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In Vitro Transdifferentiation of Mature Hepatocytes into Insulin-Producing Cells

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Abstract. Adenovirus-mediated gene transfer of pancreatic duodenal homeobox transcription factor PDX-1, especially its super-active version (PDX-1/VP16), induces the expression of pancreatic hormones in murine liver and reverses streptozotocin-induced hyperglycemia. Histological analyses suggest that hepatocytes are the major source of insulin-producing cells by PDX-1 gene transfer, although the conversion of cultured hepatocytes into insulin-producing cells remains to be elucidated. The present study was conducted to address this issue. Hepatocytes were isolated from adult rats. Then, PDX-1 or PDX-1/VP16 gene was introduced by using adenovirus vector. Two days later, the expression of insulin was detected at mRNA and protein levels. Transfection of PDX-1/VP16 was more efficient in converting hepatocytes to insulin-producing cells. Immunoreactivity of albumin was downregulated in transdifferentiated cells and some of them almost completely lost albumin expression. During the course of transdifferentiation, upregulation of mRNA for CK19 and α-fetoprotein was observed. When cultured in collagen-1 gel sandwich configuration, hepatocytes maintained their mature phenotype and did not proliferate. In this condition, transfer of PDX-1/VP16 also induced the expression of insulin. These results clearly indicate that hepatocytes possess a potential to transdifferentiate into insulin-producing cells *in vitro*.

Key words: Insulin, β cell, Hepatocyte, Transdifferentiation, PDX-1

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SINCE the report by Ferber *et al*. in 2000 [1], many researchers have demonstrated that adenovirus-mediated gene transfer of PDX-1 [2, 3] or constitutively active PDX-1 (PDX1-VP16) [4, 5] generates insulin-producing cells in normal adult liver in rodents. However, the types of adult liver cells which efficiently transdifferentiate into insulin-producing cells have not been clearly demonstrated. The immunohistological analyses done in these studies suggest that mature hepatocytes indeed are converted into insulin-producing cells by gene transfer. To our knowledge, however, there have been no reports showing that hepatocytes from adult liver convert into pancreatic cells *in vitro*, except for human hepatoma cell line HepG2 [6]. Ferber *et al.* [1] mentioned in their report that *in vitro* transfer of PDX-1 did not convert hepatocytes into insulin-producing cells. In the last decade, it was shown that hepatocytes under specialized circumstances *in vivo* undergo multiple cell division and differentiate into cholangiocytes in addition to hepatocytes [7, 8].

Consequently, mature hepatocytes may have an unlimited proliferative potential and bidirectional differentiation capacity. These cells may fulfill the criteria of progenitor cells [9]. In the present study, we examined whether or not primary hepatocytes obtained from normal rat liver transdifferentiate into insulin-producing cells by PDX-1 gene transfer.

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Materials and Methods

Isolation and culture of rat hepatocytes

All animal experiments were approved by the Animal Care and Ethical Committee of the Institute for Molecular and Cellular Regulation, Gunma University. Hepatocytes were isolated from 4–6 week-old male Sprague-Dawley rats (100–200 g, Crea Japan Co.) by the two-step liver perfusion method of Seglen [10]. The hepatocyte fraction of collagenase-digested liver was suspended in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% bovine serum albumin (BSA), 25 mM $NaHCO_3$, 30 mg/l L-proline, 0.5 mg/l insulin, 10^{-7} mol/l dexamethasone, 50 mg/l penicillin and 75 mg/l streptomycin. The viability of the isolated hepatocytes examined by trypan blue exclusion test was more than 90%. Hepatocytes (4×10^4 cells/cm^2) were plated on collagen-1-coated 60 mm dishes or collagen-1-coated glass coverslips placed in a 35 mm dish. Three hours after plating, the medium was changed to DMEM supplemented with 10% fetal bovine serum, 25 mM $NaHCO_3$, 30 mg/l L-proline, 1 mM ascorbic acid 2-phosphate (Asc-2P), 10 mM nicotinamide, 10 ng/ml epidermal growth factor (EGF), 10 μg/l insulin, 10 μg/l tranferrin, 10 ng/l selenious acid, 10^{-7} M dexamethasone (complete medium). The medium was changed to fresh complete medium every 48 hrs. In this culture condition, hepatocytes maintain a fairly higher level of albumin and tryptophan 2,3-dioxygenase (TO) expression [11], but they enter the cell cycle and proliferate, and then colonies of small hepatocytes appear as described previously [12]. To further maintain mature hepatocyte phenotype, the next set of hepatocyte culture was conducted in collagen-1 gel sandwich configuration [13]. Hepatocytes were plated on collagen-1 gel (1.5×10^5 cells/cm^2). Three hours after plating, medium was changed to modified complete medium containing 0.2% BSA without serum or Asc-2P.

Preparation of recombinant adenovirus vectors containing PDX-1 and PDX-1/VP16

Recombinant adenovirus containing PDX-1 and PDX-1/VP16 was prepared using Virapower™ Adenoviral Gateway Expression kits (Invitrogen). In brief, the coding region of mouse PDX-1 and carboxy-terminal 78 amino acids of transcriptional activation domain of herpes simplex virus VP16 [14] was cloned into an entry vector pENTR™/D-TOPO (Ad-PDX-1) or pENTR3C (Ad-PDX-1/VP16). To create expression clones and produce recombinant adenoviruses, LR Recombination Reaction, linearization of plasmids with Pac I and transfection into the adenovirus packaging cell line 293 cells were performed according to the manufacturer's instructions. The control adenovirus expressing LacZ (Ad-LacZ) was prepared in the same manner. The adenovirus titers were further increased up to 1×10^{11} plaque forming units (PFU)/ml by CsCl purification.

Infection of adenovirus to hepatocytes

After overnight culture of isolated hepatocytes, medium was changed to serum-free complete medium containing purified recombinant adenovirus at 1 multiplicity of infection (MOI) and incubated for 1 hr at 37°C. Then the complete medium was chased and further incubated. On the next day, the medium was changed to fresh complete medium and further incubated for indicated periods. Nearly 60–70% of the hepatocytes expressed the transgene at 1 MOI. In the case of collagen-1 gel sandwich configuration culture, medium containing adenovirus was aspirated, and cells were overlaid with collagen-1 gel at 8 hrs after the infection. Thirty minutes later, fresh culture medium was added and cells were further cultured for indicated periods.

Morphological analysis and immunocytochemistry

Hepatocytes cultured on collagen 1-coated glass coverslips were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), treated with 0.1% (vol/vol) Triton X-100 in PBS, and incubated sequentially with 0.3% H_2O_2 in methanol and then blocked with 1% BSA in PBS for 30 min [15]. Primary antibodies used were guinea pig anti porcine insulin (1:100, DAKO, Carpinteria, CA), rabbit anti-PDX-1 (1:500, Transgenic, Kumamoto, Japan), rabbit anti VP16 (1:500, Sigma), rabbit anti-rat albumin (1:200, Cappel, Costa Mesa, CA), rabbit anti-human transthyretin (1:200, DAKO), rabbit anti-amylase (1:400, Sigma), and mouse anti-5-bromo-2'-deoxyuridine monoclonal antibody (1:100, Amersham Bioscience, Backs, UK). Secondary antibodies used were Biotinylated anti-rabbit IgG (1:200, Vector Laboratories,

Burlingame, CA), alkaline phosphatase conjugated anti-guinea pig IgG (1:200, Chemicon, CA), and peroxydase conjugated anti-mouse IgG2a (1:70, Amersham Bioscience). Except for insulin staining, the Avidin-Biotin peroxidase complex (ABC) method (Vectastain ABC Elite Kit, Vector Lab) was used. 3,3-Diaminobenzidine (Dojindo, Kumamoto, Japan) was used as the substrate for peroxidase staining. For double staining with insulin, second series of antibody incubation with insulin and secondary antibody was performed, and then the expression of insulin was visualized using Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Lab). Counterstaining with hematoxilin was done when needed. Hepatocytes sandwiched between collagen-1 gels were fixed with 10% formalin, dehydrated in graded concentration of ethanol and embedded in paraffin blocks according to standard procedures. A series of five-micrometer sections were cut and stained. Stained cell images were observed with a Bx50 Epifluorescence microscope (Olympus Optical Co, Tokyo, Japan) equipped with a SenSys™ charge-coupled device camera (Photometrics, Tucson, AZ).

To assess cell proliferation, a cell proliferation kit (Amersham Bioshiences, UK) was used. Hepatocytes were incubated with bromodeoxyuridine (BrdU) for 24 hrs (from day 4 to day 5). Then immunocytochemistry for BrdU was carried out.

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 contamination of genomic DNA. First-strand cDNA was synthesized by SuperScript II or SuperScript III RNaseH- Reverse Transcriptase (Invitrogen). To confirm no contamination of genomic DNA, samples without reverse transcriptase (RT) treatment were prepared. Oligonucreotide primers used and reaction conditions performed in this study were as previously mentioned [16], except for insulin (5'-GGC TTT TGT CAA ACA GCA CCT TTG -3' and 5'-AGC AGA TGC TGG TGC AGC A-3'), p-Amylase (5' GGC TCA TCC TTA TGG ATT CA-3' and 5'-GAC ATC ACA GTA TGT GCC AG-3'), α-fetoprotein (AFP) (5'-TGA AAT TTG CCA CGA GAC GG-3' and 5'-TGT CAT ACT GAG CGG CTA AG-3') and CK19 (5'-TTG CGC GAC AAG ATT CTT GG-3' and 5'-CAT CTC ACT CAG GAT CTT GG-3'). The PCR products were subjected to 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. An insulin PCR product was confirmed by sequencing using an ABI Prism Dye Terminator Cycle Sequencing FS Ready Reaction Kit and ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Materials

Materials not otherwise stated were all purchased from Sigma (St. Louis, MO).

Results and Discussion

To elucidate which type of adult hepatic cells converts into insulin-producing cells by adenoviral PDX-1 gene transfer *in vivo*, we first examined the effect of transfection of the PDX-1 gene in cultured hepatocytes. Adult rat hepatocytes were isolated by collagenase digestion and plated onto collagen-1 coated dishes. The morphology of primary hepatocytes after an overnight culture is shown in Fig. 1A. After the overnight culture, hepatocytes presented typical non-proliferating morphology. All hepatocytes expressed transthyretin (TTR), one of the typical markers of mature hepatocytes [17, 18] (Fig. 1B). All of them were also strongly positive for albumin (Fig. 1C). Therefore, the mature properties were well preserved after the overnight culture. Then Ad-PDX-1 or Ad-PDX-1/VP16 (superactive version of PDX-1 [4, 5]) was introduced. Immunocytochemical analysis showed that the hepatocytes infected with Ad-PDX-1/VP16 began to express insulin on day 3 (48 hrs after infection: Fig. 1D), and clear insulin-positive cells were observed on day 5 (96 hrs after infection: Fig. 1E). The percentages of the insulin-positive cells were 5 to 15% of all PDX-1/VP16-expressing cells. The number of insulin-positive hepatocytes infected with Ad-PDX-1 was markedly less under the same observation period (<1%) (Fig. 1F). Infection of Ad-PDX-1 or Ad-PDX-1/VP16 did not significantly affect the number of cells.

In this culture condition, we assumed that hepatocytes maintained a higher level of albumin expression, yet actually multiple rounds of hepatocyte division also took place. Hepatocytes cultured for 5 days exhibited two types of albumin-positive cells, one formed

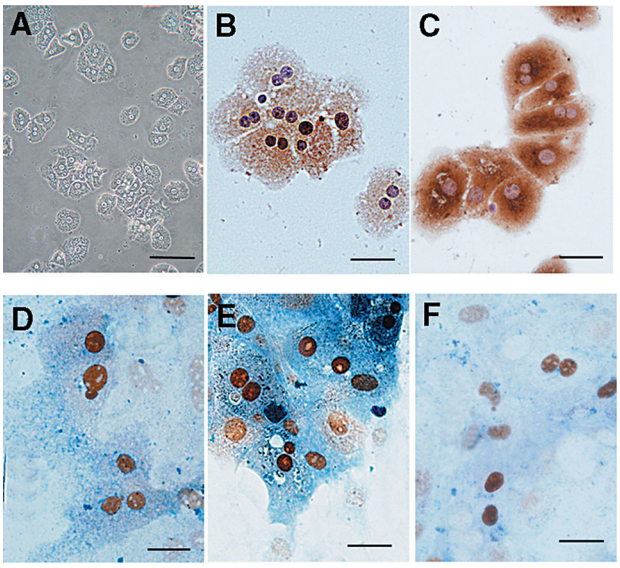

Fig. 1. Mature phenotype of rat hepatocytes and effect of PDX-1/VP16 gene transfer on the expression of insulin. Morphology of rat hepatocytes cultured on collagen-1 coated dish is shown in (A). All of the hepatocytes expressed transthyretin (B) and albumin (C) at the time of gene transfer (after overnight culture), indicating that they retained mature property of hepatocytes. Primary hepatocytes transfected with PDX-1/VP16 began to express insulin on day 3 (D) and clear insulin staining was observed on day 5 (E). In contrast, hepatocytes transfected with PDX-1 were scarcely stained with insulin on day 5 (F). D–F: double immunostaining for VP16 (D, E) or PDX-1 (F) (brown) and insulin (blue).

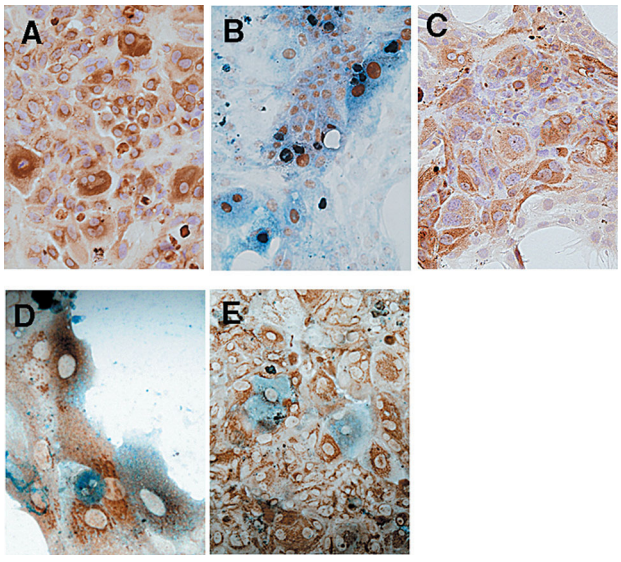

Fig. 2. Characteristics of transdifferentiated hepatocytes. In hepatocytes cultured for 5 days, two types of albumin-positive hepatocytes, mono- or binuclear hepatocytes with large cytoplasm and small cell colonies with a single nucleus, were observed (A). Both types of hepatocytes can transdifferentiate into insulin-producing cells (B). The expression of albumin was downregulated in transdifferentiated hepatocytes (C–E: hepatocytes expressing PDX-1/VP16 on day 5). A, C: Immunostaining for albumin (brown), B: Double immunostaining for VP16 (brown) and insulin (blue). D, E: Double immunostaining for albumin (brown) and insulin (blue).

small cell colonies with a single nucleus and a higher nucleus/cytoplasm ratio, and the other formed mono- or binuclear hepatocytes with large cytoplasm (Fig. 2A). Positive insulin staining was found in both types of cells (Fig. 2B). Unexpectedly, surrounding hepatocytes with large cytoplasm showed stronger insulin expression (Fig. 2B). In accordance with these findings, the number of strong albumin-positive hepatocytes with large cytoplasm was less in hepatocytes infected with Ad-PDX1-VP16 (Fig. 2C). Double immunostaining of insulin and albumin confirmed the transdifferentiation of hepatocytes into insulin-producing cells (Fig. 2D). Some of the insulin-positive cells lost albumin expression almost completely (Fig. 2E).

In order to confirm the expression of pancreatic hormones and transcription factors during transdifferentiation of hepatocytes, the expression profiles of pancreatic markers were studied by RT-PCR (Fig. 3). On day 3, mRNA expression of insulin and glucagon was induced in hepatocytes infected with Ad-PDX-1. The expression of endocrine hormones was higher in Ad-PDX-1/VP16-infected hepatocytes. Pancreatic polypeptide and somatostatin were not detected. Regarding pancreatic exocrine markers, amylase was expressed from the early period of hepatocyte culture and never upregulated by the introduction of PDX-1. Surprisingly, overexpression of PDX-1 or PDX-1/VP16 did not activate the downstream transcription factor NeuroD/Beta 2 suggesting that transdifferentiation of hepatocytes into liver did not recapitulate the differentiation program of pancreatic development.

In the culture condition mentioned above, about 40% of hepatocytes incorporated BrdU into DNA between day 4 and day 5 as previously reported [19]. Insulin-producing hepatocytes also incorporated BrdU, suggesting that they were also in proliferation (data not shown). Non-proliferating insulin-producing hepatocytes were scarcely observed.

In adult hepatocytes proliferating *in vitro*, expression

of mature hepatocyte-related markers and transcription factors are downregulated and their subpopulation begin to express ductal cell marker CK19 and immature hepatoblast marker AFP [20]. They may also regain a spectrum of progenitor markers from different germ layers [21]. It is possible that, during proliferation, hepatocytes gain the characteristics of liver progenitor cells and then differentiate into insulin-expressing cells. As for the well known liver progenitor cells, fetal hepatoblasts express AFP, albumin and CK19 [9]. Adult liver progenitor cells such as oval cells express both albumin and CK19 [9]. Therefore, we next examined the expression of CK19 and AFP during the period of transdifferentiation. Freshly isolated hepatocytes did not express these markers. They expressed CK19 mRNA from day 3 and its expression was upregulated thereafter. The same tendency was observed for AFP mRNA, although its expression level was lower (Fig. 3B).

With regard to transdifferentiation of pancreas to liver, a proportion of the hepatocytes arise directly from differentiated exocrine-like AR42J-B13 cells with no intervening cell division [22]. In our experimental conditions, primary hepatocytes maintained well-differentiated characteristics at the time of PDX-1/VP16 introduction. However, about a 150% increase in the cell number was observed between day 1 and day 5. Whether or not non-proliferating primary mature hepatocytes transdifferentiate into insulin-producing cells is also an interesting issue. To assess this, we set up another hepatocyte culture condition using collagen-1 gel sandwich configuration. Hepatocytes cultured in a sandwich configuration form functional canalicular networks and excrete organic anions and bile acids [13]. As shown in Fig. 4A, hepatocytes cultured in a collagen-sandwich configuration for up to 5 days reconstitute a trabecular network resembling liver cell plates and sinusoidal cavities in naive liver. Morphological observation showed that cell proliferative response was significantly suppressed in these gel sandwich cultures. In these gel sandwich cultures, PDX-1/VP16 also transdifferentiated hepatocytes into insulin/glucagon-expressing cells, which was confirmed by RT-PCR analysis (Fig. 4B). The expression pattern of endocrine hormones was similar to that in hepatocytes cultured on collagen-1 coated dishes. Spontaneous and transient expression of glucagon was observed at day 1 and after day 7. The expression of insulin in hepatocytes cultured in a gel sandwich configuration was also

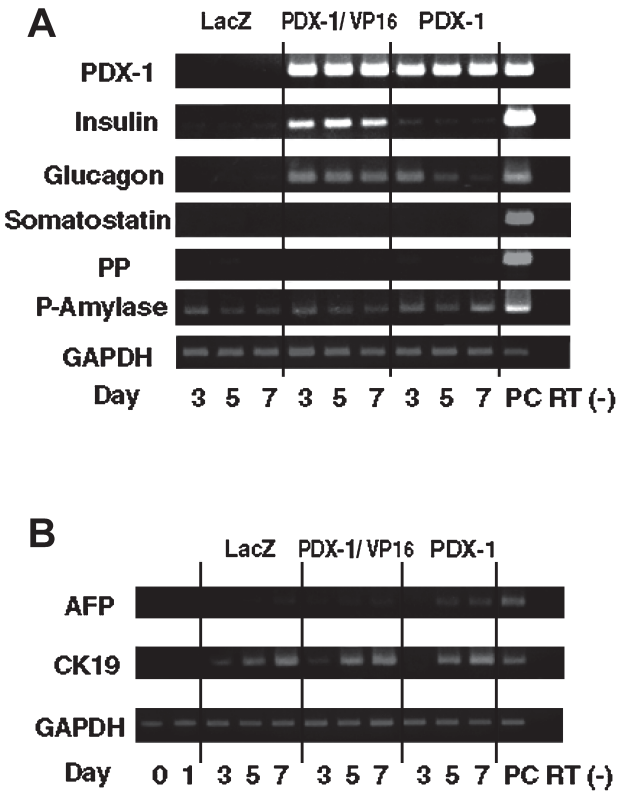

Fig. 3. Induction of the expression of pancreas-related markers and pancreatic hormones in transdifferentiated hepatocytes.
A: Adenovirus mediated gene transfer was conducted in primary hepatocytes after overnight culture. Temporal pattern of gene expression (day 3, 5, 7) was analyzed by RT-PCR. PDX-1/VP16 gene transfer induced the expression of insulin and glucagon more efficiently than PDX-1. The expression level of amylase was only slightly affected by the gene transfer. PC: positive control, RT (–): without reverse transcriptase. B: Temporal pattern of the expression of the liver progenitor cell marker AFP and the bile duct epithelial marker CK19 was studied by RT-PCR.

confirmed by immunocytochemistry (Fig. 4C). Taken together, proliferation of hepatocytes is not a prerequisite for transdifferentiation to insulin-producing cells.

Adenovirus-mediated expression of PDX-1, especially of its super-active version (PDX-1/VP16), in the liver induces the expression of pancreatic endocrine hormones and reverse streptozotocin-induced hyperglycemia in mice [1–5]. It has been shown that liver epithelial WB cells [23, 24] and small hepatocytes [25] obtained from normal adult liver also differentiate into insulin-producing cells by PDX-1 gene transfer. However, localization of both types of cells in normal liver

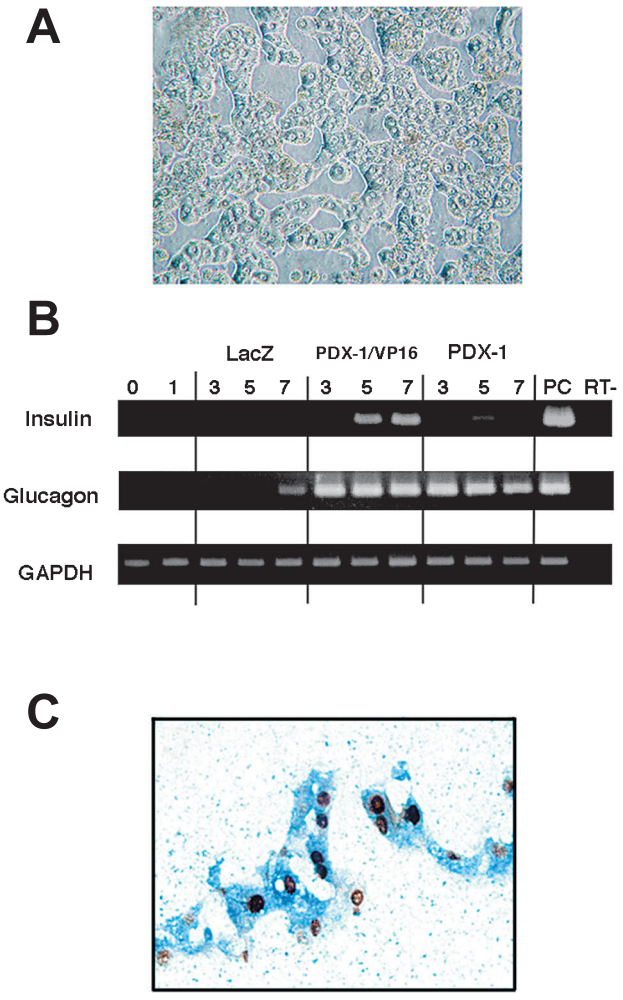

Fig. 4. Effects of PDX-1/VP16 gene transfer on hepatocytes cultured in collagen-1 gel sandwich configuration.
A: Morphology of transfected hepatocytes cultured in collagen-1 gel sandwich configuration for 5 days. B: Hepatocytes transfected with PDX-1/VP16 were cultured in collagen-1 gel sandwich for 5 days. The expression of mRNA for insulin and glucagon was measured by RT-PCR. C: Hepatocytes transfected with PDX-1/VP16 were cultured in a collagen-1 gel sandwich for 5 days and stained with anti-insulin (blue) and anti-VP-16 antibodies (brown).

is not known due to the lack of specific cell markers. These liver progenitor cell components are considered to be activated *in vivo*, only when hepatocytes are severely damaged and their proliferation is obliterated [9]. Based on these considerations, it is thought to be unlikely that the above mentioned liver progenitor cells are major components of insulin-producing cells observed in PDX-1/VP16-expressing liver.

As shown in Fig. 1A–C, hepatocytes retained mature characteristics of hepatocytes at the time of gene transfer. PDX-1/VP16 gene transfer induced the expression of insulin. Transient expression of PDX-1/VP16 was sufficient to initiate a transdifferentiation program, as previously reported in other liver progenitor cells [23, 24]. During the course of this transdifferentiation, upregulation of CK19 and AFP in mRNA level was simultaneously observed. It should be mentioned that proliferation was not a prerequisite for transdifferentiation since hepatocytes converted to insulin-producing cells when cultured in collagen-1 gel.

Collectively, we demonstrate that mature hepatocytes have potential to transdifferentiate to pancreatic endocrine cells *in vitro*. This information contributes to our understanding of what occurs in adult liver by forced expression of PDX-1 *in vivo*.

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