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Conversion of Immortal Liver Progenitor Cells Into Pancreatic Endocrine Progenitor Cells by Persistent Expression of Pdx-1

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Abstract The conversion of expandable liver progenitor cells into pancreatic beta cells would provide a renewable cell source for diabetes cell therapy. Previously, we reported the establishment of liver epithelial progenitor cells (LEPCs). In this work, LEPCs were modified into EGFP/Pdx-1 LEPCs, cells with stable expression of both *Pdx-1* and *EGFP*. Unlike previous work, with persistent expression of Pdx-1, EGFP/Pdx-1 LEPCs acquired the phenotype of pancreatic endocrine progenitor cells rather than giving rise to insulin-producing cells directly. EGFP/Pdx-1 LEPCs proliferated vigorously and expressed the crucial transcription factors involved in beta cell development, including *Ngn3*, *NeuroD*, *Nkx2.2*, *Nkx6.1*, *Pax4*, *Pax6*, *Isl1*, *MafA* and endogenous *Pdx-1*, but did not secrete insulin. When cultured in high glucose/low serum medium supplemented with cytokines, EGFP/Pdx-1 LEPCs stopped proliferating and gave rise to functional beta cells without any evidence of exocrine or other islet cell lineage differentiation. When transplanted into diabetic *SCID* mice, EGFP/Pdx-1 LEPCs ameliorated hyperglycemia by secreting insulin in a glucose regulated manner. Considering the limited availability of beta cells, we propose that our experiments will provide a framework for utilizing the immortal liver progenitor cells as a renewable cell source for the generation of functional pancreatic beta cells. J. Cell. Biochem. 104: 224–236, 2008. © 2007 Wiley-Liss, Inc.

Key words: liver progenitor cells; beta cells; transplantation; differentiation; pancreatic and duodenal homeobox gene 1

Islet transplantation represents an ideal therapeutic approach for treatment of type 1 diabetes. However, the scarcity of donor islets has precluded the widespread adoption of islet transplantation. The liver and pancreas have an intimate relationship during embryogenesis. It has been suggested that the two organs are derived from common progenitor cells and the conversion between liver and pancreas has been described in animal experiments and human pathology [Deutsch et al., 2001; Grompe, 2003; Shen et al., 2003]. Considering the limited availability of islet cells, conversion of liver cells into insulin-producing cells has drawn great attention. Ferber et al. [2000] first reported that transient adenovirus-mediated expression of pancreatic and duodenal homeobox gene 1 (*Pdx-1*) in hepatocytes activated the expression of endogenous insulin and ameliorated hyperglycemia in diabetic mice treated with streptozotocin (STZ) [Ber et al., 2003]. Recently, reprogramming of a range of liver cell types into pancreatic cells in vitro by forced expression of

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Pdx-1 has also been reported [Yang et al., 2002; Zalzman et al., 2003, 2005; Cao et al., 2004; Li et al., 2005; Sapir et al., 2005; Tang et al., 2006a,b; Fodor et al., 2007].

Although the ability of *Pdx-1* to provoke the conversion of liver cells into pancreatic phenotypes is being intensively investigated, whether the expression of Pdx-1 alone is sufficient to induce the conversion of liver cells into beta cells remains in dispute [Wu et al., 2007]. Of particular note, immortal liver progenitor cells are highly proliferative, and this may limit their capacity to differentiate into beta cells. In this work, we aim to investigate the potential of immortal liver progenitor cells as a renewable source of pancreatic beta cells.

Liver epithelial progenitor cells (LEPCs), the liver stem-like cell lines, were established in our lab as described [Li et al., 2006]. LEPCs are immortal liver progenitor cells with a normal diploid karyotype. The present studies demonstrated that Pdx-1 expression alone was insufficient to convert LEPCs into functional beta cells directly. LEPCs with persistent expression of *Pdx-1* acquired the phenotypes of pancreatic endocrine progenitor cells, which proliferated actively and expressed the crucial transcription factors involved in beta cell development, but did not secrete insulin. However, these cells could give rise to functional beta cells after treatment with high glucose/low serum medium containing a cytokine cocktail (including EGF, bFGF, and HGF) or when exposed to the in vivo diabetic microenvironment. Because of the limited availability of insulin-producing tissues and the difficulty of amplifying pancreatic beta cells in vitro, a renewable source of stem/progenitor cells must be developed before any kind of islet-precursor cells can be used therapeutically. The present studies suggest that immortal liver progenitor cells could be a renewable source of functional beta cells.

MATERIALS AND METHODS

Vector Construction

Retroviral vector *pMSCV Pdx-1 puro* was constructed by ligating the *Bgl*II/*Xho*I fragment of *pMSCV puro* (Clontech, Palo Alto, CA) and the mouse *Pdx-1* fragment released from *pEGFP Pdx-1* vector by *Bam*HI/*Xho*I digestion [Chen et al., 2006]. To construct *pPdx-1 EGFP*, the *Pdx-1* promoter (Accession No. AF192495) was amplified by PCR from mouse tail genomic DNA by primers 5′-ATGCATAAATGCAGG-TAAAACATTC-3′ and 5′-AAGCCAGGCCTTA-GGCGCTG-3′. The 4643-bp PCR product was cloned into pUCm T vector (Sangon Co., Shanghai, China) and the sequence was confirmed by sequence analysis. The *Pdx-1* promoter was released by *Not*I/*Bam*HI digestion and then ligated with the CMV promoter-removing fragment of *pEGFP N1* vector (Clontech).

Cell Culture, Cell Transfection, and Western Blot Analysis

LEPCs were derived from regenerative liver, showing distinct ductular reactions [Li et al., 2006]. LEPCs represent the cultured counterpart of liver stem-like cells with the potential to give rise to both mature hepatocytes and biliary epithelial cells. The cells were maintained in DMEM HG (high glucose; Gibco BRL, Grand Island, NY), 1% penicillin/streptomycin (Gibco BRL), 1 mM Glutamax (Gibco BRL) and 10% FBS (Hyclone Laboratories, Logan, UT) at 37°C with 5% CO_2.

pPdx-1 EGFP, used as a reporter construct, was introduced into LEPCs by electroporation. After 2 weeks selection by G418 (800 μg/ml), the individual G418 resistant clones, named EGFP LEPCs, were picked up by cloning cylinder (Corning Life Sciences, Acton, MA) and subcultured. *pMSCV PDX-1 puro* was introduced into Phoenix A retroviral package cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA). Retrovirus-rich DMEM media harvested from the late exponential phase Phoenix packaging line was sterilized through 0.45 μm filters (Millipore, Molsheim, France). Fresh retrovirus-containing media supplemented with 4 μg/ml polybrene (Sigma Aldrich, St. Louis, MO) was added to 20% confluent single-cell-derived EGFP LEPCs cultures. Virus-containing media was aspirated 12 h later and replaced with fresh DMEM supplemented with 10% FBS. 48 h later, selective media, containing 2 μg/ml puromycin (Sigma Aldrich), was added. Two weeks later, individual puromycin resistant clones, named EGFP/Pdx-1 LEPCs, were picked up by clone cylinder and subcultured. EGFP/Pdx-1 LEPCs proliferated as small, polygonal cells with a high nuclear/cytoplasmic ratio. The single-cell-derived EGFP/Pdx-1 LEPCs, showing homogeneous green fluorescence, were subjected to the following studies.

To confirm the expression of *Pdx-1* in EGFP/Pdx-1 LEPCs, the cellular proteins from each cell line were separated by SDS–PAGE using 10% Tris–HCl gels and transferred to a nitrocellulose membrane. Proteins were blotted with rabbit anti-Pdx-1 antibody (1:5,000 Chemicon, Temecula, CA), followed by HRP-conjugated goat anti-rabbit IgG (Sigma Aldrich), and then visualized by SuperSignal Western blotting luminol reagents (Pierce Biotechnology, Inc., Rockford, IL).

In Vitro Differentiation

1×10^3 cells/cm^2 EGFP LEPCs or EGFP/Pdx-1 LEPCs were plated on 60 mm dishes and cultured in DMEM HG media containing 10% FBS for 24 h. To induce differentiation, the cells were switched to serum-free DMEM HG media (containing 23 mM glucose) for 72 h, then to DMEM HG media containing epidermal growth factor (EGF, 10 ng/ml, Sigma Aldrich), hepatocyte growth factor (HGF, 20 ng/ml, Chemicon), ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, Sigma Aldrich), basic fibroblast growth factor (bFGF, 10 ng/ml, Chemicon), Nicotamide (10 mM, Sigma Aldrich), L-proline (0.3 mg/ml, Sigma Aldrich) and 2% FBS. The media was changed every 2–3 days for 3 weeks. In the third week, the ITS was omitted from the media to eliminate the possibility of insulin uptake by the cultured cells. Then the cells were cultured in serum-free DMEM HG media for 12 h to finish the induction. To elucidate the effects of FBS and cytokines on beta cell differentiation from EGFP/Pdx-1 LEPCs, EGFP/Pdx-1 LEPCs were also treated by an induction protocol with either the medium containing 10% FBS and cytokine cocktail or the medium containing 2% FBS but no supplements of EGF, bFGF, or HGF.

Fluorescence-Activated Cell Sorter Analysis

Briefly, after treatment, the cells were fixed in cold 70% ethanol for 30 min and washed twice in PBS. Then the cells were incubated for 30 min in 200 μl propidium iodide solution (containing 50 μg/ml propidium iodide, 0.3% Triton X-100 and 20 μg/ml RNase) at 37°C. All samples were analyzed on a FACStar flow cytometer (Becton Dickinson).

Glucose-Stimulated Insulin Secretion Assay

Briefly, after in vitro induction, the cells were preincubated for 1 h in Krebs–Ringer buffer (KRB) with 2.8 mM glucose, followed by incubation for 2 h in KRB containing 2.8 mM glucose and then incubation for 2 h in KRB containing 11.1 mM glucose. The amount of insulin in the incubation buffer was determined by ELISA using the Rat/Mouse Insulin ELISA Kit (Linco Research, St. Charles, MO) according to the manufacturer's manual.

RT-PCR Analysis

Total RNA was extracted from cells using the EZ-10 spin column RNA purification kit (BBI, Markham, ON, Canada) according to the manual. To eliminate genomic DNA contamination, total RNA was digested with DNase I (TaKaRa, Dalian, China) and then subjected to another round of purification by EZ-10 spin column RNA purification kit. Reverse transcription (RT) was performed with 2 μg RNA, random nonamers (TaKaRa), and murine Moloney leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's manual. The polymerase chain reaction (PCR) conditions were 95°C for 5 min, 94°C for 30 s, annealing temperature for 40 s, and 72°C for 70 s, 30–38 cycles, and then 72°C for 15 min. Of note, GC buffer II (TaKaRa) was used for amplification of *MafA*. Forward and reverse primers used for specific amplification were designed to be located in different exon(s) except *MafA* and *Ngn3* (genes have only one exon). Primer sequences and the size of the final products are provided in Table I.

Transmission Electron Microscopy

After in vitro induction, the cells were harvested with a cell scraper. For thin sections, cell aggregates were fixed with 4% phosphate-buffered paraformaldehyde (Sigma Aldrich) for 4 h at 4°C and post-fixed in 1% OsO_4 (Sigma Aldrich). Cells were then dehydrated and embedded in epoxy resin. Thin sections for electron microscopy were counterstained with uranil acetate and lead nitrate and examined with a Hitachi H-800 microscopy.

Cell Transplantation

Twelve male *SCID* mice, 8–10 weeks old, weighing 20 g were obtained from Transgenic Animal Research Center, Second Military Medical University (Specific Pathogen Free Grade). *SCID* mice were made hyperglycemic by intraperitoneal injections of STZ (Sigma Aldrich) at 180 μg/g body weight. The blood glucose levels

TABLE I. List of Primers Information for RT-PCR

Genes	Forward primer	Reverse primer	PCR size (bp)
Isl1	CACTATTTGCCACCTAGCCAC	AAATACTGATTACACTCCGCAC	255
Pdx-1	ACCAACGATAACATACCCAG	CCGAGGTCACCGCACAATCT	452
Ngn3	AGTGCTCAGTTCCAATTCCAC	AAGAAGTCTGAGAACACCAGT	558
NeuroD	GAGGCTCCAGGGTTATGAGA	ACTGGTAGGAGTAGGGATGCAC	728
Pax4	ACCCTGTGACATTTCACGGAG	GTACTCGATTGATAGAGGAC	263
Pax6	ACCAACGATAACATACCCAG	CTGAAGTCGCATCTGAGCTT	278
Nkx2.2	CATCTTGGACCTTCCGGACAC	GGCGTCACCTCCATACCTTT	545
Nkx6.1	ATCTTCTGGCCCGGAGTGATG	CAGAGAATAGGCCAAGCGTGC	214
Insulin 1	TAGTGACCAGCTATAATCAGAG	ACGCCAAGGTCTGAAGGTCC	288
Insulin 2	CCCTGCTGGCCCTGCTCTT	AGGTCTGAAGGTCACCTGCT	220
GK	GAT CAT TGG CGG AAA GTA CA	TGAAGGTGATTTCGCAGTTG	443
Glut2	GGATCTGCTCACATAGTCAC	TCTGGACAGAAGAGCAGTAG	441
Glucagon	ATTTACTTTGTGGCTGGATTG	TGTCAGTGATCTTGGTTTGAA	526
Somatostatin	CTCTGCATCGTCCTGGCTTT	CAGGATGTGAATGTCTTCCAG	310
P48	CATGCAGTCCATCAACGACG	CGATGTGAGCTGTCTCAGGA	474
PC1/3	TGATTTTGCATGGGACATCTTCTC	ACAGACTGTCTTCAGAGCCTTC	396
PC2	GAGACCCGTCTTCACGAATC	GTTGAACCAGTCATCTGTGTATCG	531
IAPP	CCACTTGAGAGCTACACCTG	GGATTCCCTATTTGGATCC	205
SUR1	GCCTTCGTGAGAAAGACCAG	GAAGCTTCTCCGGTTTGTCA	217
Kir6.2	TTGGAAGGCGTGGTAGAAAC	GGACAAGGAATCTGGAGAGAT	309
Pancreatic polypeptide	AGGATGGCCGTCGCATACTG	GAGCTGCACTCCAGGAAGTC	249
MafA	CGCAGGCCACCACGTGCGCTTGGAGGAG	CTGCGCTGGCGAGGGCTCCCGAGGGAAG	370
Albumin	CATGACACCATGCCTGCTGAT	CTCTGATCTTCAGGAAGTGTAC	451
GS	AGTTACCTGAGTGGAACTTTG	TTCGCACACCCGATGCAAGAT	523
GAPDH	AAC TTT GGC ATT GTG GAA GG	ACA CAT TGG GGG TAG GAA CA	222

Isl1, ISL1 transcription factor, LIM/homeodomain; *Pdx-1*, pancreatic and duodenal homeobox factor 1; *Ngn3*, neurogenin 3; *NeuroD*, neurogenic differentiation; *Pax4*, paired box gene 4; *Pax6*, paired box gene 6; *Nkx2.2*, NK2 transcription factor related, locus 2; *Nkx6.1*, NK6 transcription factor related, locus 1; *GK*, glucokinase; *Glut2*, glucose transporter 2; *PC1/3*, prohormone convertase 1/3; *IAPP*, islet amyloid polypeptide; *SUR1*, sulfonylurea receptor 1; *Kir6.2*, potassium inwardly rectifying channel, subfamily J, member 11; *MafA*, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A; *GS*, glutamine synthetase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

were monitored daily by using an Accu-CHEK glucose detector (Roche Diagnostics, Mannheim, Germany). The *SCID* mice with blood glucose levels >350 mg/dl were transplanted with 2×10^6 EGFP LEPCs (n = 3) and EGFP/Pdx-1 LEPCs (n = 6) into the left subrenal capsule spaces. The same volume of PBS was transplanted into the diabetic mice as a negative control (n = 3). The blood glucose levels were monitored every 2 days post-transplantation after 6 h of fasting at the end of their dark (feeding) cycle. The mice transplanted with EGFP/Pdx-1 LEPCs (n = 6) were killed at 6 weeks post-transplantation. Left kidneys were removed for detecting insulin-producing cells using immunohistochemical analysis and for detecting the possibility of teratomas formation. All animal procedures were performed in accordance with institutional guidelines and the Principles of Laboratory Animal Care issued by the NIH.

Intraperitoneal Glucose Tolerance Testing (IPGTT)

Before they were killed, the mice (n = 3) implanted with EGFP/Pdx-1 LEPCs were fasted for 6 h by removal to a clean cage without food at the end of their dark (feeding) cycle. After 6 h of fasting, a fasting glucose level was obtained from the venous blood from a small tail clip. Then, 1 mg/g body weight glucose was injected intraperitoneally and the blood glucose values were obtained at 5, 15, 30, 60, and 120 min after injection.

Immunocytochemistry and Immunohistochemistry

The cells, growing on a cover glass, were fixed in cold 4% phosphate-buffered paraformaldehyde for 30 min. Fixed cells were incubated with monoclonal rat anti-insulin antibody (1:200, R&D Systems Inc., Minneapolis) or goat anti-rat C-peptide antiserum (1:500, Linco Research) for 16 h at 4°C, and then visualized with HRP-conjugated goat anti-rat IgG (1:200, KPL, Gaithersburg, MD) or Alex-555 conjugated rabbit anti-goat IgG (Molecular Probes, Eugene, OR).

The removed kidneys from EGFP/Pdx-1 LEPCs transplant group were fixed in cold 4% phosphate-buffered paraformaldehyde overnight and embedded in OCT compound. Kidney cryostat sections (10 µm in thickness) were incubated with monoclonal rat anti-insulin

antibody (1:200, R&D Systems, Inc.) for 16 h at 4°C, and then stained with Alexa Fluor 568 goat anti-rat IgG (Molecular Probes). Nuclei were revealed by DAPI staining.

RESULTS

Generation of Pdx-1 Positive Single-Cell-Derived LEPCs

In order to investigate the potential of LEPCs to give rise to pancreatic beta cells, we generated stably transfected LEPCs, which expressed both *Pdx-1* and *EGFP* reporter gene. By electroporation, LEPCs were first transfected with the reporter construct *pPDX-1 EGFP*, in which the expression of *EGFP* is controlled by *Pdx-1* promoter. These transfected cells, called EGFP LEPCs, showed no green fluorescence (data not show), which suggested that there was no endogenous Pdx-1 expression in these LEPCs. A single clone was selected and used in all of the following studies. EGFP LEPCs were subsequently transduced with recombinant retrovirus carrying a *Pdx-1* expression cassette, establishing EGFP/Pdx-1 LEPCs. Unlike EGFP LEPCs, EGFP/Pdx-1 LEPCs showed green fluorescence, resulting from expression of *EGFP* under the control of the *Pdx-1* promoter. The expression of the *EGFP* reporter gene in EGFP/Pdx-1 LEPCs suggested that transgenic Pdx-1 induced the expression of an otherwise silent endogenous Pdx-1 in cultured LEPCs. The clonal EGFP/Pdx-1 LEPCs, with homogenous green fluorescence, were expanded and used in the following studies (Fig. 1A,B). The expression of Pdx-1 in EGFP/Pdx-1 LEPCs was further confirmed by Western blot, with no signal detected in EGFP LEPCs (Fig. 1C). EGFP LEPCs and EGFP/Pdx-1 LEPCs were cultured persistently in vitro without any notable change in their proliferative capacity. The doubling time of LEPCs, EGFP LEPCs, and EGFP/Pdx-1 LEPCs was 22.2 ± 2.5, 21.1 ± 1.7, and 23.5 ± 2.1 h, respectively.

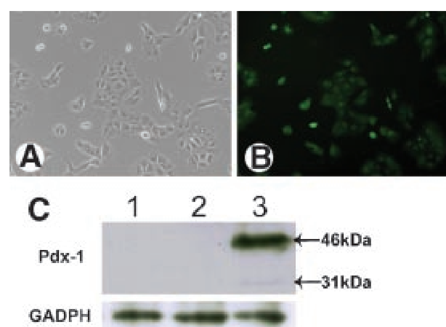

Fig. 1. The generation of Pdx-1 positive and single-cell-derived EGFP/Pdx-1 LEPCs. EGFP/Pdx-1 LEPCs showed homogenous green fluorescence (**A**,**B**). Frame A represents the corresponding phase contrast photograph for B. The expression of Pdx-1 in EGFP/Pdx-1 LEPCs was confirmed by Western blot (**C**). Arrows indicate the positions for active Pdx-1 (46 kDa) and inactive Pdx-1 (31 kDa). 1, LEPCs; 2, EGFP LEPCs; 3, EGFP/Pdx-1 LEPCs. GADPH was used as loading control. Relative magnification: A and B (200×).

LEPCs Acquired Pancreatic Endocrine Progenitor Cell Phenotypes After Pdx-1 Expression

RT-PCR was used to characterize the gene expression profiles of the newly generated EGFP/Pdx-1 LEPCs. The expression of various genes, related to pancreatic beta cell development and function, was examined (Fig. 2A–C). As demonstrated in Figure 2, EGFP/Pdx-1 LEPCs acquired pancreatic endocrine progenitor cell phenotypes, which was implicated by (1) the expression of *Ngn3* (Fig. 2A), an early marker for endocrine progenitor cells [Chakrabarti and Mirmira, 2003; Habener et al., 2005], (2) the expression of transcription factors involved in pancreatic development and beta-cell differentiation (Fig. 2A), such as *NeuroD*, *Nkx2.2*, *Nkx6.1*, *Pax4*, *Pax6*, and *Isl1* [Chakrabarti and Mirmira, 2003; Habener et al., 2005], and (3) the down-regulation of hepatocyte markers, such as *Albumin* and *Glutamine Synthetase* (*GS*; Fig. 2C). In contrast, EGFP LEPCs did not express these pancreas-enriched transcription factors. It was noteworthy that, although beta-cell transcription factors were activated, the persistent *Pdx-1* expression did not elicit expression of beta cell functional genes, such as *insulin* 1, *Kir6.2*, *SUR1*, and *Glut-2* (Fig. 2B). The pancreatic endocrine hormones, such as *glucagon*, *pancreatic polypeptide*, *somatostatin*, and the pancreatic exocrine specific transcripts *p48* and *elastase*, were also not detected in EGFP/Pdx-1 LEPCs (Fig. 2C). Although both EGFP LEPCs and EGFP/Pdx-1 LEPCs expressed *insulin 2*, as shown by RT-PCR analysis (Fig. 2B), insulin was not detected by immunocytochemistry (data not show). In addition, the LEPCs without gene-deliveries (for both EGFP and EGFP/Pdx-1) were same as EGFP LEPCs for these gene expression profiles (data not shown). Taken

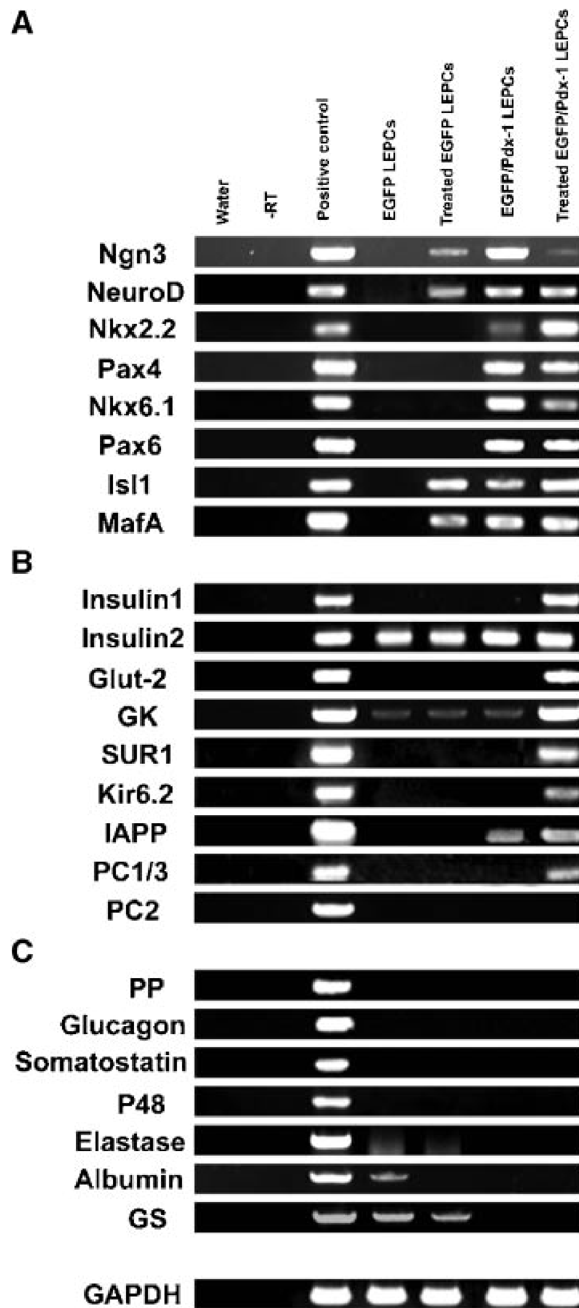

Fig. 2. RT-PCR analysis of EGFP/Pdx-1 LEPCs after *Pdx-1* expression and in vitro induction. Normal mouse pancreas cDNA served as positive controls for RT-PCR analysis except *Ngn3* (mouse brain cDNA was used), *albumin* and *Glutamine Synthetase* (mouse liver cDNA was used). Total RNA samples of EGFP/Pdx-1 LEPCs not treated with reverse transcriptase were performed to exclude DNA contamination (-RT). Water was used as blank control (W).

together, our findings suggested that EGFP/Pdx-1 LEPCs acquired pancreatic endocrine progenitor cell phenotypes but without beta cell functions.

EGFP/Pdx-1 LEPCs Became Functional Beta Cells When Cultured in High Glucose/Low Serum Medium Containing a Cytokine Cocktail

To investigate whether the EGFP/Pdx-1 LEPCs possessed the potential to give rise to functional pancreatic beta cells, EGFP/Pdx-1 LEPCs were subjected to in vitro induction in high glucose/low serum medium containing EGF, HGF, bFGF, Nicotamide, and L-proline, which has been shown to favor pancreatic beta cell differentiation [Nakajima-Nagata et al., 2004; Fujikawa et al., 2005; Zalzman et al., 2005]. During the in vitro induction, the proliferation of EGFP/Pdx-1 LEPCs was arrested, and the cell size increased. RT-PCR analysis suggested that the cytokine-treated EGFP/Pdx-1 LEPCs differentiated into functional beta cells (Fig. 2B), which not only expressed *insulin 1* and *insulin 2* but also expressed the molecular components of glucose sensing (*GK*, and *Glut-2*), insulin secretion-coupling machinery (*Kir6.2*, *SUR1*, and *IAPP*), and *PC1/3*, which processes proinsulin into insulin. In agreement with the transcription factor cascade during pancreatic development, the expression of *Ngn3* was down-regulated significantly; meanwhile the expression of *Isl1* was up-regulated and the expression of *NeuroD*, *Pax4*, *Pax6*, and *MafA* remained unaltered (Fig. 2A). Importantly, the treated EGFP/Pdx-1 LEPCs did not express hepatocyte markers, such as *Albumin* and *Glutamine Synthetase*, exocrine pancreatic molecules, such as *p48* and *elastase*, and other islet hormones, such as *glucagon*, *pancreatic polypeptide*, and *somatostatin*. The expression of oval cell markers, such as *CK*19 and *Thy-1*, was significantly decreased in treated EGFP/Pdx-1 LEPCs, but the expression of *c-Kit* remained unaltered (data not show). Under the same induction condition, EGFP LEPCs expressed pancreatic transcription factors such as *Ngn3*, *NeuroD*, and *Isl1*, but did not express beta cell functional genes. This RT-PCR profile indicated EGFP/Pdx-1 LEPCs gave rise to functional beta cells specifically after in vitro induction. Beta cell differentiation of EGFP/Pdx-1 LEPCs was further confirmed by immunocytochemistry and ultrastructural analysis using electronic microscopy. EGFP/Pdx-1 LEPCs expressed insulin and C-peptide in the cytoplasm after treatment (Fig. 3A,B). Ultrastructurally, treated EGFP/Pdx-1 LEPCs showed scattered cytoplasmic globular structures, with a beta cell-characteristic

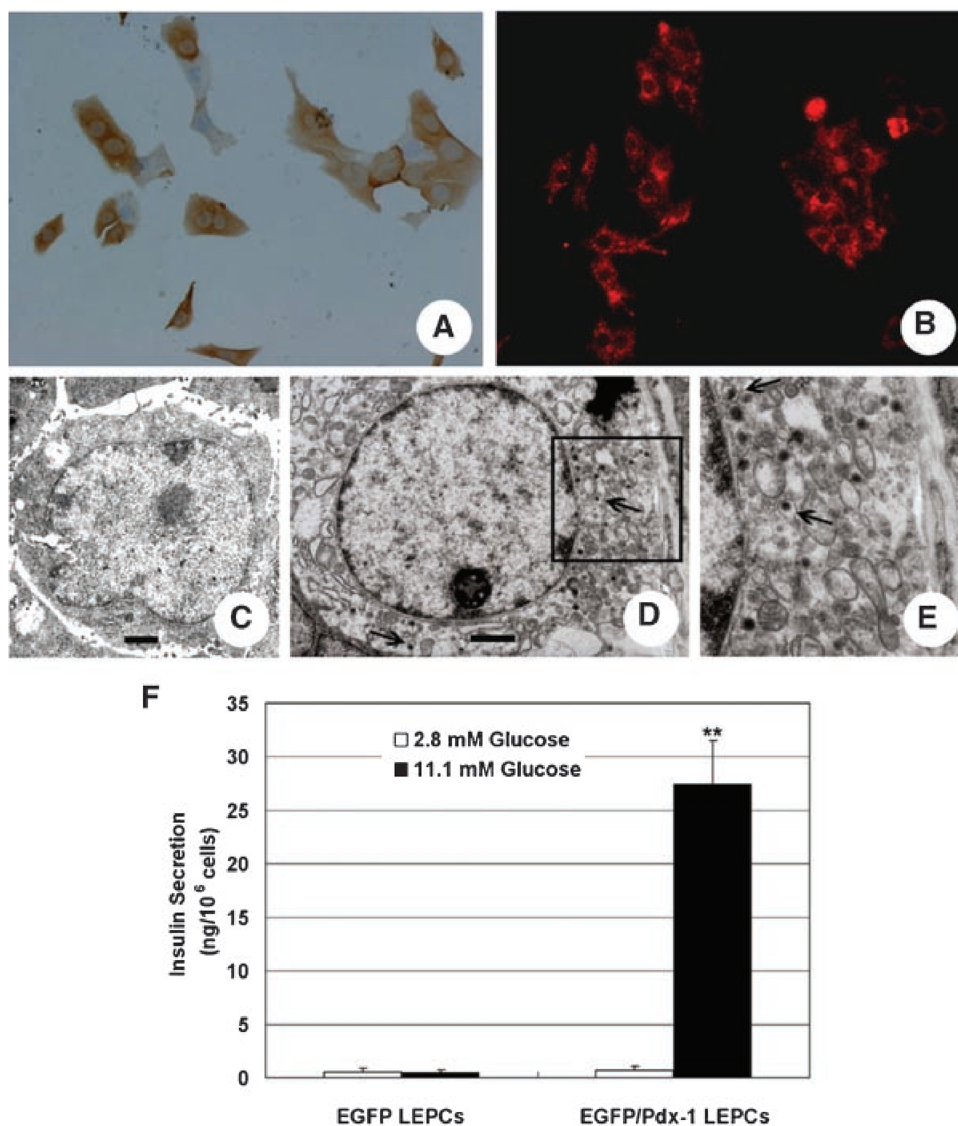

Fig. 3. EGFP/Pdx-1 LEPCs differentiated into pancreatic beta cells after induction by cytokines. After in vitro induction, EGFP/Pdx-1 LEPCs gave rise to insulin-producing cells (**A**); the cells also expressed C-peptide in the cytoplasm (**B**). **C**: The transmission electron microscopy photography of untreated LEPCs. Treated EGFP/Pdx-1 LEPCs showed scattered cytoplasmic globular structures (**D**,**E**). Some granules contained the beta cell characteristic dense core as those of in intact pancreatic beta cells in vivo (showed by arrow). Frame E was a magnification of the outlined area in D. Treated EGFP/Pdx-1 LEPCs can secrete insulin in a glucose-regulated manner (**F**). Insulin in the media was quantitated by ELISA and normalized to cell number. Data are means ± SEM (n = 3). Relative magnification: A and B (200×). Bar, 1 μm; $^{**}P < 0.01$.

dense core, as seen in intact pancreatic beta cells in vivo (Fig. 3C–E). The presence of positive immunostaining of insulin/C-peptide and secretory globules indicated that the cells were capable of storing insulin. The ability to secrete insulin in a glucose-regulated manner was further established by ELISA. The insulin secretion of treated EGFP/Pdx-1 LEPCs was 0.71 ± 0.38 ng per 10^6 cells in static condition and increased to 27.42 ± 4.09 ng per 10^6 cells after exposure to 11.1 mM glucose (Fig. 3F). Taken together, these data provide convincing evidence that EGFP/Pdx-1 LEPCs can differentiate into functional beta cells after in vitro induction without evidence of exocrine and other islet cell lineage differentiation. LEPCs clonal lines 2, 4, 8, and 10 [Li et al., 2006] were all subjected to the above experiments, and responses were similar in all these lines. The above results represent the experiments using LEPCs clonal 2.

To elucidate the key factors in beta cell differentiation from EGFP/Pdx-1 LEPCs, EGFP/Pdx-1 LEPCs (derived from LEPCs clonal 2)

were treated by an induction protocol with either medium containing 10% FCS and cytokine cocktail or the medium containing 2% FCS but no supplements of EGF, bFGF, or HGF. By FACS analysis, the growth of EGFP/Pdx-1 LEPCs was arrested when cultured in high glucose/low serum medium (2% FCS) containing a cytokine cocktail (Fig. 4A–C). However, this growth arrest might be essential for the beta cell differentiation from EGFP/Pdx-1 LEPCs, which was revealed by the expression of *insulin 1* in the results of RT-PCR analysis (Fig. 4D). In addition, RT-PCR analysis also suggested bFGF and HGF, instead of EGF, were crucial factors in inducing EGFP/Pdx-1 LEPCs to give rise to insulin-producing cells (Fig. 4D). The underlying mechanism need to be further investigated.

EGFP/Pdx-1 LEPCs Ameliorated Hyperglycemia of Diabetic Mice

In order to determine whether the EGFP/Pdx-1 LEPCs can become fully functional when exposed to the in vivo diabetic microenvironment, 2×10^6 EGFP/Pdx-1 LEPCs were transplanted into the left renal subcapsular space of STZ-induced diabetic *SCID* mice. As demonstrated in Figure 5A, the EGFP/Pdx-1 LEPCs transplant group were capable of reducing blood glucose levels from ~400 to ~100 mg/dl within 2 weeks and maintained this level up to 6 weeks post-implantation. In contrast, diabetic mice in the EGFP LEPCs transplant group and PBS transplant group were all dead within 2 weeks. Before the mice of EGFP/Pdx-1 LEPCs transplant group were killed at 6 weeks post-transplantation, left nephrectomy (n = 3) and intraperitoneal glucose tolerance testing (IPGTT; n = 3) were performed. Removal of the transplanted cells by left nephrectomy resulted in rebound hyperglycemia 24 h later, indicating that amelioration of the hyperglycemic effect was the direct consequence of the transplanted EGFP/Pdx-1 LEPCs. An IPGTT test showed a glucose clearance rate similar to that of normal *SCID* mice (Fig. 5B). This finding suggested that transplanted cells indeed further differentiated and matured into functional pancreatic beta cells, which secreted insulin in a glucose-regulated manner. The left kidneys from the EGFP/Pdx-1 LEPCs transplant group were subjected to immunohistochemistry analysis to detect transplanted cells (Fig. 6A–D). No tumors were detected in any of the experimental

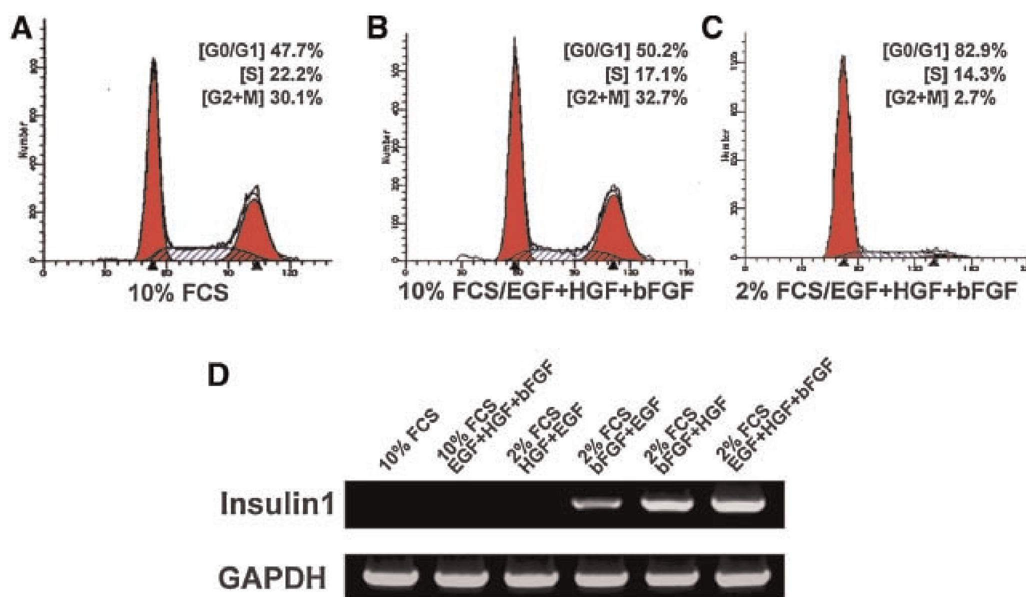

Fig. 4. The cell growth arrest, combined with bFGF and HGF, were essential for EGFP/Pdx-1 LEPCs to give rise to insulin-producing cells. The cell cycle of EGFP/Pdx-1 LEPCs was analyzed in different culture condition by FACS after propidium iodide staining (**A**–**C**). **Panel A** showed the FACS results of EGFP/Pdx-1 LEPCs kept in the medium containing only 10% FCS without additional cytokine treatment. **B**: The LEPCs detected after culture of 2 weeks under the indicated condition of treatment (10% FCS supplemented with EGF, HGF and bFGF). **C**: The LEPCs detected after culture of 2 weeks under another condition (2% FCS supplemented with EGF, HGF, and bFGF). The expression of *insulin 1* in EGFP/Pdx-1 LEPCs was revealed by RT-PCR analysis after different induction protocol (**D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

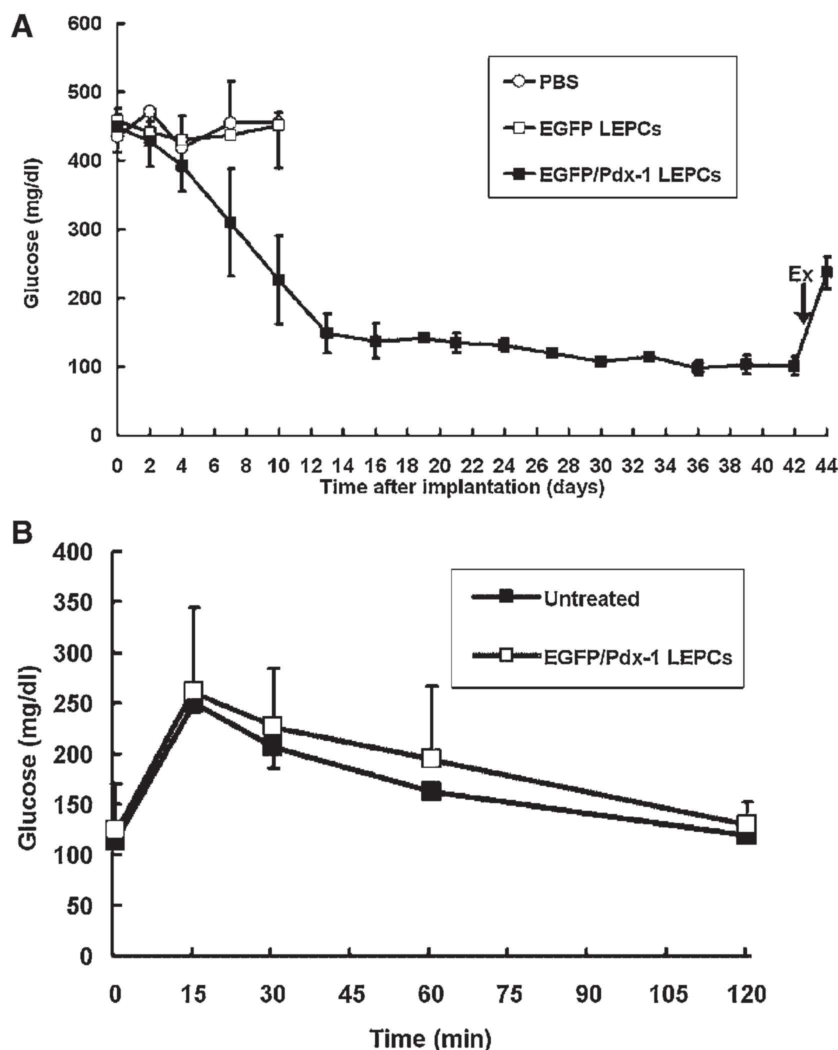

Fig. 5. EGFP/Pdx-1 LEPCs ameliorated hyperglycemia of diabetic mice. The EGFP/Pdx-1 LEPCs transplant group (n = 6) were capable of reducing blood glucose levels from ~400 to ~100 mg/dl within 2 weeks (**A**). In contrast, diabetic mice in the EGFP LEPCs transplant group (n = 3) and PBS transplant group (n = 3) were all dead within 2 weeks. Values are mean ± SEM. The EGFP/Pdx-1 LEPCs transplant mice were subjected to left nephrectomy (n = 3, downward arrows, indicated by Ex) and intraperitoneal glucose tolerance testing (n = 3) before killed. Removal of the transplanted cells by left nephrectomy resulted in rebound hyperglycemia 24 h later (A). An IPGTT test demonstrated a normal rate of glucose clearance rate similar to that of normal *SCID* mice (**B**). Values are mean ± SEM. There was no significant difference between transplanted and normal mice in the rate of glucose clearance ($P > 0.05$).

animals (n = 6). EGFP positive cells, indicating engrafted cells with endogenous *Pdx-1* expression, were detected in the recipient mice (Fig. 6B). Serial sections stained with an anti-insulin antibody (red) clearly showed that part of the EGFP-positive donor cells were also positive for insulin (Fig. 6B,C). However, the transplanted cells did not show proliferation in vivo, which was revealed by the negative Immunostaining of Ki-67 protein antigen in the kidney paraffin-embedded sections at 6 weeks post-transplantation (data not show). Taken together, these observations suggested that EGFP/Pdx-1 LEPCs could differentiate into beta cells and ameliorate hyperglycemia after transplantation into recipient diabetic animals.

DISCUSSION

Replacing the missing insulin-producing beta cells is considered to be the optimal treatment for type 1 diabetes [Cozar-Castellano and Stewart, 2005]. However, large-scale application of islet cell implantation is hindered by the limited availability of insulin-producing tissues or functional beta cells. As a result, there has

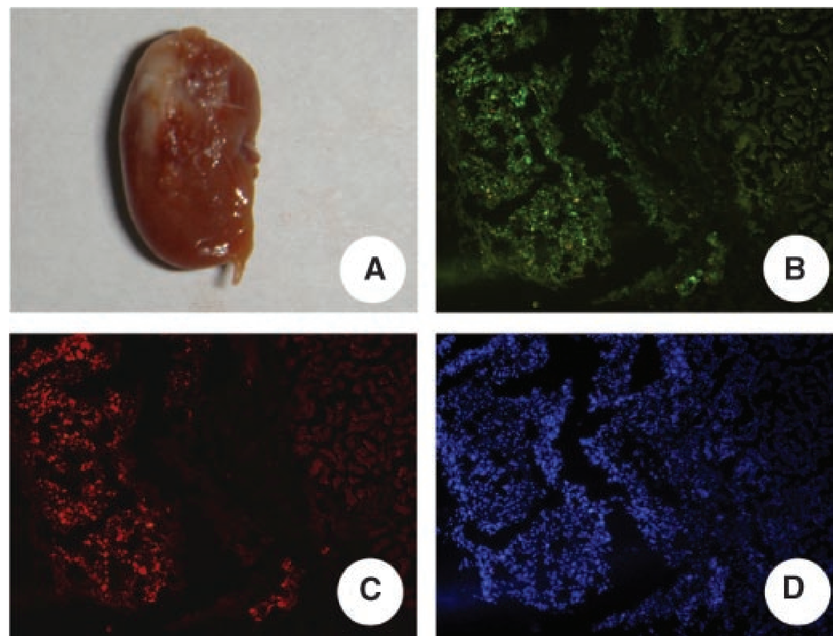

Fig. 6. EGFP/Pdx-1 LEPCs differentiated into insulin producing cells after transplanted into diabetes mice. The left kidneys of EGFP/Pdx-1 LEPCs group were removed at about 6 weeks after implantation. Frame **A** shows the implanted tissues below the subrenal capsule. EGFP positive cells, indicating the engrafted cells, were detected in the kidney cryostat sections (**B**). Serial sections stained by an anti-insulin antibody (red) clearly showed that part of the EGFP-positive donor cells were also positive for insulin (**C**). **D**: The DAPI staining of the same vision field of C to reveal nuclei. Relative magnification: B–D (200×).

been a great deal of interest in utilizing stem cells to treat type 1 diabetes. Embryonic stem cells have been proposed to be a potential source of beta cells, but the risk of teratoma formation needs to be seriously eliminated before therapeutic utilization [Fujikawa et al., 2005]. Tissue specific stem cells are an alternative source of pancreatic beta cells, but little success has been achieved so far in proliferating and culturing pancreatic progenitor cells (or pancreatic beta cells) in vitro.

In the present studies, LEPCs with the persistent expression of *Pdx-1* were reprogrammed into pancreatic endocrine progenitor cells. Contrary to previous reports [Yang et al., 2002; Zalzman et al., 2003, 2005; Cao et al., 2004; Li et al., 2005; Tang et al., 2006a,b], enforced expression of *Pdx-1* did not convert EGFP/Pdx-1 LEPCs into insulin producing cells directly. However, this result was consistent with the previous developmental studies, in which the ectopic Pdx-1 expression in the posterior small intestine, stomach, and liver changed the phenotypes of cells in these tissues during development into the phenotype of pancreatic progenitor cells [Grapin-Botton et al., 2001]. The expression of *Pdx-1* in LEPCs activated a set of transcription factors that were essential to pancreatic beta cell development and maturation, but did not elicit beta-cell function. Importantly EGFP/Pdx-1 LEPCs can be cultured persistently in vitro. After being treated with high glucose/low serum medium supplemented with cytokines, EGFP/Pdx-1 LEPCs stopped proliferating and further differentiated into beta cells specifically, without any evidence of exocrine differentiation or other islet cell lineage differentiation. Usually, cells that proliferate well don't produce insulin efficiently, and those that do produce insulin don't proliferate well. This "two-step differentiation" maintained the delicate balance between growth and differentiation. It was also the first report, to our knowledge, of *Pdx-1*-dependent reprogramming of immortal liver progenitor cells into pancreatic endocrine progenitor cells, which can further differentiate into functional beta cells under the appropriate conditions. In vivo studies indicated that EGFP/Pdx-1 LEPCs can differentiate into functional beta cells when exposed to the in vivo diabetic microenvironment, they can restore and

maintain euglycemia for prolonged periods, and they can secrete insulin in a glucose-regulated manner. Our results provide convincing evidence that immortal liver progenitor cells can be a renewable cell source for pancreatic beta cell. Recently, great advance has been achieved to isolate and long-term culture human liver stem/progenitor cells, which could be derived from both fetal and adult human livers [Harmeet et al., 2002; Nowak et al., 2005; Maria et al., 2006; Cédric et al., 2007]. We propose that our experiments will provide a framework for utilizing the human liver progenitor cells to generate functional beta cells.

Glucose has a number of positive effects on pancreatic beta cells, including stimulation of insulin secretion, insulin gene transcription and translation, and cell growth. Glucose can promote translocation and modification of an inactive cytoplasmic Pdx-1 of 31-kDa to the active nuclear Pdx-1 of 46-kDa in the beta-cell-derived MIN6 cells [Rafiq et al., 1998]. Previous studies indicated that high glucose was necessary for complete maturation of Pdx-1-expressing hepatic cells into insulin-producing cells [Cao et al., 2004]. In the present studies, Pdx-1-expressing LEPCs became functional beta cells cultured in high glucose/low serum medium containing a cytokine cocktail. When cultured in low glucose/low serum medium containing the same cytokine cocktail, EGFP/Pdx-1 LEPCs could not survive after treatment for three weeks (data not show), which suggested that glucose exerted beneficial effects on the survival of Pdx-1 expressing liver progenitor cells. However, when beta cells are exposed to elevated levels of glucose for prolonged periods of time, glucose becomes toxic to insulin secretion, gene expression, and cell survival. This phenomenon is referred to as glucotoxicity [Robertson et al., 1992]. Whether the long-term culture under high glucose condition could exert toxic effect on EGFP/Pdx-1 LEPCs still need to be investigated.

Previous reports demonstrated that persistent expression of *Pdx-1* in liver cells could not only elicit the expression of insulin but also the expression of the other pancreatic islet hormone, such as *pancreatic polypeptide* and *glucagons*. Unlike previous studies [Yang et al., 2002; Zalzman et al., 2003, 2005; Cao et al., 2004; Li et al., 2005; Sapir et al., 2005; Tang et al., 2006a,b; Fodor et al., 2007], EGFP/Pdx-1 LEPCs only gave rise to insulin producing cells after treatment. It is noteworthy that the expression of NK homeobox factors *Nkx2.2* and *Nkx6.1* was robustly induced in EGFP/Pdx-1 LEPCs. Both *Nkx2.2* and *Nkx6.1* appear to be crucial for directing pancreatic endocrine precursors to the beta-cell lineage and to the final differentiation of beta cells [Sander et al., 2000; Chakrabarti and Mirmira, 2003; Habener et al., 2005]. Recent studies revealed that *Nkx6.1* could maintain the beta cell phenotype in part through suppression of *glucagon* expression and control of glucose-stimulated insulin secretion [Schisler et al., 2005]. The vigorous upregulation of *Nkx2.2* and *Nkx6.1* may contribute to the establishment of pancreatic beta cell phenotypes of treated EGFP/Pdx-1 LEPCs. Now, little is known about the molecular mechanism of Pdx-1 induced conversion of liver cells to pancreatic endocrine cells. EGFP/Pdx-1 LEPCs may be useful tools as in vitro models to study molecular events in pancreatic conversion.

It was noteworthy that EGFP LEPCs expressed *insulin 2*, as revealed in our study (Fig. 2B). This result was consistent with in vivo, developmental studies demonstrating that *insulin 2* mRNA, but not *insulin 1*, could be detected in fetal and adult rat livers, just as in fetal and adult brains [Giddings and Carnaghi, 1990; Devaskar et al., 1993].

Under our experimental conditions, not all EGFP/Pdx-1 LEPCs differentiated into insulin producing cells after in vitro treatment (Fig. 3A) and in vivo transplantation (Fig. 6). This incomplete conversion has been also observed in other studies [Sapir et al., 2005; Zalzman et al., 2005]. RT-PCR analysis revealed EGFP/Pdx-1 LEPCs still expressed *Ngn3* weakly after treatment. Usually, beta cells do not co-express *insulin* and *Ngn3* in adult pancreas [Gradwohl et al., 2000]. These data suggest that a portion of cells still remain in an immature state after treatment. The basis of this heterogeneity needs to be further investigated. However, from a therapeutic view point it may be desirable to retain some progenitor cells for long-term benefits as these cells may represent a reservoir which can continue to produce beta cells.

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