT (threshold cycle) values are normalized to β-actin gene expression within the same cDNA sample ($n \geq 9$ in three independent experiments). Results are presented as the fold increase in the mean ± SD compared to untreated liver cells. $^{*}p < 0.01$, $^{**}p < 0.05$.

repressed the glucagon promoter and decreased PDX-1-induced activation and glucagon gene expression (Fig. 6a and b, respectively). Glucagon gene repression by NKX6.1 in PDX-1-treated cells was not associated with a decrease in α-cell differentiation. The expression of NKX6.1 did not decrease α-cell-specific transcription factors; on the contrary, it modestly promoted the expression of these factors when combined with ectopic PDX-1 expression (Fig. 6c). These findings suggest that the inhibition of glucagon gene expression by NKX6.1 resides at the level of glucagon promoter activation, as suggested by Gauthier et al. (2007), without preventing α-cell lineage differentiation.

Discussion

Our data suggest that adult human liver cells in culture constitute a useful experimental system for analyzing the individual and concerted roles of pancreatic transcription factors by a gain-of-function approach. Using this system, we demonstrate a restricted individual role of NKX6.1 in the activation of pancreatic marker expression. NKX6.1 activated several pancreatic transcription factors that are expressed in the early stages of pancreatic development, such as NGN-3 and ISL-1, which mark pancreatic endocrine progenitors. The activation of NGN-3 and ISL-1 expression in liver may suggest an individual potential role for NKX6.1 in activating pancreatic progenitors in extra-pancreatic tissues. Ectopic NKX6.1 expression very modestly activated insulin but not other pancreatic hormones. Interestingly, NKX6.1 did not activate endogenous PDX-1 in liver cells, which in turn may explain the restricted reprogramming capacity of NKX6.1 in liver. Due to the central role of PDX-1 in pancreatic organogenesis, adult β-cell function, and reprogramming along the endocrine pancreatic lineage, we analyzed the developmental consequences of complementing its ectopic expression with that of NKX6.1.

Combined PDX-1 and NKX6.1 expression resulted in the generation of functional β-like function in the liver that was higher than that of PDX-1 alone. The improved β-cell-like function is manifested by increased insulin promoter activity, insulin gene expression, and augmented insulin production, and pro-hormone processing. Finally, NKX6.1 improved glucose-regulated C-peptide secretion compared to liver cells treated with PDX-1 alone. The improved β-cell-like function was associated with an increased expression of markers that are associated with pro-hormone processing, vesicle assembly, glucose sensing, and an increase in specific pancreatic transcription factors expression. However, the β-cell like phenotype and function was not associated by increased numbers of reprogrammed liver cells (Fig. 3). These data may suggest an additional role for NKX6.1 in the maturation of PDX-1 reprogrammed liver cells, along the β-cell-like lineage.

Along with promoting the β-cell lineage, NKX6.1 repressed glucagon gene expression induced by PDX-1 without repressing α-cell-specific transcription factor expression, which suggests that NKX6.1 suppresses glucagon gene expression at the promoter level as previously suggested (Gauthier et al., 2007).

Schisler et al. (2005) suggested that NKX6.1 maintains the mature β-cell phenotype in dedifferentiated insulinoma cell lines clones (INS), in part through participation in suppression of glucagon expression. Poorly differentiated insulinoma cells clones were characterized by low NKX6.1 expression, whereas overexpression of NKX6.1 in these cells decreased glucagon mRNA levels and improved insulin production and its glucose regulated secretion (Schisler et al., 2005). A similar process may occur also in PDX-1-treated liver cells. PDX-1-induced reprogrammed cells are expected to be at heterogeneous level of differentiation along the β-cell-like lineage and function. Overexpression of NKX6.1 in poorly differentiated reprogrammed cells could have promoted these cells maturation along the β-cell-like lineage and at the same time decrease glucagon gene expression. An additional role that was suggested for NKX6.1 in promoting β-cells proliferation (Schisler et al., 2008) was not recapitulated in reprogrammed liver cells. NKX6.1 did not increase liver cells proliferation either when expressed alone or in combination with PDX-1 (BrdU, data not presented). This is in agreement with the data in Figure 3, which suggest a reprogramming promoting effect of NKX6.1, which is not associated by increased number of reprogrammed cells.

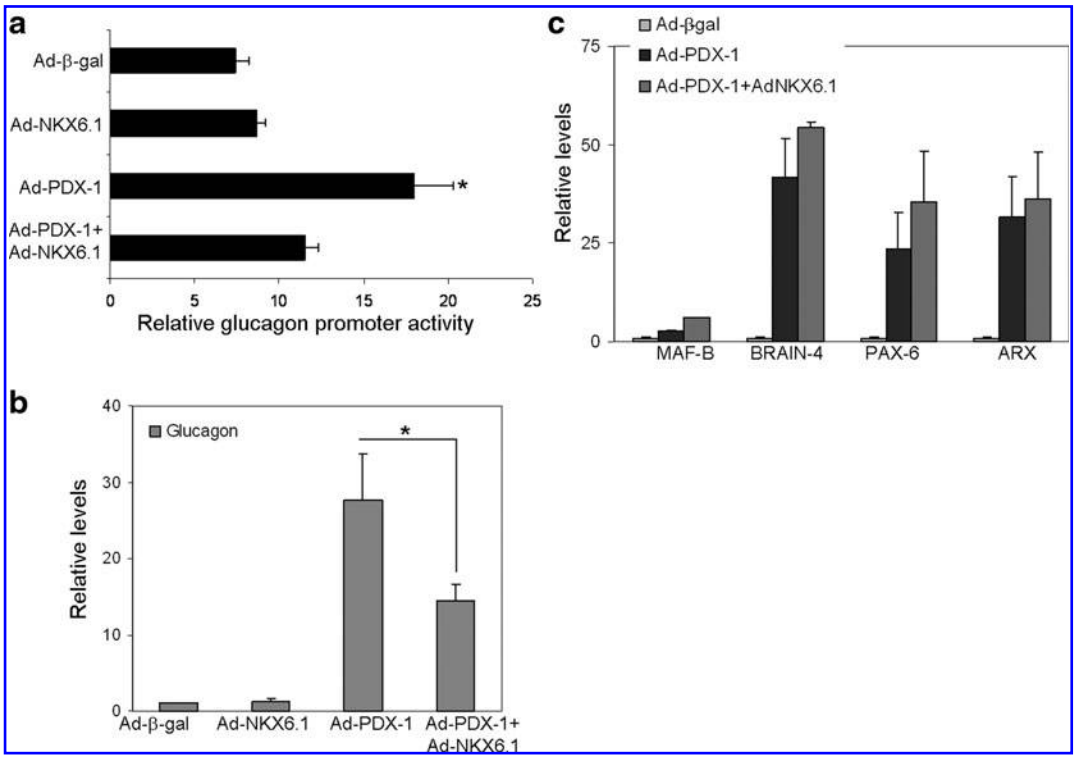

FIG. 6. Ectopic coexpression of PDX-1 and NKX6.1 induces β-cell differentiation in liver cells while decreasing glucagon expression. Cultured adult human liver cells were infected with *Ad-CMV-PDX-1* and/or *Ad-CMV-NKX6.1* supplemented with adenoviruses carrying a reporter gene under the control of the glucagon promoter: *Ad-GluP-Luciferase.* **(a)** Luciferase activity of the infected liver cells expressed as the ratio of luciferase activity observed in each treatment compared to the control treated with only *Ad-GluP-Luciferase.* Each data point represents the mean ± SE of at least three independent experiments ($n = 9$). **(b)** Quantitative RT-PCR analyses of glucagon and **(c)** MAF-B, BRAIN-4, ARX, and PAX-6 gene expression. CT (threshold cycle) values are normalized to β-actin gene expression within the same cDNA sample ($n \geq 10$ in three independent experiments). Results are presented as the fold increase of the mean ± SD compared to control treated liver cells. $^*p < 0.05$.

These data may suggest that not all the β-cell-specific characteristics are recapitulated in the reprogrammed liver cells.

The role of NKX6.1 expression in the developing pancreas is controversial. Traditionally, NKX6.1 was suggested to be crucial for terminal differentiation of β-cells (Sander et al., 2000), but recent studies have demonstrated that ectopic NKX6.1 expression in PDX-1-positive cells prior to NGN3 activation rescues the β-cell phenotype in NKX6.1−/−mice (Henseleit et al., 2005; Nelson et al., 2007). NKX6.1 expression, which precedes that of NGN3, suggests that it may play an additional role in pancreatic development prior to the determination of β-cell fate. This concept is in agreement with our findings; we demonstrated that NKX6.1 alone is capable of activating both NGN-3 and ISL-1, which are known to be expressed in the early stages of pancreatic organogenesis. Moreover, our data suggest that this activation occurs also in the absence of PDX-1 expression in liver cells.

The ability of the combined NKX6.1 and PDX-1 treatment to promote endocrine pancreas differentiation in liver cells compared to the effect of PDX-1 alone can be attributed in part, also to their unique concerted effect in inducing *de novo* MYT1 expression. Neither PDX-1 nor NKX6.1 alone activated MYT1 expression in liver cells. Previous studies suggested that MYT1 and NGN3 positively regulate each other (Wang et al., 2008), and, along with the activation of MYT1 in PDX-1 and NKX6.1-treated liver cells, the expression of NGN3 and other pancreatic transcription factors increased in the present study over the expression induced by each of the factors alone. This increase in pancreatic transcription factor expression by the concerted action of PDX-1 and NKX6.1 is best exemplified in the twofold activation of NGN3 expression and six- to sevenfold increase in ISL-1 expression compared to either NKX6.1 or PDX-1 alone. A role for NGN3 expression in the induction of the trans-determination of hepatic precursor cells along the β-cell lineage in mice livers was recently demonstrated (Yechoor et al., 2009).

In summary, our data demonstrate that in the hepatic milieu, NKX6.1 plays a dual role in individually activating early pancreatic progenitor markers and in promoting the β-cell-like maturation of PDX-1 reprogrammed cells in the expense of glucagon gene expression. The decrease in glucagon gene expression by NKX6.1 is not associated by α-cells dedifferentiation, because the expression of α-cell-specific transcription factors is even slightly increased by the concerted action of both factors. The individual and concerted roles of additional pancreatic transcription factors in activating pancreatic lineage and function in adult human liver cells will be further analyzed. Primary culture of adult liver cells may prove a useful experimental system in unraveling distinct steps in pancreatic differentiation using a gain-of-

function approach, in addition to its potential therapeutic merit in cell replacement therapy for diabetic patients.

Acknowledgments

This work was supported, in part, by Juvenile Diabetes Research Foundation Grant 1-2006-221, Israel Science Foundation Grant ISF -99-05, and EFSD-Lilly award 2005 (all to S.F.).

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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