Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (1): 20-29
Published online February 28, 2015 (http://www.cellmolbiol.com)
Received on December 5, 2014, Accepted on January 20, 2015.
doi : 10.14715/cmb/2015.61.1.4

Forced expression of PDX-1 gene makes hepatoma cells to acquire glucose-responsive insulin secretion while maintaining hepatic characteristic

H. Hashimoto[1]✉, Y. Higuchi[1] and K. Kawai[2]

[1] Laboratory Animal Research Department, Central Institute for Experimental Animals, 3-25-12, Tonomachi, Kawasaki-ku, Kawasaki, 210-0821, Japan.
[2] Pathological Analysis Center, Central Institute for Experimental Animals, 3-25-12, Tonomachi, Kawasaki-ku, Kawasaki, 210-0821, Japan.

Corresponding author: Haruo Hashimoto, Central Institute for Experimental Animals, Japan. Tel: +044-201-8530, Fax: +044-201-8541, Email: hashimot@ciea.or.jp

Abstract

Evidence shows that forced expression of the PDX1 gene converts hepatoma cells, mouse liver epithelial cells (MLECs) and HepaRG cells, into insulin-producing cells, β-cells, or islets of Langerhans. However, no reports have investigated the characteristics of mouse or human hepatocytes introduced with the PDX1 gene over prolonged observation periods. In this study, we immunohistologically and molecularly investigated the alternative processes induced by PDX1 gene introduction in mouse and human hepatocytes over prolonged observation periods using immunocytochemistry, immunofluorescence, polymerase chain reaction (PCR), Western blotting, and flow cytometry (FCM) analysis. Immunocytochemical and immunofluorescent observations showed that MLECs and HepaRG cells on 2 and 21 days after introduction of the PDX1 gene comprised cells double-positive for insulin and albumin. Additionally, they showed MAFA expression and glucose-responsive insulin secretion with glucokinase expression. However mouse embryonic fibroblasts introduced with PDX1-GFP could not acquire glucose-responsive insulin secretion and glucokinase expression. Subsequently, we hypothesized that the number of albumin-positive MLECs and HepaRG cells would decrease after introduction of PDX1 due to the conversion of MLECs and HepaRG cells into insulin-producing cells. However, FCM analysis indicated that the number of albumin-positive MLECs and HepaRG cells was not altered by the introduction of PDX1. We thought that MLECs and HepaRG cells introduced with the PDX1 gene could acquire a functional insulin secretory capacity without conversion to β-cells, or islets of Langerhans, and the acquisition could need glucokinase expression.

Key words: Albumin, HepaRG cells, insulin secretion, mouse liver epithelial cells, PDX-1.

Introduction

According to the International Diabetes Federation (http://www.idf.org/worlddiabetesday/news/wdd-14-healthy-eating-diabetes), 382 million individuals live with diabetes worldwide. By 2035, 592 million, or one in 10 individuals, will have the disease. Type 1 diabetes mellitus, an autoimmune disorder, is characterized by the loss of insulin-producing β-cells (1). Pancreatic islet transplantation is one of the most successful therapeutic strategies for diabetes, but several obstacles must be overcome in islet transplantation, including the adverse effects caused by treatment with immunosuppressive drugs, preservation of islet function, and a shortage of islet donors. Therefore, generating functional β-cells would be a promising strategy to provide adequate numbers of these cells for transplantation. In recent years, attention has focused on gene or cell therapy options for diabetes mellitus using artificially prepared non-β-cell-derived β-cells. In particular, the advent of induced pluripotent stem (iPS) cell technology enabled the conversion of skin fibroblast cells (2) and iPS cells (3) into insulin-producing cells. However, conversion using iPS cell technology requires more than 3 weeks (2, 3) and requires expense. Consequently, we turned our attention to forced expression of the PDX1 gene in hepatocytes using Lipofectamine-mediated transfection and adeno-associated viruses. Many research groups have found that the forced expression of PDX1 into various cells, such as hepatocytes (4), neural stem cells (5), small intestinal epithelial cells (6), and keratinocytes (7), induced insulin-producing cells. Most research studies have used hepatocytes. However, to our knowledge, no studies have reported on hepatocyte characteristics after introduction with the PDX1 gene over prolonged observation periods, despite the more than 3 weeks required for iPS cell technology (2, 3). We are sure that it is significantly to understand the prolonged characteristic of the cell converted to β cell. In addition, no reports exist indicating that hepatocytes from adult human liver are converted into β-cells in vitro, with the exception of the human hepatoma cell line HepG2 (8).

A pancreatic progenitor cell is defined by the expression of PDX1, and we thus immunohistologically and molecularly investigated the alternative processes induced by PDX1 gene introduction in mouse and human hepatocytes over prolonged periods. We expected our results to reveal information on the alternative processes involved in cell formation and acquisition of insulin secretory capacity induced by expression of the PDX1 gene.

Materials and methods

Culture of MLECs and mouse embryonic fibroblasts (MEFs)

Mouse liver epithelial cells (MLECs) derived from C3H mice established by Lee et al. (9) and MEFs derived from IQI/Jic mice established at our institute were used in this study. The hepatocytes and MEFs

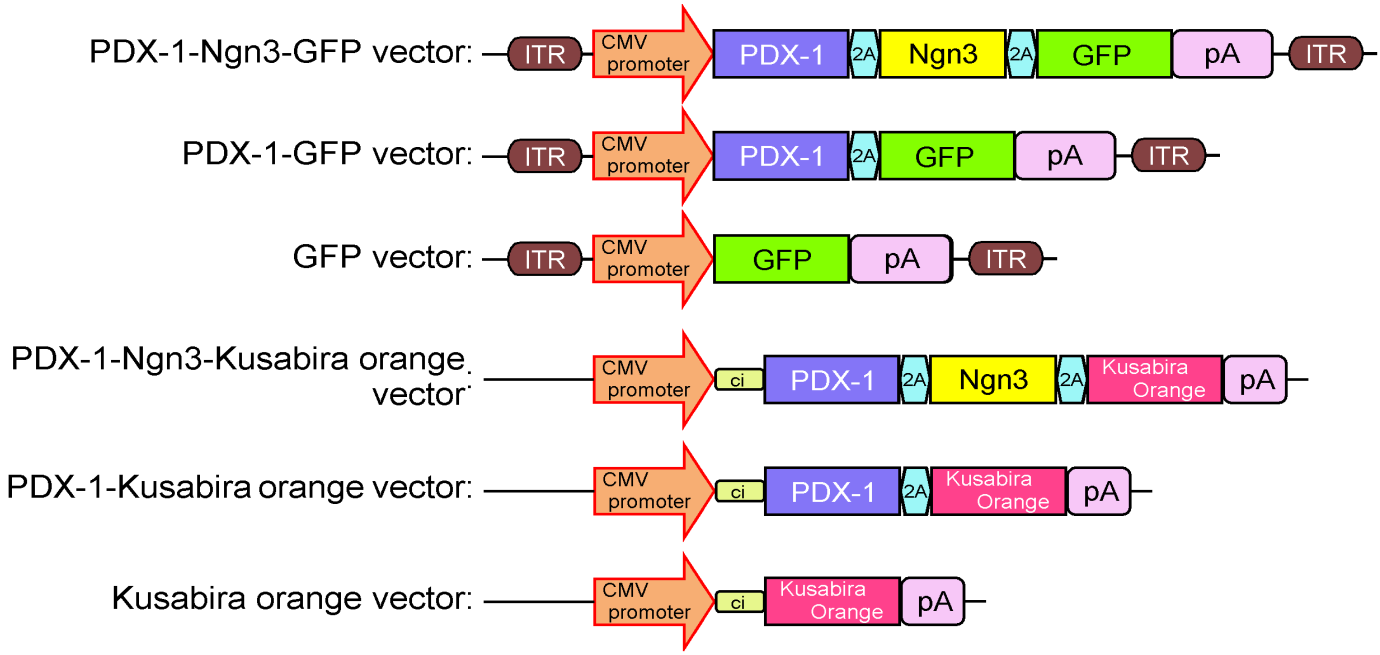

Figure 1. Expression vectors used in this study. PDX1-Ngn3-GFP, PDX1-GFP, and GFP vectors were used for transfection and AAV production. PDX1-Ngn3-Kusabira-Orange, PDX1-Kusabira-Orange, and Kusabira-Orange vectors were used for transfection. Fluorescent protein genes encoding GFP or Kusabira-Orange within expression vectors were adapted to suit the different experimental conditions.

were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 mg/l penicillin, and 75 mg/l streptomycin. MLECs and MEFs (1×10^6 cells/dish) were plated on collagen 1-coated 35-mm dishes or collagen 1-coated glass coverslips placed in the dish.

Culture of HepaRG cells

HepaRG cells were provided by Merck Millipore (Cat No. MMHPR116, Billerica, MA, USA) and maintained as described by Gripon et al. (10). Culture medium comprised Williams' Medium E supplemented with 2 mM L-glutamine, 1% (v/v) pen/strep (all from Invitrogen, Carlsbad, CA, USA), 5 µg/ml bovine insulin, 50 µM hydrocortisone (both from Sigma-Aldrich, St. Louis, MO, USA), and 10% (v/v) FetalClone (HyClone, Logan, UT, USA). The 2-3 week differentiation process was induced by adding 2% (v/v) dimethyl sulfoxide to the culture medium.

Preparation and cell transfection of PDX1, Ngn3, and fluorescent protein expression vectors

Six different DNA constructs were prepared (Figure 1), including "CMV promoter-human PDX1 gene (PDX1)-2A-human neurogenin 3 (Ngn3)-2A- fluorescent protein gene (PDX1-Ngn3-fluorescent protein)," "CMV promoter-human PDX1-2A-fluorescent protein gene (PDX1-fluorescent protein)," and "CMV promoter-fluorescent protein gene." The fluorescent protein genes in these vectors encoded either green fluorescent protein (GFP) or Kusabira-Orange and were used appropriately, according to the purpose of the experiment.

Transient transfections

MLECs, mouse MEFs, and HepaRG cells were plated in 35-mm dishes 48 h before transfection. The standard culture medium was switched to reduced serum medium 2 h before transfection. A 8-µg conjugate of each expression vector shown in Figure 2 with 48 µg FuGENE HD (Cat No. E2311 and E2312, Promega, Madison, WI, USA) was transfected into the cells. The transfected cells were refreshed with standard medium 6 h after transfection.

Virus production and infection in HepaRG cells

AAV2-PDX1-(2A)-GFP and AAV-GFP were produced using a standard triple transfection method employing a transgene vector, the pAAV-RC2 vector (Cat No. 340201, Cell Biolabs, Inc., San Diego, CA, USA), and the pHelper vector (Cat No. 340202, Cell Biolabs, Inc.) with FuGENE HD and were packaged into adeno-associated virus (AAV) serotype 2 capsids. Subsequently, the AAVs were purified using the ViraBind™ AAV Purification Kit (Cat No. VPK-140, Cell Biolabs, Inc.) and titered using the QuickTiter™ AAV Quantitation Kit (Cat No. VPK-145, Cell Biolabs, Inc.) according to the manufacturer's protocols.

AAVs (1.0×10^9 GC) were added to HepaRG cells plated at 80% confluence in 35-mm dishes and incubated for 24 h, after which the HepaRG cells were washed with phosphate-buffered saline (PBS) and 2 ml fresh standard medium.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen). RNA samples were pretreated with DNase I (Invitrogen) to remove any contaminating genomic DNA. First-strand cDNA was synthesized using SuperScript III RNase H-Reverse Transcriptase (Invitrogen). To confirm the absence of genomic DNA contamination, samples with no reverse transcriptase treatment were prepared. The oligonucleotide primers used in this study were as follows: mouse PDX1 (sense: 5′-GGACATCTCCCCATACGAAG-3′, antisense: 5′-TTCAACATCACTGCCAGCTC-3′; 365 bp), human PDX1 (sense: 5′-GCCTTTCCCATGGATGAAGTC-TAC-3′, antisense: 5′-GTCCCGCCGCCGCGCTTC-3′; 287 bp), mouse Ngn3 (sense: 5′-CTCAGCAAA-CAGCGAAGAAG-3′, antisense: 5′-CTGAGTCAG-

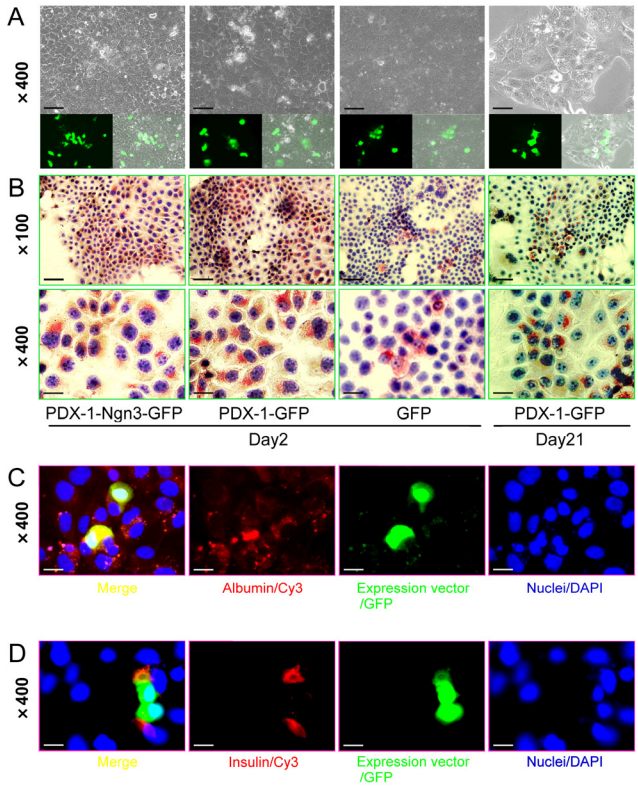

Figure 2. Albumin- and insulin-positive MLECs introduced with PDX1-Ngn3- fluorescent protein or PDX1-fluorescent protein. A: Fluorescence photomicrographs and immunochemical double-staining of MLECs 2 and 21 days after the introduction of PDX1-Ngn3-GFP, PDX1-GFP, or GFP using FuGENE HD. B: Albumin and insulin are depicted in red and brown, respectively. The upper photomicrographs were taken at a magnification of ×100. The lower photomicrographs were taken at a magnification of ×400. The calibration bars in the ×100 images represent 200 μm, and those in the ×400 images represent 50 μm. C: Immunofluorescent observations of albumin (Cy3) and GFP in MLECs 2 days after introduction of the PDX1- GFP-expression vector using FuGENE HD. The red fluorescence signal represents albumin. The green fluorescence signal represents GFP from the expression vector. The yellow fluorescence represents the overlaying signals from albumin and GFP. The calibration bars in the ×400 images represent 50 μm. D: Immunofluorescent observations of insulin (Cy3) and GFP present in MLECs 2 days after introduction of the PDX1- GFP -expression vector using FuGENE HD. The red fluorescence represents insulin. The green fluorescence represents GFP from the expression vector. The yellow fluorescence represents the overlaying signals from insulin and GFP. The calibration bars in the ×400 images represent 50 μm.

TGCCCAGATGT-3′; 190 bp), human Ngn3 (sense: 5′-AATGCACAACCTCAACTCG-3′, antisense: 5′-TACAAGCTGTGGTCCGCTAT-3′; 155 bp), mouse MAFA (sense: 5′-CTTCAGCAAGGAGGAGGTC-3′, antisense: 5′-CGCCAACTTCTCGTATTTCT-3′; 216 bp), human insulin (sense: 5’-GAGGCCATCAAG-CACCATCAC-3, antisense: 5’-GGCTGCGTCTAGT-TGCAGTA-3’; 412 bp), and mouse insulin (sense: 5’-CAGCCCTTAGTGACCAGCTA-3’, antisense: 5’-ATGCTGGTGCAGCACTGATC-3’; 340 bp). PCR amplification was performed in 20 μl EmeraldAmp Max PCR Master Mix (Cat No. RR320A, TaKaRa, Tokyo, Japan) via 25-30 cycles consisting of 95°C for 30 s, 63.2°C (mouse PDX1), 67.5°C (human PDX1), 62.8°C (mouse Ngn3), 60.0°C (human Ngn3, human insulin), 60.8°C (mouse MAFA), or 68.0°C for 25-35 s, and 72°C for 5 min. The PCR products were subjected to 1.0-1.2% agarose gel electrophoresis and visualized by Gel Red staining (Cat No. WK04529795, Wako, Osaka, Japan).

Western blot analysis

Total proteins from cells were extracted from the separated red phase generated by TRIzol reagent extraction and washed with 2-propanol and $GuCl_2$. A total of 40 μg protein extract in 1% sodium dodecyl sulfate (SDS) solution were separated electrophoretically on 7% polyacrylamide gels and blotted onto nitrocellulose membranes (Hybond™ ECL™, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The membranes were incubated with primary antibodies, followed by the ECL™ anti-rabbit immunoglobulin (IgG), horseradish peroxidase (HRP)-linked $F(ab')_2$ fragment (Cat No. NA9340V, GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, UK). The intensities of the separated bands were quantified using the ImageQuant LAS 4000 Mini (GE Healthcare UK, Ltd.). Antibodies used in this study included anti-human PDX1 (28 kDa; Cat No. ab72324, Abcam, Cambridge, UK; 1:200), anti-human Ngn3 (27 kDa; Cat No. H-80, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1500), anti-mouse Ngn3 (27 kDa; Cat No. M-80; Santa Cruz Biotechnology; 1:400), anti-human MAFA (37 kDa; Cat No. ab26405, Abcam; 1:300), anti-mouse MAFA (52kDA; Cat No. ab17976, Abcam; 1:5000), and anti-mouse/human GAPDH (37kDA; Cat No. ab9485, Abcam; 1:2500).

Morphological analysis and immunocytochemistry

Cells cultured on collagen 1-coated glass coverslips were fixed with 4% paraformaldehyde in PBS, incubated sequentially with 0.3% hydrogen peroxide (H_2O_2) in methanol, and then blocked with 1% rabbit serum for 30 min. The primary antibodies used included guinea pig anti-porcine insulin (Cat No. A0564, Dako, Glostrup, Denmark; 1:100) and goat anti-mouse albumin (Cat No. A90-234A, Bethyl, Montgomery, TX, USA; 1:1000). The secondary antibodies used included guinea pig/IgG/HRP (Cat No. P0141, DAKO; 1:40) and donkey anti-goat IgG H&L (AP) (Cat No. ab6886, Abcam; 1:100).

Immunofluorescence analysis

MLECs and HepaRG cell cultures on glass cover slides in 35-mm dishes were introduced with the PDX1-fluorescent protein gene, PDX1-Ngn3-GFP gene, or GFP gene alone for 2-21 days. The cells were fixed using 4% paraformaldehyde phosphate buffer solution (Cat No. 163-20145, Wako). Slides were washed with PBS and blocked with either 10% normal goat serum (Cat No. H0809, Nichirei Bioscience Inc., Tokyo, Japan) or 10% normal rabbit serum (Cat No. H1212, Nichirei Bioscience Inc.) for 30 min. For insulin detection in MLECs, biotin-labeled insulin antibody (Cat No. 13-9769-80, eBioscience, San Diego, CA, USA). For albumin detection in MLECs, the primary antibody used was biotin-labeled mouse albumin antibody (Cat No. A90-234B, Bethyl). For human albumin detection in HepaRG cells, the primary antibody used was biotin-labeled human albumin antibody (Cat No. A80-229A, Bethyl). The secondary antibody used for insulin, mouse albu-

min, and human albumin detection was Cy™3-conjugated AffiniPure goat anti-mouse IgG (H+L) (Cat No. 115-165-003, Jackson ImmunoResearch, West Grove, PA, USA). Detection of GFP and Kusabira-Orange did not require antibodies because they were expressed via plasmids. Finally, the cells were stained and mounted with 4′,6-diamidino-2-phenyl-indole (DAPI, Immuno-Select Antifading Mounting Medium DAPI, Cat No. SCR-38448, Dianova, Hamburg, Germany). The slides were analyzed using a fluorescence microscope (AXIO Imager, M1, Cat No. 18355, Carl Zeiss International, Barcelona, Spain).

Northern blot analysis

Ten micrograms of total RNA from each sample was separated by electrophoresis on a 1.5% agarose gel containing 2% formaldehyde. RNA was transferred to a positively charged nylon membrane (GE Healthcare UK, Ltd.) and hybridized with digoxigenin (DIG)-labeled cDNA probes specific to the mouse glucokinase, human glucokinase, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and human GAPDH genes. The membrane was incubated further with an alkaline phosphatase-labeled anti-DIG antibody (Cat No. 11093274910, Roche Diagnostics, Basel, Switzerland). The blots were developed using CDP-star (Cat No. 11655884001, Roche Diagnostics). The ImageQuant LAS 4000 Mini (GE Healthcare UK, Ltd.) was used to detect the bands for each gene evaluated.

FCM analysis

Protocol for the detection of albumin in MLECs

Two or 7 days after introduction of PDX1-Kusabira-Orange, PDX1-Ngn3-Kusabira-Orange, or Kusabira-Orange, MLECs (6×10^6) were collected and washed with PBS. The MLECs were added to IC Fixation Buffer (Cat No. 00-8222-491, eBioscience Inc., San Diego, CA, USA), and then to a permeabilization solution (Cat No. 421002, BioLegend, San Diego, CA, USA). After removing the supernatant by centrifugation, the MLECs were blocked with 10% normal rabbit serum (Cat No. H1212, Nichirei Bioscience Inc.) for 15 min. After further removal of the supernatant by centrifugation, MLECs were treated with goat anti-mouse albumin FITC (Cat No. A90-23F, Bethyl) and diluted with permeabilization solution (Cat No. 421002, BioLegend). After replacing the cells in PBS, the MLECs were analyzed by FACSCanto. Detection of Kusabira-Orange did not require antibodies because it was expressed via plasmids. Data were recorded using the BD FACS Diva Software program (BD Biosciences, Tokyo, Japan) and analyzed using the Flowjo program (Tree Star Inc., Ashland, OR, USA).

Protocol for the detection of albumin in HepaRG cells

Two or 7 days after introduction of PDX1-Kusabira-Orange, PDX1-Ngn3- Kusabira-Orange, or Kusabira-Orange, HepaRG cells (6×10^6) were collected and washed with PBS. The HepaRG cells were added to IC Fixation Buffer (Cat No. 00-8222-491, eBioscience) and then to a permeabilization solution (Cat No. 421002, BioLegend). After removal of the supernatant by centrifugation, the HepaRG cells were blocked with 10% normal rabbit serum (Cat No. H1212, Nichirei Bioscience Inc.) for 15 min. After further removal of the supernatant by centrifugal separation, the HepaRG cells were treated with biotin-labeled human albumin antibody (Cat No. A80-229A, Bethyl) as the primary antibody, followed by APC-streptavidin (Cat No. 554067, BD Biosciences) as the secondary antibody. After replacing the cells in PBS, the HepaRG cells were analyzed by FACSCanto. Data were recorded using the BD FACS Diva Software program (BD Biosciences) and analyzed using the Flowjo program (Tree Star Inc.).

Results

Insulin secretion from albumin-positive MLECs and MEFs introduced with the PDX1-Ngn3 or PDX1 gene

Morphological observations of MLECs introduced with the PDX1-Ngn3-GFP, PDX1-GFP, or GFP genes are shown in Figure 2-A. Cell morphology was observed for 21 days after introducing PDX1-GFP or PDX1-Ngn3-GFP. The expression of the fluorescent proteins in the same cells was used to monitor the expression of the introduced gene. The morphologies were identical to those of the control cells that expressed only GFP (Figure 2-A). Furthermore, the MLECs introduced with PDX1-Ngn3-GFP or PDX1-GFP, but not with GFP, were albumin-and insulin-positive in immunochemical observations (Figure 2-B). Immunofluorescent observations also confirmed the albumin (Cy3) and the GFP after introduction of PDX1-GFP expression vector (Figure 2-C). In addition, immunofluorescent staining confirmed that the expression of insulin (Cy3) and the GFP in MLECs occurred within the same cells (Figure 2-D).

Furthermore, when the PDX1-Ngn3-Kusabira orange or PDX1- Kusabira orange gene was introduced into MEFs established from IQI/Jic mice, their morphology was also identical to that of MEFs expressing Kusabira orange only, which were used as the control (Figure 3). Immunochemical observations confirmed the insulin-positivity after the PDX1-Ngn3-Kusabira orange the PDX1-Ngn3-Kusabira orange and PDX1-Kusabira orange introduction (Figure 3, lower panels).

Comparison of insulin concentrations secreted from MLECs and MEFs introduced with the PDX1-Ngn3 or PDX1 gene: MAFA gene and protein expression in MLECs and MEFs introduced with the PDX1-Ngn3 or PDX1 gene

Insulin secreted into the culture medium from MLECs was measured on days 2, 7, and 21 after introduction of the PDX1-GFP or PDX1-Ngn3-GFP gene (Figure 4-A). The concentrations of insulin (mean ± SD) secreted by MLECs were 0.91 ± 0.20, 0.85 ± 0.30, and 0.14 ± 0.04 ng/ml after introduction of PDX1-GFP and 0.89 ± 0.26, 0.92 ± 0.18, and 0.12 ± 0.03 ng/ml after introduction of PDX1-Ngn3-GFP on days 2, 7, and 21, respectively. No insulin was detected in the culture medium of MLECs introduced with GFP as the control. Insulin concentrations were also measured in the culture medium of MEFs derived from the IQI/Jic mouse strain on days 2, 7, and 21 after introduction of the PDX1-GFP or PDX1-Ngn3-GFP gene (Figure 4-B). In MEFs, the insulin concentrations were 20.08 ± 1.10, 18.77 ± 0.78, and 4.24 ± 1.59 ng/ml after introduction with PDX1-

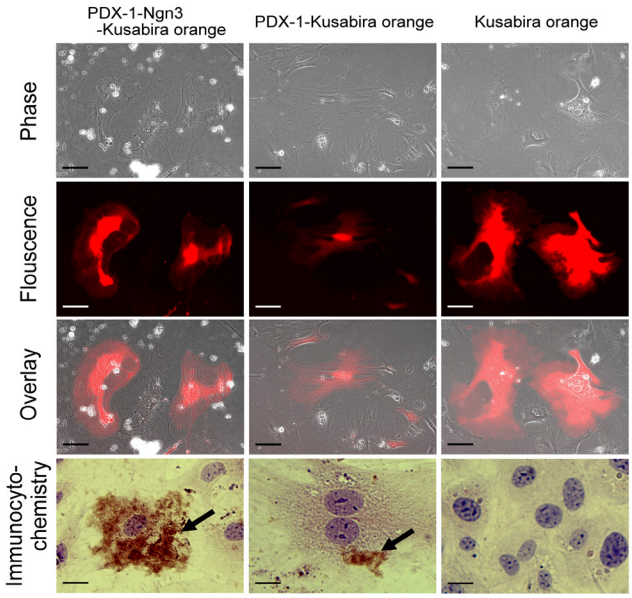

Figure 3. Fluorescence and immunochemistry photomicrographs of MEFs introduced with PDX1-Ngn3-Kusabira orange, PDX1-Kusabira orange, or Kusabira orange using FuGENE HD. Insulin is depicted in brown in the lower images. The calibration bars represent 50 μm and 200μm (Lower pannels). Arrows indicate insulin.

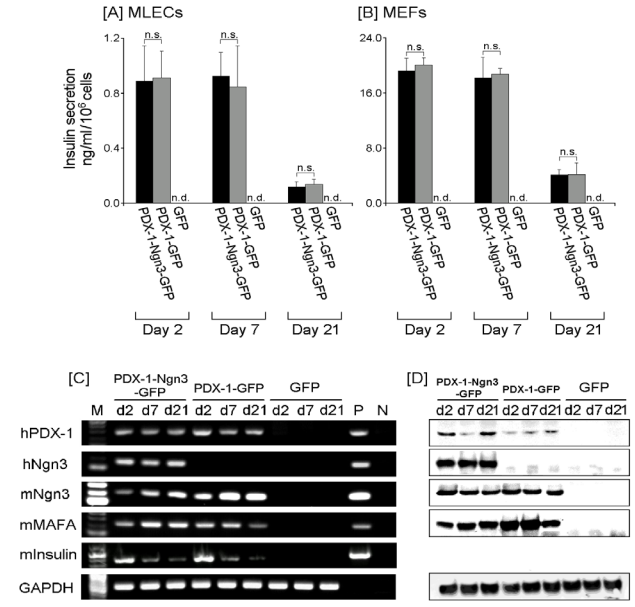

Figure 4. Characteristics of MLECs introduced with PDX1-Ngn3-GFP, PDX1-GFP, or GFP alone. A, B: The concentrations of insulin secreted from MLECs (A) and MEFs (B) 2, 7, and 21 days after introduction of PDX1-Ngn3-GFP, PDX1-GFP, or GFP using FuGENE HD. The insulin concentrations in the medium are expressed as means ± SD (ng/ml/10^6 cells). The threshold for statistical significance was $p < 0.05$. n.d., not detectable; n.s., not significant. C: RT-PCR was performed to evaluate the expression of human PDX1, human Ngn3, mouse Ngn3, mouse MAFA, mouse insulin. Lane 1 contains a DNA ladder (Cat No. 313-06961, Nippongene Co., Ltd., Tokyo, Japan). Total RNA was isolated from MLECs introduced with PDX1-Ngn3-GFP (lanes 2-4), PDX1-GFP (lanes 5-7), or GFP (lanes 8-10). P represents the positive controls, which comprised human PDX1, human Ngn3, mouse Ngn3, and mouse MAFA expressed from DNA fragments previously cloned into the T-vector (pCR2.1, Invitrogen). N represents the negative control, which was the T-vector alone. D: Western blotting was performed to detect human PDX1, human Ngn3, mouse Ngn3, and mouse MAFA protein expression.

GFP and 19.21 ± 1.86, 18.24 ± 2.94, and 4.14 ± 0.79 ng/ml after introduction with PDX1-Ngn3-GFP on days 2, 7, and 21, respectively. No insulin secretion was detected in the culture medium of MEFs introduced with GFP only.

At these times, gene (Figure 4-C) and protein (Figure 4-D) expression in hepatocytes introduced with PDX1-Ngn3-GFP, PDX1-GFP, or GFP were investigated. The MLECs introduced with the PDX1-Ngn3-GFP and PDX1-GFP genes expressed both the human PDX1 and Ngn3 plasmid genes and their corresponding proteins, as well as both the mouse Ngn3 and MAFA genes and their corresponding proteins. Additionally, PDX1-Ngn3-GFP and PDX1-GFP genes expressed mouse insulin gene. Expression of these genes and proteins was not detected in MLECs introduced with the GFP gene only.

Glucose-responsive insulin secretion from MLECs and MEFs introduced with the PDX1-Ngn3 or the PDX1 gene

MLECs that were introduced with the PDX-1-GFP showed glucose-responsive insulin secretion (Figure 5-A). The insulin concentration produced by MLECs cultured in standard medium (22.2 mmol/l glucose) was 0.68 ± 0.32 ng/ml. It significantly decreased to 0.13 ± 0.09 ng/ml by culture in a medium containing 11.1 mmol/l glucose but significantly increased to 0.96 ± 0.35 ng/ml by culture in a medium containing 33.3 mmol/l glucose. In contrast, insulin concentrations produced by MEFs that were introduced with PDX-1-GFP, cultured in media containing 22.2, 11.1, and 33.3 mmol/l glucose, were 17.40 ± 3.36, 20.56 ± 0.81, and 18.95 ± 1.60 ng/ml, respectively (Figure 5-B). These insulin concen-

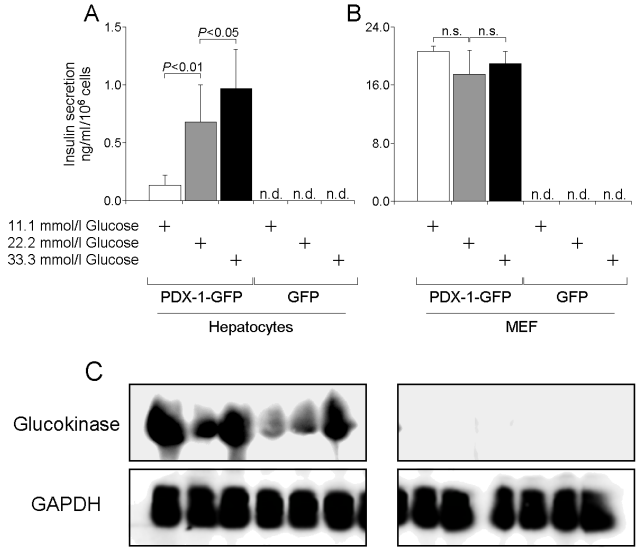

Figure 5. Evaluation of glucose-responsive insulin secretion from MLECs and MEFs introduced with PDX1-GFP or GFP using FuGENE HD. A, B: Three days after the introduction of PDX1-GFP or GFP, the hepatocytes (A) and MEFs (B) were switched from a standard medium containing 22.2 mmol/l glucose to medium containing either 11.1 or 33.3 mmol/l glucose; the glucose-responsive insulin concentrations were evaluated after 2 days. C: Northern blot analysis of the hepatocytes and MEFs corresponding to the insulin secretion levels shown in (A) and (B). The insulin concentrations in the media are expressed as means ± SD (ng/ml/10^6 cells). The threshold of statistical significance was $p < 0.05$. n.d., not detectable; n.s., not significant.

trations were not altered by the concentration of glucose in the medium; rather, they showed excess secretion.

To confirm the expression of the glucokinase gene in MLECs and MEFs introduced with PDX1-GFP or GFP and cultured in media containing 11.1, 22.2, or 33.3 mmol/l glucose, we examined glucokinase expression by Northern blot analysis (Figure 5-C). Glucokinase was highly expressed in hepatocytes, but not MEFs, under these conditions.

Insulin secretion from albumin-positive HepaRG cells introduced with the PDX1-Ngn3 or PDX1 gene

Next, we investigated the effects of PDX-Ngn3 or PDX1 gene on HepaRG cells as human hepatocyte because HepaRG cells were absolutely applied to drug screening test for hepatic metabolites. Immunofluorescence observations of HepaRG cells 2 days after introduction of the PDX1-Ngn3-GFP, PDX1-GFP, or GFP genes are shown in Figure 6-A. The morphologies of these cells were identical to those of cells expressing GFP alone. In addition, 2 days after the introduction of the PDX1-Ngn3-GFP or PDX1-GFP gene, HepaRG cultures were confirmed for immunofluorescence-positive cells, demonstrating the presence of both albumin and insulin. Immunofluorescence observations in HepaRG cultures 2 and 21 days after infection with AAV2 carrying PDX1-GFP or GFP alone are also shown in Figure 6-B. The morphology of cells infected with AAV2 carrying the PDX1-GFP gene was identical to that of cells infected with AAV2 carrying the GFP gene alone. In addition, immunofluorescence analysis of HepaRG cultures 2 and 21 days after infection with AAV2 carrying the PDX1-GFP or GFP gene showed positive expression of albumin. Detections of human insulin were omitted in HepaRG cells because the corresponding of insulin and PDX1-GFP-expression vector was confirmed in MLECs introduced with the PDX1-GFP gene (Figure 2-D).

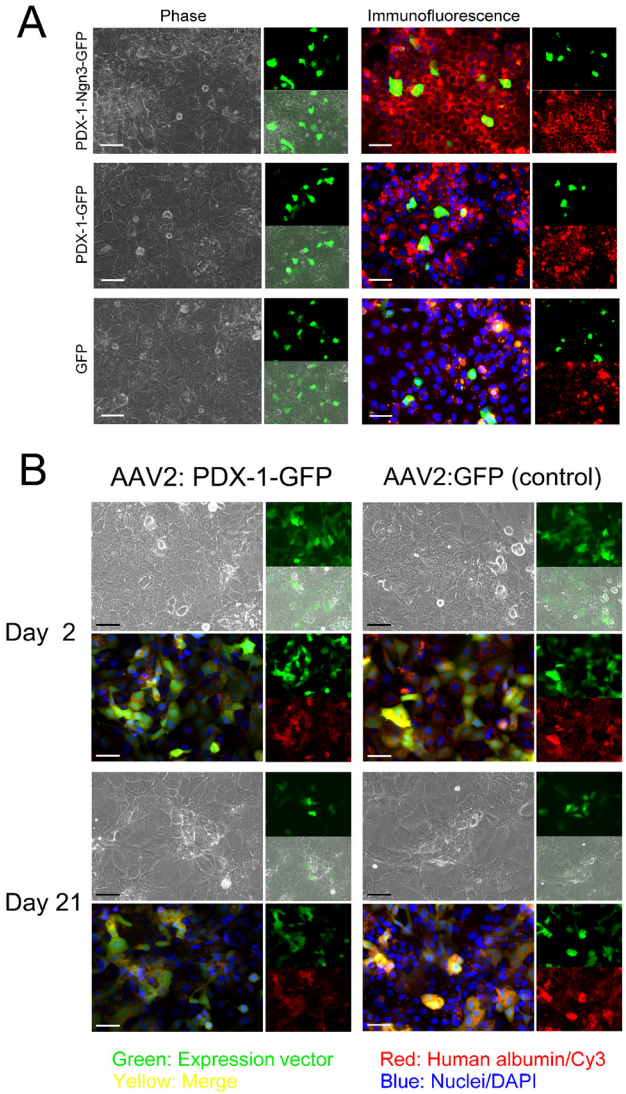

Figure 6. Immunofluorescence photomicrographs of HepaRG cells. A: HepaRG cells 2 and 21 days after the introduction of PDX1-Ngn3-GFP, PDX1-GFP, or GFP using FuGENE HD. B: HepaRG cells 2 and 21 days after infection of PDX1-Ngn3-GFP, PDX1-GFP, or GFP using AAV2. The green fluorescence in the phase images is emitted from the GFP in the expression vectors. The red fluorescence represents human albumin. The yellow fluorescence represents the overlaying GFP and human albumin signals. Calibration bars in all images represent 50 μm.

Comparison of insulin concentrations secreted from HepaRG cells introduced with the PDX1-Ngn3 or PDX1 gene

HepaRG cultures secreted insulin into the culture medium 2, 7, and 21 days after introduction of the PDX1-GFP or PDX1-Ngn3-GFP gene (Figure 7-A). HepaRG cells introduced with the PDX1-Ngn3-GFP gene secreted insulin up to concentrations (mean ± SD) of 0.35 ± 0.07, 0.39 ± 0.06, and 0.15 ± 0.07 ng/ml on days 2, 7, and 21, respectively, after gene introduction. HepaRG cells introduced with the PDX1-GFP gene secreted insulin up to concentrations of 0.34 ± 0.04, 0.41 ± 0.11, and 0.19 ± 0.05 ng/ml on days 2, 7, and 21, respectively. No insulin was secreted from HepaRG cells introduced with the GFP gene only.

HepaRG cells infected with AAV2 carrying PDX1-GFP secreted insulin into the culture medium on days 2, 7, and 21 after infection (Figure 7-B). HepaRG cells infected with AAV2 carrying the PDX1-GFP gene secreted insulin at concentrations of 1.29 ± 0.04, 1.20 ± 0.13, and 0.86 ± 0.12 ng/ml on days 2, 7, and 21 after infection, respectively. No insulin secretion was detected from cells that had been infected with AAV2 carrying only the GFP gene.

At these times, human insulin gene (Figure 7-C) expression in HepaRG cells introduced with PDX1-Ngn3-GFP, PDX1-GFP, or GFP were confirmed. The HepaRG cells infected with AAV2 carrying PDX1-GFP expressed human insulin gene. Furthermore, the HepaRG cells introduced with PDX1-Ngn3-GFP and PDX1-GFP, but not with GFP alone, also expressed human insulin genes

Protein expression in HepaRG cells infected with AAV2-expressing PDX1-GFP or GFP alone was investigated (Figure 7-D). The HepaRG cells infected with AAV2 carrying PDX1-GFP expressed human PDX1 and human Ngn3 proteins from the AAV vectors, as well as human MAFA protein. Expression of these proteins was not detected in the HepaRG cells infected with AAV2 carrying GFP alone. Furthermore, the HepaRG cells introduced with PDX1-Ngn3-GFP and PDX1-GFP, but not with GFP alone, also expressed human PDX1, Ngn3, and MAFA proteins.

Glucose-responsive insulin secretion from HepaRG cells introduced with the PDX1-Ngn3 or PDX1 gene

HepaRG cells introduced with PDX1-GFP demonstrated glucose-responsive insulin secretion (Figure 8-A). The concentration of insulin secreted by HepaRG cells cultured in medium containing 11.1 mmol/l glucose was 0.38 ± 0.09 ng/ml and it significantly increased to 0.63 ± 0.22 ng/ml and 0.66 ± 0.14 ng/ml after culturing in media containing 22.2 and 33.3 mmol/l glucose, respectively. Furthermore, HepaRG infected with AAV2 carrying PDX1-GFP and cultured in medium containing 11.1 mmol/l glucose showed glucose-responsive insulin secretion at a concentration of 1.16 ± 0.09 ng/ml (Figure 8-B). The concentration of insulin increased significantly to 2.12 ± 0.02 ng/ml when these cells were cultured in medium containing 22.2 mmol/l glucose.

To confirm the expression of the glucokinase gene in HepaRG cells introduced with PDX1-GFP or GFP and cultured in media containing 11.1, 22.2, or 33.3 mmol/l glucose, we examined glucokinase expression by Northern blot analysis (Figure 8-C). Glucokinase was highly expressed in HepaRG cells cultured in media containing 11.1, 22.2, and 33.3 mmol/l glucose.

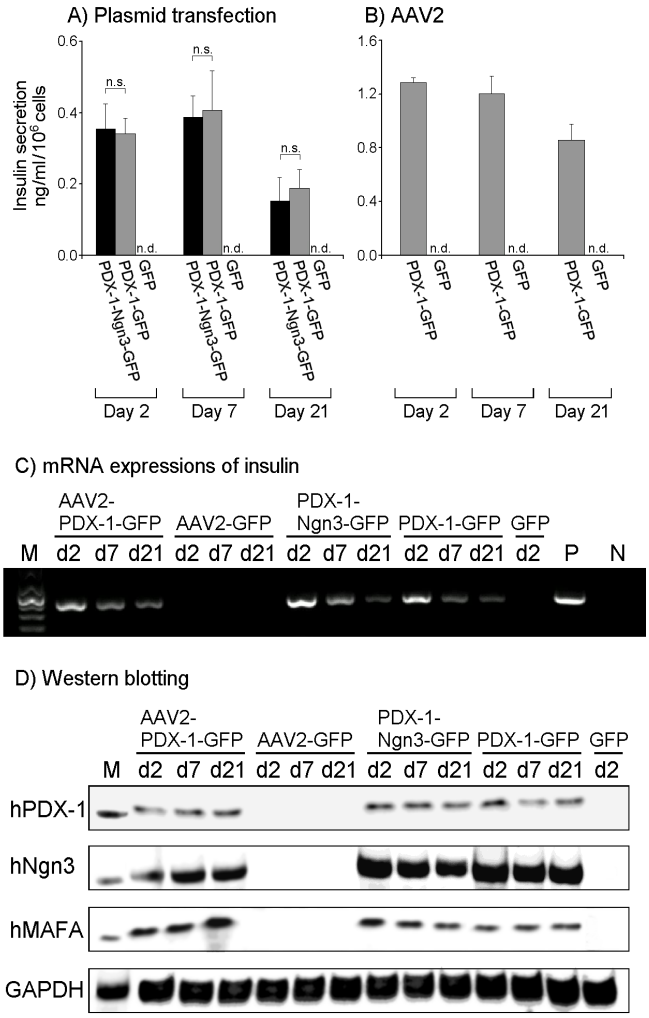

Figure 7. Evaluation of insulin concentrations and expression of hPDX1, hNgn3, and hMAFA in HepaRG cells. A: HepaRG cells 2, 7, and 21 days after introduction of PDX1-Ngn3-GFP, PDX1-GFP, or GFP using FuGENE HD. B: HepaRG cells 2, 7, and 21 days after infection with AAV2 carrying PDX1-GFP or GFP. C: RT-PCR was performed to evaluate the expression of human insulin. Lane 1 contains a DNA ladder (Cat No. 313-06961, Nippongene Co., Ltd., Tokyo, Japan). Total RNA was isolated from HepaRG cells 2, 7, and 21 days after infection with AAV2 carrying PDX1-GFP (lanes 2-4) or GFP (lanes 5-7) and after introduction of PDX1-Ngn3-GFP (lanes 8-10), PDX1-GFP (lanes 11-13), or GFP alone (Lane 14) using FuGENE HD.
P represents the positive controls, which comprised human insulin expressed from DNA fragments previously cloned into the T-vector (pCR2.1, Invitrogen). N represents the negative control, which was the T-vector alone. D: Protein expression in HepaRG cells 2, 7, and 21 days after infection with AAV2 carrying PDX1-GFP or GFP and after introduction of PDX1-Ngn3-GFP, PDX1-GFP, or GFP alone using FuGENE HD. Insulin concentrations in the medium were expressed as means ± SD (ng/ml/10^6 cells). The protein marker used was Wide View™ Prestained Protein Size Marker III (Cat No. 230-024-61, Wako). The threshold of statistical significance was $p < 0.05$. n.d., not detectable; n.s., not significant.

FCM analysis of albumin- and expression vector-positive MLECs and HepaRG cells

FCM analysis showed that MLECs introduced with the PDX1-Kusabira-Orange gene via plasmid transfection included albumin-positive, Kusabira-Orange-positive, and albumin-Kusabira-Orange double-positive cells (Figure 9-A). The percentages of albumin-positive cells included albumin-Kusabira-Orange double-positive in mouse hepatocyte cultures 2 and 21 days after introduction of the PDX1-GFP or PDX1-Ngn3-GFP gene were not altered by the differences of culture day and DNA construction as compared with control.

HepaRG cells introduced with the PDX1 gene were also analyzed by FCM, which showed albumin-positive, Kusabira-Orange-positive, and albumin-Kusabira-Orange double-positive cells (Figure 9-B). The percentages of albumin-positive cells included albumin-Kusabira-Orange double-positive in HepaRG cultures2 and

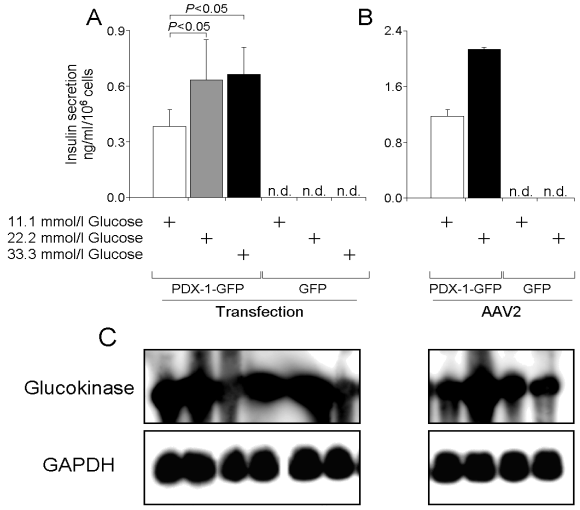

Figure 8. A, B: Evaluation of glucose-responsive insulin secretion from HepaRG cells introduced with PDX1-GFP or GFP using FuGENE HD (A) and from HepaRG cells infected with AAV2 carrying PDX1-GFP or GFP (B). The HepaRG cells 3 days after introduction or infection with PDX1-GFP or GFP were switched from 11.1-mmol/l glucose medium as the standard medium to 22.2- or 33.3-mmol/l glucose medium, and the glucose-responsive insulin concentrations were evaluated after 2 days. C: Northern blot analysis of the HepaRG cells corresponding to the insulin secretion levels shown in (A) and (B). The insulin concentrations in the medium are expressed as means ± SD (ng/ml/10^6 cells). The threshold of statistical significance was $p < 0.05$. n.d., not detectable; n.s., not significant.

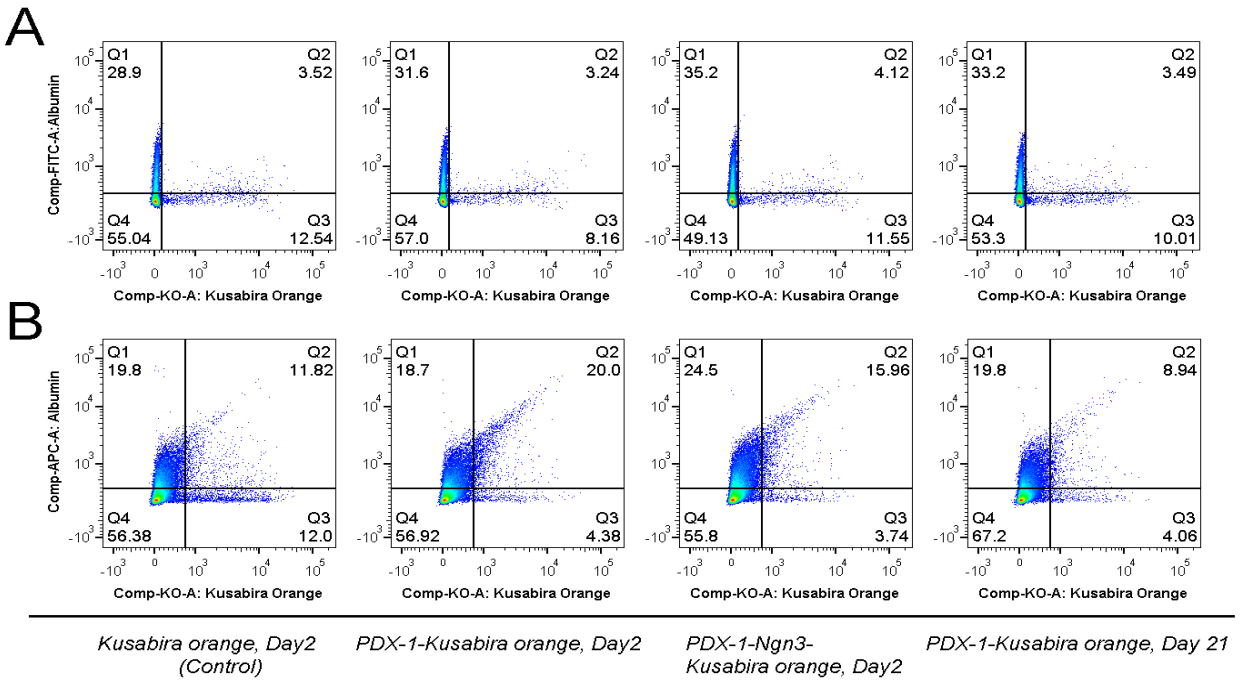

Figure 9. FCM analysis of albumin-positive and Kusabira-Orange-positive MLECs. A: Calculations (%) of the albumin-positive cells among the Kusabira-Orange-positive cells after introduction with PDX1-Kusabira-Orange or PDX1-Ngn3-Kusabira-Orange in MLECs. **B**: Calculations (%) of the albumin-positive cells among the Kusabira-Orange-positive cells after introduction with PDX1-Kusabira-Orange or PDX1-Ngn3-Kusabira-Orange in HepaRG cells.

21 days after introduction of the PDX1-GFP or PDX1-Ngn3-GFP gene were not altered by the differences of culture day and DNA construction as compared with control.

Discussion

Numerous studies havereported the transdifferentiation to beta cells or Langerhan's island from hepatoma cells through the introduction of PDX1-Ngn3 or PDX1 genes. Horb *et al*. (2003) reported that HepG2 in which the PDX1 gene had been introduced differentiated to beta cells or Langerhan's island while secreting albumin (13). However, Lu *et al*. (2005) denied the albumin secretions in the report by Horb *et al*. (2003) (14). Yamada et al. also detected cells expressing both albumin and insulin in rat hepatocytes (4). However, they came to the conclusion that these double-positive cells emerged during the middle of the conversion process from hepatocytes to insulin-producing cells, following the introduction of the PDX1 gene. We hypothesized that forced expression of the PDX1 gene, as reported by Yamada *et al*. (4), would result in the acquisition of an insulin secretory capacity in rat hepatocytes rather than in the conversion of hepatocytes into β-cells.

From 2 to 21 days after the introduction of the PDX1-GFP transgene, the morphologies of both MLECs and HepaRG did not differ from control hepatocytes introduced with the GFP gene. Although approximately 3 weeks are required for the transformation of iPS cells into insulin-producing cells (11), the MLECs and HepaRG cells introduced with the PDX1-GFP or PDX1-Ngn3-GFP gene secreted insulin 2 and 21 days after introduction. Cellular differentiation of MLECs and HepaRG cells after introduction of PDX1 or PDX1-Ngn3 mimicked that of pancreatic stem cells according to the expression of MAFA and other proteins not delivered by the vectors. It suggests that even though the developmental processes of both MLECs and HepaRG cells after the introduction of PDX1 or PDX1-Ngn3 were normal, they were very short in duration with unusual features that were noted during the observation period. FCM analysis also showed that the MLECs and HepaRG cells were not converted into insulin-producing cells by the introduction of PDX1-Kusabira orange or PDX1-Ngn3-Kusabira orange (Figure 9). Albumin is a hepatocyte marker in both mice (12) and humans (15, 16). If the cells had been converted into β-cells or insulin-producing cells, the number of albumin-positive cells in the cultures would be expected to decrease. However, the numbers of albumin-positive cells remained the same after introduction of the PDX1 gene in both cell types. The immunocytochemical observations, together with the results from the fluorescence staining, gene/protein expression, and FCM analyses, suggest that the introduction of the PDX1 gene results in hepatocyte acquisition of an insulin secretory capacity rather than conversion to β-cells.

hTERT-expressing fetal liver cells-derived insulin-producing human hepatocytes were reported to acquire glucose-responsive insulin secretion (17). In this study, MLECs and HepaRG cells introduced with the PDX1-GFP or PDX1-Ngn3-GFP gene showed glucose-responsive insulin secretion. In contrast, MEFs in which the PDX1 or PDX1-Ngn3 genes had been introduced did not show glucose-responsive insulin secretion, although the concentrations of insulin secreted by these cells were higher than those of similarly treated MLECs.

Forced expression of the PDX1 gene was reported to induce glucokinase gene expression in small intestinal epithelial cells (6) and the pancreas[18]. In addition, glucokinase expression caused the release of insulin from granules via mitochondrial processes, the mass-action ratio of ATP, and voltage-dependent Ca^{2+} influx

into β-cells (19). However, IEC-6 cell-derived insulin-producing cells displayed insulin secretion that was not responsive to glucose, even though IEC-6 cells introduced with the PDX1 gene express glucokinase (7). In this study, after introduction of the PDX1-GFP or GFP gene, we confirmed glucokinase gene expression originally in MLECs (Figure 5-C, left) and HepaRG cells (Figure 8-C), but not in MEFs (Figure 5-C, right). Glucokinase expression may have been induced in MEFs by forced expression of the PDX1 gene if the observation period had been prolonged. However, we speculate that the most important aspect related to acquisition of glucose-responsive insulin secretion in various cells is not only PDX1-induced glucokinase gene expression, but also the ability of the original cells introduced with PDX1 to release insulin from granules via the glucokinase, mitochondrial processes, the mass-action ratio of ATP, and voltage-dependent Ca^{2+} influx(19). In addition, glucokinase expression would allow hepatocytes to acquire glucose-responsive insulin secretion more easily compared to cells without the expression of the glucokinase gene (e.g., neural stem cells (5) and small intestinal epithelial cells (6) because the glucokinase gene possesses two distinct promoters, referred to as the liver-specific promoter (20) and β-cell-specific promoter (21, 22).

In conclusion, in this study, the morphology observation and IHC analysis of the PDX1-Ngn3 or PDX1 gene-expressed hepatoma cells confirmed the acquisition of glucose-responsive insulin secretion while maintaining hepatic characteristics. Almost of the studies on hepatocytes introduced PDX1 gene has insisted on the conversion to beta cells or Langerhans's islands based on the expressions of pancreatic marker such as MAFA, insulin and so on. However, we confirmed also insulin secretions of albumin-positive hepatoma cells on 21 days after introduction of PDX1-Ngn3 or PDX1 gene. The 21 days are the period that makes iPS cells convert to beta cells (3). This point is unique method which have not been reported by the past study. So, our findings could be verified by these results (Figure 2 and Figure 6). The acquisition of the new function acquired by the hepatocytes is better for a living body more than a cell of the different with liver such as the pancreas is generated in the liver.

Many research groups have found that the forced expression of PDX1 into various cells, such as hepatocytes (4), neural stem cells (5), small intestinal epithelial cells (6), keratinocytes (7), and iPS cells (3) induced to insulin-producing cells, but they could not acquire the glucose- responsive insulin secretion. Our results indicate that the cells could need some requirements to acquire the glucose- responsive insulin secretion judged from comparison of HepaRG, MLECs and MEF, and need glucokinase, mitochondrial processes, the mass-action ratio of ATP, and voltage-dependent Ca^{2+} influx into β-cells (19) such as hepatocytes.

There are no studies on the possibility of PDX1 gene expressed hepatoma cells with hepatic characteristic and the appropriate cells to acquire the glucose- responsive insulin secretion. We expect that the investigation of mechanism to acquire the glucose- responsive insulin secretion that initially triggered this study could lead to the pioneering of new resources for diabetic treatment by other cells except for a liver.

Acknowledgments

We thank Dr. Hiroshi Suemizu, Central Institute for Experimental Animals, for his critical reading of the manuscript. We thank Ikumi Katano, Central Institute for Experimental Animals, for assistance with Northern blot analysis. This study was supported by a Grant-in-Aid for Scientific Research (C) (No. 24500500) to Haruo Hashimoto from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Skyler JS, Pugliese A. Immunotherapy trials for type 1 diabetes: the contribution of George Eisenbarth. *Diabetes Technol Ther* 2013; Suppl 2:S2-13-S2-20. doi: 10.1089/dia.2013.0107.

2. Pennarossa G, Maffei S, Campagnol M, Tarantini L, Gandolfi F, Brevini TA. Brief demethylation step allows the conversion of adult human skin fibroblasts into insulin-secreting cells. *Proc Natl Acad Sci USA* 2013; 110:8948-8953. doi: 10.1073/pnas.1220637110.

3. Higuchi Y, Shiraki N, Yamane K, Qin Z, Mochitate K, Araki K, Senokuchi T, Yamagata K, Hara M, Kume K, Kume S. Synthesized basement membranes direct the differentiation of mouse embryonic stem cells into pancreatic lineages. *J Cell Sci* 2010; 123:2733-2742. doi: 10.1242/jcs.066886.

4. Yamada S, Yamamoto Y, Nagasawa M, Hara A, Kodera T, Kojima I. In vitro transdifferentiation of mature hepatocytes into insulin-producing cells. *Endocr J* 2006; 53:789-795. doi.org/10.1507/endocrj.K06-116

5. Wang H, Jiang Z, Li A, Gao Y. Characterization of insulin-producing cells derived from PDX1-transfected neural stem cells. *Mol Med Rep* 2012; 6:1428-1432. doi: 10.3892/mmr.2012.1089.

6. Yoshida S, Kajimoto Y, Yasuda T, Watada H, Fujitani Y, Kosaka H, Gotow T, Miyatsuka T, Umayahara Y, Yamasaki Y, Hori M. PDX1 induces differentiation of intestinal epithelioid IEC-6 into insulin-producing cells. *Diabetes* 2002; 51:2505-2513. doi: 10.2337/diabetes.51.8.2505

7. Mauda-Havakuk M, Litichever N, Chernichovski E, Nakar O, Winkler E, Mazkereth R, Orenstein A, Bar-Meir E, Ravassard P, Meivar-Levy I, Ferber S. Ectopic PDX1 expression directly reprograms human keratinocytes along pancreatic insulin-producing cells fate. *PloS One* 2011; 6:e26298. doi: 10.1371/journal.pone.0026298.

8. Li WC, Horb ME, Tosh D, Slack JM. In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. *Mech Dev* 2005; 122:835-847. doi:10.1016/j.mod.2005.01.001

9. Lee GH, Sawada N, Mochizuki Y, Nomura K, Kitagawa T. Immortal epithelial cells of normal C3H mouse liver in culture: possible precursor populations for spontaneous hepatocellular carcinoma. *Cancer Res* 1989; 49:403-409.

10. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA* 2002; 99:15655-15660. doi: 10.1073/pnas.232137699

11. Alipio Z, Liao W, Roemer E, Waner M, Fink LM, Ward DC, Ma Y. Reversal of hyperglycemia in diabetic mouse models using induced-pluripotent stem (iPS)-derived pancreatic β-like cells. *Proc Natl Acad Sci USA* 2010; 107:13426-13431. doi: 10.1073/pnas.1007884107

12. Jones EA, Tosh D, Wilson DI, Lindsay S, Forrester LM. Hepatic differentiation of murine embryonic stem cells. *Exp Cell Res* 2002; 272:15-22. doi:10.1006/excr.2001.5396

13. Horb ME, Shen CN, Tosh D, Slack JM. Experimental conversion of liver to pancreas. *Curr Biol* 2003; 13: 105-115. doi: 10.1016/S0960-9822(02)01434-3

14. Lu S, Wang WP, Wang XF, Zheng ZM, Chen P, Ma KT, Zhou CY. Heterogeneity in predisposition of hepatic cells to be induced into pancreatic endocrine cells by PDX-1. World J Gastroenterol 2005; 11: 2277-2282. doi:10.3748/wjg.v11.i15.2277

15. Suemizu H, Hasegawa M, Kawai K, Taniguchi K, Monnai M, Wakui M, Suematsu M, Ito M, Peltz G, Nakamura M. Establishment of a humanized model of liver using NOD/Shi-scid IL2Rgnull mice. *Biochem Biophys Res Commun* 2008; 377:248-252. doi: 10.1016/j.bbrc.2008.09.124.

16. Hasegawa M, Kawai K, Mitsui T, Taniguchi K, Monnai M, Wakui M, Ito M, Suematsu M, Peltz G, Nakamura M, Suemizu H. The reconstituted 'humanized liver' in TK-NOG mice is mature and functional. *Biochem Biophys Res Commun* 2011; 405:405-410. doi: 10.1016/j.bbrc.2011.01.042.

17. Zalzman M, Anker-Kitai L, Efrat S. Differentiation of human liver-derived, insulin-producing cells toward the beta-cell phenotype. *Diabetes* 2005; 54:2568-2575. doi: 10.2337/diabetes.54.9.2568

18. Kaneto H, Miyatsuka T, Kawamori D, Matsuoka TA. Pleiotropic roles of PDX1 in the pancreas. *Rev Diabet Stud* 2007; 4:209-225. doi: 10.1900/RDS.2007.4.209.

19. Matschinsky FM. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 1996; 45:223-241. doi: 10.2337/diab.45.2.223.

20. Magnuson MA, Shelton KD. An alternate promoter in the glucokinase gene is active in the pancreatic beta cell. *J Biol Chem* 1989; 264:15936-15942.

21. Liang Y, Jetton TL, Zimmerman EC, Najafi H, Matschinsky FM, Magnuson MA. Effects of alternate RNA splicing on glucokinase isoform activities in the pancreatic islet, liver, and pituitary. *J Biol Chem* 1991; 266:6999-7007.

22. Hughes SD, Quaade C, Milburn JL, Cassidy L, Newgard CB. Expression of normal and novel glucokinase mRNAs in anterior pituitary and islet cells. *J Biol Chem* 1991; 266: 4521-4530.