Adult mouse intrahepatic biliary epithelial cells induced *in vitro* to become insulin-producing cells

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Abstract

Transdifferentiation of cells from a patient's own liver into pancreatic β -cells could be useful for β -cell replacement. We hypothesized that intrahepatic biliary epithelial cells (IHBEs) could become a new source of insulin-producing cells. IHBEs isolated from adult mice were expanded using our novel culture method termed, collagen-embedded floating culture method (CEFCM). With CEFCM, IHBEs formed three-dimensional ductal cysts and rapidly expanded their number by about 15-fold within 2 weeks. Over 90% of cells were positive for cytokeratin 7 and 19. At day 14, IHBEs were transfected with adenoviral (Ad)-pancreas duodenum homeobox 1 (*Pdx-1*), *NeuroD* or *Pdx-1/VP16*. After 7 additional days in serum- and insulin-free differentiation medium (DM), cell phenotypes were determined by RT-PCR, immunostaining and ELISA for insulin. In DM control IHBEs started to express some endocrine progenitor

genes (*Neurog3*, *NeuroD*, *Nkx6.1*, and *Pdx-1*) but lacked insulin gene (*Ins*) mRNA. Transduced expression of PDX-1, NEUROD or PDX-1/VP16 led to expression of not only INS but also *GLUT2* and prohormone convertase 1 and 2. About 3% of 4000 cells counted in PDX-1/VP16 transduced cultures stained strongly for C-peptide suggesting that a subpopulation may have the capacity for differentiation. Transduced cells released insulin (Ad-PDX-1 0.08 ± 0.05 , Ad-NEUROD 0.33 ± 0.09 , Ad-PDX-1/VP16 0.37 ± 0.14 ng/ 1×10^5 cells after 48 h in culture). IHBEs can be markedly expanded, and then with molecular manipulation a subpopulation of these cells can differentiate towards a β -cell phenotype. This approach may lead to a new source of β -cells that can be used for transplantation in diabetes.

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Introduction

There is great hope that transplantation of insulin-producing cells will be a cure-equivalent for type 1 diabetes (Shapiro *et al.* 2000, Ricordi & Strom 2004), however, two major challenges remain: the shortage of available tissue and the need for immunosuppressive drugs. To resolve the first problem, efforts are underway to produce sufficient quantities of β -cells for transplantation (Bonner-Weir & Weir 2005). Promising strategies include differentiation of embryonic stem cells (Kroon *et al.* 2008), conversion of either pancreatic adult stem/progenitor cells (Bonner-Weir *et al.* 2000), expansion of existing β -cells (Gershengorn *et al.* 2004), and xenotransplantation (Cardona *et al.* 2006, Hering *et al.* 2006). Transdifferentiation of cells from a patient's own tissue into pancreatic β -cells could address one obstacle of islet transplantation. This tissue could be obtained from a diabetic individual by endoscopic surgery, followed by *in vitro* expansion and differentiation into insulin-producing cells for subsequent autologous transplantation. Some researchers have pursued transdifferentiation using liver

tissue both *in vivo* and *in vitro* (Ferber *et al.* 2000, Yang *et al.* 2002, Kojima *et al.* 2003, Sapir *et al.* 2005, Zalzman *et al.* 2005, Tang *et al.* 2006). The advantage of the liver is that both liver and pancreas develop embryologically from very similar pathways via the foregut endoderm (Wilson *et al.* 2003, Lemaigre & Zaret 2004, Zaret 2008). Ectopic expression of NEUROD and PDX-1 has been found to promote the differentiation of liver towards pancreas (Ferber *et al.* 2000, Kojima *et al.* 2003, Sapir *et al.* 2005, Zalzman *et al.* 2005). Among the cell populations in the liver, the bile duct epithelium seems to have considerable promise as a new source of insulin-producing cells. It is clear that β -cells can exist surrounding the common bile duct of the liver (Sumazaki *et al.* 2004, Dutton *et al.* 2007), but the use of extrahepatic duct cells is problematic because the removal of sufficient amounts of tissue would be very dangerous (Antolovic *et al.* 2007). Oval cells are considered to be transit-amplifying hepatic progenitor cells (Factor *et al.* 1994, Yang *et al.* 2002, Kofman *et al.* 2005) and it has also been suggested that they can be precursors of insulin producing cells (Yang *et al.* 2002). However, they only appear in

response to several types of pathologic liver injury (Factor *et al.* 1994, Kofman *et al.* 2005).

We focused our attention on intrahepatic biliary epithelial cells (IHBECs) for the following reasons: 1) A considerable amount of liver tissue can be surgically removed without much difficulty. 2) Liver has considerable capacity for regeneration (Michalopoulos & DeFrances 1997). 3) IHBECs can serve as progenitors in response to certain stimuli; this can be shown in biliary ductules and even in the normal liver (Nagaya *et al.* 2006). We used normal liver for this study; therefore, there is little chance that oval cells were included in the preparation.

Cells were transduced with adenoviruses (Ad) expressing the transcription factors pancreas duodenum homeobox 1 (PDX-1), NEUROD, or PDX-1/VP16. PDX-1 plays a crucial role in pancreatic development (Jonsson *et al.* 1994), β -cell differentiation (Ferber *et al.* 2000, Sapir *et al.* 2005, Zalzman *et al.* 2005, Noguchi *et al.* 2006), and the maintenance of normal β -cell function (Ahlgren *et al.* 1998). PDX-1 overexpression in pancreatic ductal cells (Noguchi *et al.* 2006) or the liver (Ferber *et al.* 2000, Sapir *et al.* 2005, Zalzman *et al.* 2005), has been found to promote insulin gene expression. NEUROD is important for regulating insulin gene transcription and for the terminal differentiation of islet cells (Chae *et al.* 2004). In *NeuroD* gene targeting experiments, the combination of NeuroD and betacellulin selectively induced islet related genes in the liver (Kojima *et al.* 2003). PDX-1/VP16, a fusion protein of PDX-1 and the activating domain of the viral VP16 transcription factor, can markedly increase insulin biosynthesis and induce various pancreas-related markers in the liver and ameliorate glucose tolerance of streptozotocin-induced diabetic mice (Kaneto *et al.* 2005).

Herein, we demonstrated that a meaningful amount of transdifferentiation has taken place from IHBECs through the use of these transcriptional factors. The cells can express proteins characteristic of β -cells and secrete insulin.

Materials and Methods

Animals and surgery

Adult 8 week old male C57/BL6 mice were obtained from Taconic Farms (Germantown, NY, USA). All animals were bred and maintained under specific pathogen-free conditions. Joslin Diabetes Center housing and husbandry guidelines were adapted from requirements in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals in accordance with National Institutes of Health guidelines.

Isolation and culture of IHBECs

To isolate the IHBECs, a two-step liver perfusion was performed (Nagaya *et al.* 2006). The perfused liver was removed and hepatocytes were selectively removed by forcing them with gentle pressure through an incision in the liver

capsule. Then, the remnant containing the intact biliary tree was shaken in the flask with Hanks solution for 5 min and washed several times. This treatment serves to remove residual hepatic parenchymal cells from biliary tissue fragments. The remnant tissues were collected and minced, transferred into a flask, and then treated with 0.02% soybean trypsin inhibitor (Gibco, Invitrogen Corporation) and 0.04% collagenase solution (collagenase D, Roche) for 7 min. The digested tissues were suspended in DMEM (Gibco) with 10% FBS and 100 mg/l penicillin and 100 mg/l streptomycin, and centrifuged at 150 *g* for 1.5 min. The pellet was resuspended in the medium, filtered sequentially through 250, 100, 40 μ m nylon mesh; small cell aggregates on the 40 μ m mesh were retrieved. Essentially, pure isolate of biliary tissue were obtained. Typical recovery was 1×10^5 viable cells with essentially pure biliary tissue obtained.

Collagen-embedded floating culture method

Cells were suspended in DMEM/F12 (Gibco) on ice containing 0.3 mg/ml collagen (Collagen type I rat tail, Becton Dickinson (BD), Franklin Lakes, NJ, USA). Then 1.0×10^4 cells/ml per well were plated in 12-well dishes. The collagen-containing aggregates were allowed to solidify at 37 °C for 2 h; media was then changed to DMEM/F12 supplemented with 5% FBS, 5% NuSerum IV (BD), 0.5 μ g/ml insulin–transferrin–sodium selenite (Gibco), 10 mmol/l nicotinamide, 1 mmol/l ascorbic acid 2-phosphate, 10^{-7} M dexamethasone (Sigma-Aldrich Corp.), 10 ng/ml EGF (Fitzgerald Industries International, Inc., Concord, MA, USA), 10 ng/ml HGF (R&D, Minneapolis, MN, USA) 4 μ g/ml forskolin (Calbiochem, San Diego, CA, USA), and 3.4 μ g/ml 3,3,5-triiodo-L-thyronine (Calbiochem). This medium is a modification of that described by Alpini *et al.* (2003), which we term growth medium (GM); it was changed every 2 days. With culture, the collagen gel (CG) containing the aggregates gradually contracted as shown in Fig. 2.

Monolayer culture for IHBECs

After 14 days in collagen-embedded floating culture method (CEFCM) culture, the aggregates were washed in Ca^{2+} -free HEPES-buffered saline, then a 0.1% collagenase solution, for 10 min to dissolve the CG and release the aggregated IHBECs fragments. The dispersed cells were counted on the hemocytometer with trypan blue and allowed to settle, then resuspended in GM, and plated onto collagen-coated, 35 mm plastic dishes (BD) or 12-well dishes (BD) and maintained at 37 °C in a humidified 5% CO_2 incubator. After 3 days in GM, the media was changed to serum- and insulin-free differentiation medium (DM), which consisted of DMEM/F12 (Gibco) supplemented with 10 mM nicotinamide (Sigma), 1 mM ascorbic acid 2-phosphate (Sigma), 10 mM MEM Vitamin Solution (Gibco), 10 mM MEM non-essential amino acids solution, 10 mM GlutaMA-I Supplement (Gibco), 4 mg/ml transferrin (Invitrogen), 10 ng/ml

betacellulin (R&D), 10 ng/ml IGF-I (R&D), and 50 ng/ml exendin-4 (Sigma). The IHBECs were cultured for an additional 2 days and randomly divided into three groups; the cells were maintained in this condition (Group 1), transduced with Ad expressing – green fluorescent protein (GFP; Group 2), or transduced with Ad expressing – *Pdx-1*, –*Neurogenin 3* (NEUROG3), –*NeuroD*, –*Pax 4*, and –*Pdx-1/VP16* (Group 3). The media were changed every 2 days.

Preparation of recombinant adenoviruses

Recombinant Ad expressing *Pdx-1*, –*Neurog3*, –*NeuroD*, and –*Pdx-1/VP16* driven by a cytomegalovirus (CMV) promoter were prepared with the AdEasy system as previously described (Kaneto *et al.* 2005). These AdVs all carried the reporter GFP. The control Ad (Ad-GFP) was prepared in the same manner. Ad expressing *Pax4* (Ad-Pax4) was kindly provided by Dr St-Onge (DeveloGen AG, Gottingen, Germany) and contains the construct of CMV-Pax4-IRES-GFP.

The integration of each gene into the Ad was done by transfection into the Ad packing cell line 293 cells according to the manufacture's instructions. Ad titers were further increased up to 1×10^{10} plaque forming units/ml with Vivapure AdenoPACK 100 purification kits (Vivascience, Edgewood, NY, USA).

Transduction of adenovirus to IHBECs

For transduction, media were changed to serum-free DMEM containing purified recombinant Ad –PDX-1, –NGN 3, –NEURO D, –PAX4 or –PDX-1/VP16 and incubated for 1 h at 37 °C. To test which multiplicity of infection (MOI) was most effective for transduction, IHBECs were infected at MOIs of 1, 10, 25, 50, 100 or 200 for 2 h at 37 °C. Two-days after transduction, cells were counted to determine the percentage of GFP⁺ cells. Seven days after transduction, the cells were harvested and evaluated.

RNA isolation, cDNA synthesis, RT-PCR

Total RNA was isolated from cultured cells at day 26 using RNeasy Mini Kits (Qiagen) according to manufacturer-suggested protocols, and RT-PCR were conducted. The extracted total RNA was reverse-transcribed into cDNA with Omniscript RT Kit (Qiagen) using standard procedures. The primers were complementary to the mRNA sequences of the genes of interest and are listed in Supplementary Table 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol201/issue1/>. All the primers were designed to cross exon–exon boundaries to exclude the possibility that genomic DNA was amplified. The exception was MafA that lacks an intron. Twenty nanograms cDNA were applied to each well. PCR was performed with the following parameters: 36 cycles of 30 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C.

Cell sorting

All samples were analyzed on an ARIA cell sorter with the Summit software (BD). The cells were dispersed to mostly single cells. Propidium iodide (PI, Sigma) was used for exclusion of dead cells; we gated both PI[–] GFP⁺/PI[–] GFP[–] populations to retrieve living cells from PDX-1/VP16 transduced cells. Both GFP⁺ and GFP[–] single cells were placed into 96-well dishes. Group 1 and Group 2 cells were also dispersed into single cells and served as controls.

Analysis of single cells by RT-PCR

To detect our genes of interest from single cells, nested PCR was performed. Multiplex single-cell RT-PCR analysis was performed according to a previously described method with some modifications (Miyamoto *et al.* 2002). Briefly, we first performed reverse-transcription followed by the first PCR. Sorted single cells were deposited into 96-well U-bottom plates (BD) with 7.5 µl lysis and RT buffer containing gene-specific reverse primers for *Krt19*, *NeuroD*, *Pdx-1*, *Ins 1*, *Ins 2*, *Glucagon*, *Somatostatin*, *PP*, and *HPRT* (Supplementary Table 1): the concentration of lysis buffer was the following: (1×First, Strand Buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 1 mM dNTPs (New England BioLabs, Ipswich, MA, USA), 0.5% Triton X-100 (Sigma), 0.1% BSA, 10 U/µl M-MLV reverse transcriptase (Invitrogen), 0.1 U/µl RNase inhibitor (Invitrogen), 0.4 pM reverse primers). Retrieved cells were lysed by pipetting several times in the plate, and then cell lysates were transferred to 96-well optical Reaction Plate with Barcode (Applied Biosystem, Foster City, CA, USA). After incubation at 37 °C for 90 min, the samples were incubated at 94 °C for 30 s to inactivate the enzyme. This step is reverse-transcription and cDNAs are generated simultaneously. The first-round of PCR was carried out in the same plate by addition of premixed PCR buffer containing the gene-specific forward primers (1× GeneAmp PCR Gold Buffer (Applied Biosystem), 2.5 mM MgCl₂, AmpliTaq Gold 0.1 U/µl, 0.1 pM forward primers). The total volume of the first PCR reactions were 20 µl, and PCR was performed with the following parameters: 36 cycles of 30 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C. For nested PCR, the second round PCR was performed as follows: 1 µl of the first-round PCR reactions were plated into new PCR plates, which contained mixed buffer (1×GeneAmp PCR Gold Buffer (Applied Biosystem), 2.5 mM MgCl₂, AmpliTaq Gold (Applied Biosystem) 0.1 U/µl, and 0.25 pM forward primers). For the second-round PCR, each gene was analyzed separately using nested gene-specific primers with the following parameters: 35 cycles of 36 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C. Aliquots of second-round PCR products were subjected to gel electrophoresis. Two hundred pg of total RNA isolated from mouse islets was used as a positive control.

Immunostaining

The methods used for immunostaining have been previously described (Nagaya *et al.* 2006). Briefly, cells on dishes were fixed in cold absolute ethanol after three rinses with PBS. Primary antibodies included mouse anti-cytokeratin 7 (1:25; CK7; Dako Cytomation), rabbit anti-cytokeratin 19 (1:1000; KRT19; a gift of Dr A Miyajima, Tokyo University, Japan), rabbit anti-NeuroD (1:200; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-Pdx-1 (1:2000; IPF-1C antibody, a gift from Dr J M W Slack, University of Minnesota) and rabbit anti-C-peptide (1:400; Cell Signaling). Alexa⁴⁸⁸-conjugated and Alexa⁵⁹⁴-conjugated antibodies (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Cell nuclei were counter-stained with PI (VECTASHIELD Mounting Medium with PI; Vector Laboratories, Ltd., Peterborough, England) or DAPI (VECTASHIELD Mounting Medium with DAPI). Cultures processed with secondary antibodies only were used as negative controls. Liver and pancreas sections were stained as positive controls.

Measurement of insulin production

Insulin levels in culture supernatants were measured using the ultrasensitive insulin ELISA (Crystal Chem, Inc, Downers Grove, IL, USA; detection limit of 0.04 ng/ml). Media from cultures from day 5 to day 7 after transduction were collected and measured as basal secretion. Subsequently, insulin secretion in response to secretagogues was measured, the cells were subjected to static incubation in Krebs–Ringer bicarbonate buffer (KRB; 133 mM NaCl, 4.69 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM HEPES, 2.52 mM CaCl₂, 2H₂O, 5 mM NaHCO₃) supplemented with 5 mM glucose and 0.2% BSA. Plated in 12-well culture

plates (BD), cells were washed four times with KRB. They were continuously incubated in KRB with low glucose (5 mM), high glucose (25 mM) or high glucose with 45 mM KCl for 2 h at 37 °C. Between each incubation, they were washed four times with KRB and incubated in KRB for 2 h. IHBECs (Group 1, *n*=3), control cells (Group 2, *n*=3), transduced cells (Group 3, the number of independent experiments with Pdx-1 was *n*=6; NeuroD, *n*=7; and Pdx-1/VP16, *n*=7). Fold stimulation was calculated for each culture (1×10⁵ cells/well per ml) by dividing the insulin concentration in the stimulation supernatant by the insulin concentration in the basal supernatant.

Statistical analysis

Experimental results were expressed as the mean±S.E.M. Student’s *t*-test, ANOVA and Fisher’s protected least significant difference test were used, and *P*<0.05 was considered significant. All experiments were repeated at least three times.

Results

Generation of insulin-producing cells from IHBECs

We have developed a five step-protocol for inducing the differentiation of IHBECs to insulin producing cells (Fig. 1). In Step 1, the IHBEC-rich fraction was isolated from adult mouse liver with 1×10⁵ viable cells/mouse being recovered, then in Step 2 their number was expanded with CEFCM. Figure 2A left and B left show macroscopic and phase contrast microscopic views respectively. After about 5 days with CEFCM, IHBECs formed three-dimensional ductal cysts (Fig. 2B, right) and rapidly expanded their number about

Figure 1 General outline of the culture system and protocols for inducing the differentiation of IHBECs.

15-fold within 2 weeks (Fig. 2E). The CG containing the expanding cells gradually shrank over 14 days (Fig. 2D). In cultures without CEFCM (CEFCM-), IHBECs initially expanded but then started to die at around 8 days (Fig. 2E).

Initial experiments with IHBECs were performed to determine their behavior in tissue culture and their potential for transdifferentiation. RT-PCR and immunofluorescent staining were carried out with hepatocyte, biliary epithelial cell (BEC), and islet markers. Cells at day 0 expressed BEC markers but neither hepatocyte nor islet markers (Fig. 2F). In addition, IHBECs expressed a number of hepatocyte nuclear factor (HNF) family genes, (*HNF1 β* , *HNF3 β* , *HNF4 α* , *HNF6*) considered to be endoderm progenitor markers (Lemaigre & Zaret 2004). The cells were also positive for both CK7 (blue; Fig. 2G) and KRT19 (data not shown).

At day 14, dispersed IHBECs were seeded onto collagen-coated dishes and cultured for 3 more days (Step 3). These

IHBECs formed colonies and expanded about 2.4-fold during these 3 days. At day 17, cultured IHBECs without CEFCM displayed signs of senescence (large, multinucleated flat cells; Fig. 3A, black arrows) or numerous cytoplasmic vacuoles (Fig. 3A, white arrow heads). IHBECs subjected to CEFCM culture had fewer senescent cells or cytoplasmic vacuoles (Fig. 3B). These monolayers of homogenous appearing cells maintained their BEC character even at day 17 (Fig. 2F) and proliferated efficiently in culture (Fig. 2G, right). The purity of IHBECs averaged >90% at day 17 as assessed by immunostaining for CK7 and KRT19.

For Step 4, the media was changed to DM and the IHBECs were cultured for an additional 2 days. We focused first on generating insulin-expressing cells by testing soluble factors including activins, exendin-4, HGF, IGFs, nicotinamide, and betacellulin. After seven additional days (Step 5) in DM Control (Group 1), IHBECs started to express

Figure 2 (A–G) IHBECs cultured with collagen embedded floating culture method (CEFCM). (A) Macroscopic appearance of IHBECs with CEFCM. Isolated IHBECs were suspended in rat tail collagen plus GM; the collagen gel containing the IHBECs gradually contracted. Scale bar, 10 mm. (B) Nomarski differential interference contrast (DIC) image of cultured IHBECs. Day 0 with CEFCM (B, left); Very small aggregates of IHBECs. After 5 days with CEFCM, IHBECs formed three-dimensional ductal cysts (B, right). (C) IHBECs at 14 day with CEFCM. Asterisk shows the same position as in panel A. IHBECs rapidly expanded to form ductal structures. Scale bar, 100 μ m. (D) The diameter of collagen gel gradually shrank with time. (E) The number of IHBECs in each group. Bars show mean $\pm$ S.E.M. of 5 independent experiments. # $P < 0.001$. (F) Gene expression profiles of IHBECs and immunofluorescence staining for cytokeratin 7. RT-PCR was carried out to clarify the character of IHBECs at day 0 and day 17. Hepatocyte markers (*tryptophan oxygenase (TO)*, *tyrosine aminotransferase (TAT)*, *cytochrome P450 1A2 (CYP1A2)*), BEC markers (*Cytokeratin (CK)7* and *19*, *γ -glutamyltranspeptidase (γ -GTP)*, islet markers (*Insulin 1*, *Glucagon*, *Somatostatin*, and *Pancreatic polypeptide (PP)*), and what are considered to be endoderm progenitor markers (*HNFs1 β* , *3 β* , *4 α* , and *6*). The oligonucleotide primers and cycle number used for semiquantitative PCR are shown in Supplementary Table 1. Mouse liver and islets were used as controls. Gene expression studies in all groups were repeated at least three times independently. (G) Immunofluorescent analysis for CK7 in IHBECs. IHBECs were positive for both for CK7 (blue) at days 0 (G, left) and 17 (G, right). Propidium iodide (PI, red) was used to show nuclei. Scale bar, 100 μ m.

Figure 3 (A) Nomarski differential interference contrast (DIC) image of IHBECs at day 17. Left panel: when the cells were cultured for 17 days without CEFCM, they displayed signs of senescence. Black arrowheads show large, multinucleated flat cells; white arrowheads show numerous cytoplasmic vacuoles. Right panel: cells were cultured with CEFCM (CEFCM+). The cells were cultured with CEFCM for 14 days, then dispersed and seeded onto collagen-coated dishes and cultured for 3 days (total 17 days), and then form a monolayer of homogenous appearing cells. Scale bar, 100 µm. (B) Quantification of senescent cells at day 17 as judged by the presence of senescence and cytoplasmic vacuoles. The number of aggregates that contain signs of senescence or cytoplasmic vacuoles were counted and compared between CEFCM- and CEFCM+ cultures at day 17. At least three colonies per well that were at least 1 mm apart were selected in each of five independent experiments. A total of fifty colonies were checked in each of the group. Data are depicted as mean $\pm$ S.E.M. of five independent experiments. # $P=0.016$.

some endocrine progenitor genes (*Ngn 3*, *NeuroD*, *Nkx6.1* and *Pdx-1*), but lacked *Ins* mRNA (Fig. 5A). Therefore, we decided to transduce the IHBECs with *Ad-Pdx-1*, *-Neuro3*, *-Neuro D*, *-Pax4* and *-Pdx-1/VP16*.

Transduced IHBECs with *Ad-Pdx-1*, *NeuroD*, or *Pdx-1/VP16*

MOI of 50 MOI for *Pdx-1*, *Neuro3*, *NeuroD*, *Pax4* or 100 for *Pdx-1/VP16* with recombinant Ad resulted in efficient

expression of the transgenes with 30–50% transduction efficiency and high cell survival (Supplementary Figure 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol201/issue1/>). We found that MOIs of 100–200 resulted in toxicity and loss of cells.

Gene expression profiles of IHBECs-derived insulin-producing cells

To analyze the molecular events occurring in IHBECs during the series of culture steps, gene expression profiles of transcription factors and pancreas-related genes were determined by RT-PCR. IHBECs at Step 1 only expressed BEC markers and early liver and pancreas markers (Fig. 2F). At the end of Step 4, Group 1 cells expressed the pancreatic progenitor markers *Neurog3*, *NeuroD*, *Nkx6.1*, *Pdx-1*, and pancreas-specific transcription factor 1 α (*Ptf1 α* ; Fig. 4A). These genes were not observed in cultured IHBECs at day14, therefore, the IHBECs assumed these characteristics in DM culture. Transduced expression of *Pdx-1*, *NeuroD* or *Pdx-1/VP16* led to expression of not only both *Ins 1* and 2 but also *Glut2* and prohormone convertases 1 and 2 (*PC1* and 2). Glucokinase was also expressed at this stage (data not shown). *Pdx-1* or *Pdx-1/VP16* transduced cells faintly expressed amylase (Fig. 4A). Expression of *Ptf1 α* was found in all groups. Neither Ad-NEUROG3 nor Ad -PAX4 led to express *Ins*, therefore, their use were discontinued.

Immunostaining of IHBECs 7 days after transduction with *Ad-Pdx-1/VP16*

PDX-1/VP16 transduction led to the appearance of endocrine precursor and islet related markers. Using immunofluorescence analysis, cells stained for NEUROD⁺ (nuclear) co-expressed both GFP and CK7 (cytoplasm; Fig. 5A). There was complete overlap of NEUROD and DAPI in the nuclei (Fig. 5B). A relatively small number of NEUROD⁺ GFP[−] cells were seen (data not shown). Cells with nuclear *Pdx-1* staining also co-expressed GFP and CK7 (Fig. 5C). As expected, due to the *Pdx-1/VP16* transduction the number of *Pdx-1*⁺ cells was larger than that of NEUROD⁺ cells (Fig. 5C). C-peptide staining confirmed the synthesis of insulin by IHBECs-derived insulin-producing cells. We found that 30.1 and 2.8% of 4000 cells counted in *Pdx-1/VP16* transduced cultures were GFP⁺ and C-peptide⁺ respectively (Fig. 5E and F). Of the GFP⁺ cells, about 6% were strongly stained for C-peptide.

Insulin release into the culture medium by clusters of IHBECs-derived insulin-producing cells

Insulin release into the culture medium was measured at 7 days after transduction (Fig. 5G). From day 5 to day 7 after transduction, *Ad-Pdx-1*, *NeuroD*, and *Pdx-1/VP16* transduced IHBECs released insulin (*Ad-Pdx-1* 0.08 $\pm$ 0.05,

Figure 4 Gene expression profiles in each group. β -cell related markers (*Insulin 1* and *2*, *glucagon*, *somatostatin*, and *pancreatic polypeptide*, *prohormone convertase* (*PC 1* and *2*), *Glucose transporter 2* (*GLUT2*)), transcription factors (*Neurogenin3* (*NEUROG3*), *NeuroD*, *Nkx2.2*, *Paired box4* (*Pax4*), *Nkx6.1*, *MafA*, *Pdx-1*, and *Islet-1* (*Isl1*)), exocrine cell related markers (*pancreas-specific transcription factor 1 α* (*Ptf1 α*), *Amylase*) and β -actin mRNA transcripts were assessed. IHBECs at day 14 in culture were used as a control. Mouse islets, insulinoma cell line MIN6, pancreas were controls. Findings are representative of at least three independent experiments for each. The oligonucleotide primers and cycle number used for semiquantitative PCR are shown in Supplementary Table 1.

Ad-*NeuroD* 0.33 ± 0.09 , Ad-*Pdx-1/VP16* 0.37 ± 0.14 ng/ 1×10^5 cells after 48 h in culture). As controls, the cells from Groups 1 and 2 did not have detectable insulin release (Fig. 5G).

We also monitored the release of insulin into the culture medium in response to various stimuli supplied sequentially over 2 h intervals for group 3 cells. No basal insulin release could be detected for any of the transduced cells, but after exposure to 25 mM glucose or 45 mM KCl, barely measurable amounts of insulin from *Pdx-1/VP16* transduced cells were found in a small number for experiments. Little can be concluded from these inconsistent results. Questions could be raised about, whether 25 mM glucose is high enough to have a glucose toxicity effect. Because such a concentration has been used successfully so often in other studies, we doubt this type of toxicity is contributing much to the low secretion.

Characterization of transduced IHBECs with single cell PCR

From the previous results, it was clear that some IHBECs transduced with PDX-1, NEURO D, PDX-1/VP16 produced insulin. We therefore attempted to characterize these cells on a single cell level. GFP⁺ and GFP[−] single cells from PDX-1/VP16 transductions were placed into wells of 96-well dishes by the ARIA sorter. We gated both PI[−] GFP⁺ populations to retrieve only living cells (Fig. 6B). Group 1 cells were also dispersed into single cells and used as controls

(Fig. 6A). Both PI[−] GFP⁺ populations from Group 2 were also analyzed by the same manner. Twenty three percent of GFP⁺ cells expressed both the insulin 1 and 2 genes in Ad-PDX-1/VP16 transduced IHBECs; cells always expressed both or neither insulin genes. These *Ins 1* and *2* expressing cells also expressed endogenous *NeuroD* and *Pdx-1* genes (data not shown). Importantly, these cells expressed other marker genes including islet genes (*Glucagon*, *Somatostatin*, and *Ppy*) and KRT19 (Table 1C). Neither cells in control group 1 nor GFP negative cells in PDX-1/VP16 transduced cells exhibited insulin gene expression (Table 1A and B). Cells in group 2 were similar to the result of group 1. Twenty eight percent of cells from group 2 expressed *Pdx-1*, however, they lacked *Ins* gene expression.

Discussion

We characterized the differentiation process of transduced IHBECs at the RNA level using RT-PCR, and at protein levels with immunofluorescence and ELISA. To convincingly show that IHBECs produced insulin, exogenous insulin was eliminated from step 4 because apoptotic cells can take up exogenous insulin from the culture medium (Rajagopal *et al.* 2003). We found both insulin mRNA as well as *de novo* synthesis of C-peptide by immunocytochemistry. We have not yet determined the relative proportions of proinsulin to

insulin, but expect that full processing has not taken place in these cells that are not fully differentiated. Our findings demonstrated that IHBECs can be transdifferentiated to insulin-producing cells.

The transcription factors HNF1 β , HNF3 β , HNF4 α , HNF6 are islet progenitor markers (Wilson *et al.* 2003), which form a transcriptional network that coordinates β -cell development and function (Rausa *et al.* 1997, Sumazaki *et al.* 2004, Zaret 2008). Even though HNF1 β in such cells is

downstream of HNF6, it has a crucial role for BECs development (Clotman *et al.* 2002, Lemaigre & Zaret 2004). Moreover, the expression of HNF1 β to define a cellular population that forms the primitive pancreatic ducts (Rausa *et al.* 1997). Such embryonic duct HNF1 β ⁺ cells are phenotypically distinct from earlier pancreatic bud cells, and evidence suggests that they are direct precursors of the NEUROG3⁺ endocrine progenitor cells required for endocrine cell formation (Jacquemin *et al.* 2000, Xu *et al.* 2008). We found that IHBECs even at an early stage possessed these important initiation transcripts (Fig. 2) and that *Neurog3* gene was induced in IHBECs at step 4 even without molecular engineering.

Even though IHBECs are attractive targets for transdifferentiation, the previous techniques for isolation of IHBECs have been cumbersome and associated with a low yield of cells (Alvaro *et al.* 1993, Alpini *et al.* 1997, 2003). Furthermore, *in vitro* studies with freshly isolated cells or intrahepatic bile duct units fragments are limited by the lack of long-term (> 24 h) viability (Alpini *et al.* 2003). Therefore, expansion to obtain significant quantities of healthy IHBECs was an important goal. We thought that maintenance of the polarized characteristics of biliary epithelium might facilitate that goal and postulated that a three-dimensional matrix might be helpful. It has been found that three-dimensional culture conditions can allow polarized cells to keep their original character (Grünert *et al.* 2003, Matsuoka *et al.* 2006). As indicated in Fig. 2E, many cells died within 2 weeks when cultured in two-dimensional conditions. Because the media with its growth factors and collagen were the same with two dimensions versus three-dimensional cultures, we assume there is some effect of the three-dimensional geometry that promoted expansion and improved viability.

From step 4, our serum- and insulin-free DM was used in hopes of priming the cells for transdifferentiation. Indeed, these cells began to express the pancreatic progenitor markers

Figure 5 Immunofluorescence analysis and Insulin release into the culture medium at 7 days after transduction. (A–F) Immunofluorescence analysis of Ad-Pdx-1/VP16 transduced IHBECs at 7 days after transduction. (A–D) Immunofluorescence analysis of transfections from endocrine precursor to hormone-expressing cells. (A) Triple staining for NeuroD (red), GFP (green), and CK7 (blue). (B, D) Nuclear staining for NeuroD and Pdx-1. Fluorescence micrographs showing expression of NeuroD (B) or Pdx-1 (D). DAPI shown in blue. There was a complete overlap between transcription factors and DAPI in the nuclei. (E, F) C-peptide staining for Ad-Pdx-1/VP16 transduced IHBECs 7 days after transduction. Triple staining was for C-peptide (red), GFP (green), and CK7 (blue). Of 4000 cells counted, 2.8% in Pdx-1/VP16 transduced cultures stained strongly for C-peptide. 4000 counted cells from five independent experiments. Scale bars, 50 μ m. (G) Insulin release into the culture medium after 48 h incubation of Ad-Pdx-1/VP16 transduced IHBECs at 7 days after transduction. The insulin levels in IHBECs (Group 1), control cells (Group 2), transduced cells (Group 3, Ad-Pdx-1: white column, Ad-NeuroD: dotted column, Ad-Pdx-1/VP16: black column) were assessed. #*P* = 0.0495. Data are means $\pm$ S.E.M. of 3–7 independent experiments.

Figure 6 (A, B) Flow cytometry analysis of Ad- PDX-1/VP16 transduced IHBECs 7 days after transduction. (A) Flow cytometry analysis of group 1. (1) The cells from IHBECs in DM (non-transduced). (B) Flow cytometry analysis of Ad-PDX-1/VP16 transduced IHBECs 7 days after transduction. (2) Ad-PDX-1/VP16 transduced IHBECs, but non-emitter cells. (3) Ad-PDX-1/VP16 transduced IHBECs. DM, differentiation medium.

NEUROD, NEUROG3, NKX6.1, PDX-1, and PTF1 α (Fig. 4A). If the cells were exposed to GM at this stage, they proliferated and did not express these transcripts (data not shown). Work by Gershengorn *et al.* (2004) showed that to induce insulin mRNA in cytokeratin positive cells, elimination of EGF and the use serum free medium was important. During stage 4 in DM culture, markers for both pancreatic epithelial progenitors and endocrine precursors were found. Recent reports find that *Ptf1 α* is expressed in the progenitors of pancreatic ducts, exocrine, and endocrine cells, rather than

being an exocrine-specific gene as previously thought (Kawaguchi *et al.* 2002). Thus, co-expression of PTF1 α and PDX-1 is required for proper pancreatic determination and subsequent differentiation (Kawaguchi *et al.* 2002, Afelik *et al.* 2006, Wiebe *et al.* 2007). IHBECs cultured for 14 days did not express PTF1 α ; however, all groups of cells expressed PTF1 α after DM culture. It is possible that expression of PTF1 α is a prerequisite for transdifferentiation. The combined use of a two-dimensional culture environment and DM accerelated differentiation pathways toward pancreatic fate but failed to induce insulin gene expression (Fig. 4A). Seven days after transduction with Ad-PDX-1, NEUROD, or PDX-1/VP16, insulin expression was found by RT-PCR, immunofluorescence and ELISA. To further characterize these cells, we examined islet hormone gene expression in single cells that had been transduced with PDX-1/VP16 and found that 91% of *Ins1* and 2 positive single cells expressed all four islet hormone genes (Table 1). This expression of multiple islet hormones likely reflects immaturity as previous studies have found that islet cells can express more than one of these islet hormones simultaneously (Alpert *et al.* 1988, De Krijger *et al.* 1992, Teitelman *et al.* 1993). It is clear that some of these transduced cells have insulin gene expression and contain insulin protein. We can be confident that this insulin resulted from biosynthesis rather than uptake from the medium, as judged by the following criteria: 1) no insulin was detected in GFP transduced IHBECs in the same conditions, 2) insulin was detected in transduced IHBECs in DM that was not

Table 1 Single cell analysis with nested PCR; heterogeneity of islet hormone gene expression in adenoviral (Ad)- pancreas duodenum homeobox 1 (Pdx-1)/VP16 transduced intrahepatic biliary epithelial cells (IHBECs) 7 days after transduction. Separate primers were used for *Ins1*+*Ins2*, but all insulin expressing cells expressed both *Ins1* and *Ins2*

	<i>HPRT</i>	<i>CK19</i>	<i>Pdx-1</i>	<i>Ins1 + Ins2</i>	Number/(%)		
<i>(A) IHBECs in DM (Group 1)</i>							
	+	—	—	—		2 (3)	
	+	+	—	—		46 (70)	
	+	+	+	—		18 (27)	
	+	+	+	+		0 (0)	
Number/%	66 (100)	64 (97)	18 (27)	0 (0)			
<i>(B) GFP negative cells from Pdx-1/VP16 transduced cell population (Group 3)</i>							
	+	—	—	—		1 (2)	
	+	+	—	—		39 (59)	
	+	+	+	—		26 (39)	
	+	+	+	+		0 (0)	
Number/%	66 (100)	65 (98)	26 (39)	0 (0)			
	<i>HPRT</i>	<i>CK19</i>	<i>Ins1 + Ins 2</i>	<i>Glucagon</i>	<i>Somatostatin</i>	<i>PP</i>	Number/(%)
<i>(C) Pdx-1/VP16 GFP positive transduced cells (Group 3)</i>							
	+	—	+	—	—	—	0 (0)
	+	—	—	—	—	—	6 (6)
	+	+	—	—	—	—	71 (71)
	+	+	+	—	—	—	2 (2)
	+	+	+	+	+	+	21 (21)
Number/%	100 (100)	94 (94)	23 (23)	21 (21)	21 (21)	21 (21)	

DM, differentiation medium.

supplemented with insulin, 3) both *Ins1* and 2 mRNA were detected in the cells 4) C-peptide was detected in the cells by immunofluorescence. There are differences among the cells that express insulin, which likely reflects variation in differentiation. Thus, about 23% of GFP+ cells Pdx-1/VP16 transduced cells express Insulin by nested PCR. Of these GFP+ cells, about 6% were strongly stained for C-peptide. We were unable to demonstrate glucose-induced secretion from transduced cells, but future *in vitro* and *in vivo* experiments will determine the capacity of these cells for further differentiation.

Data are only presented from the experimental conditions described in the methods. We have also used various doses and combinations of other agents including nicotinamide, betacellulin, activins, hepatocyte growth factor, and exendin-4, but these efforts have not provided improved results. Work on finding optimal differentiation conditions is continuing.

In summary, it has been possible to force the differentiation of IHBECs towards a β -cell phenotype, which supports the possibility that these cells have the potential to be useful for β -cell replacement therapy.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Author contribution statement

M N: Conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.; H K: Provision of study materials. S B W: Conception and design, financial support, data interpretation, manuscript writing, final approval of manuscript; G C W: Conception and design, financial support, data interpretation, manuscript writing, final approval of manuscript.

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