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World J Gastroenterol 2005;11(15):2277-2282
World Journal of Gastroenterology ISSN 1007-9327

• *BASIC RESEARCH* •

Heterogeneity in predisposition of hepatic cells to be induced into pancreatic endocrine cells by PDX-1

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Supported by the National Natural Science Foundation of China, No. 30240087; the National High Technology Research and Development Program of China, No. 2003AA205090
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Received: 2004-05-12 Accepted: 2004-06-24

Abstract

AIM: The role of Pancreatic and Duodenal Homeobox-1 (PDX-1) as a major regulator of pancreatic development determines the function and phenotype of β cell. In this study, potential plasticity of liver cells into pancreatic endocrine cells induced by PDX-1 was evaluated.

METHODS: Human hepatoma cell line HepG2 was stably transfected with mammalian expression plasmid pcDNA3-PDX encoding human PDX-1 gene. Ectopic expression of PDX-1 and insulin were detected by RT-PCR, Western blot and/or immunostaining. PDX-1$^+$ HepG2 cells were transplanted under renal capsule of STZ-induced diabetic nude mice (n = 16) to examine the inducing effect *in vivo*.

RESULTS: Exogenous PDX-1 transgene was proved to express effectively in HepG2 cell at both mRNA and protein levels. The expression of endogenous insulin and some β cell-specific differentiation markers and transcription factors were not induced in PDX-1$^+$ HepG2 cells. When transplanted under renal capsule of STZ-induced diabetic nude mice, PDX-1$^+$ HepG2 cells did not generate insulin-producing cells. These data indicated that stable transfected PDX-1 could not convert hepatoma cell line HepG2 to pancreatic cells *in vitro* or *in vivo*. Mature hepatocytes might need much more complicated or rigorous conditions to be shifted to insulin-producing cells.

CONCLUSION: The expression of exogenous PDX-1 is not sufficient to induce relatively mature hepatocytes differentiating into insulin-producing cells.

Key words: PDX-1; HepG2, Insulin; Transgene; Diabetes

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(15): 2277-2282
http://www.wjgnet.com/1007-9327/11/2277.asp

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is characterized by insulin deficiency due to autoimmune destruction of pancreatic β cells. Gene therapy or cell-replacement therapy might be an optimal strategy[1,2]. Recently, increasing studies focused on inducing non-β-cell derived cells into surrogate β cells that can produce and secrete insulin, by genetic modification in most cases[3-5]. In addition to several types of stem cells[6-8], the most commonly used cell type for this purpose is liver cells[9-11].

Both liver and pancreas emerge from ventral foregut endoderm and specifically at about the same time during embryonic development. Although different set of transcription factors and specialized gene profiles are expressed in liver and pancreas buds, some gene products are common to both tissues[12], including the essential factors in glucose sensing machinery, such as GLUT-2 and glucokinase.

As a regulator located upstream in the insulin gene transcription cascade, pancreatic and duodenal homeobox-1 (PDX-1) is considered as a promising candidate transgene in gene therapy for diabetes. PDX-1 was originally identified as an insulin gene transcription factor[13]. It also activates expression of other islet-enriched genes, including GLUT-2[14], glucokinase[15], islet amyloid polypeptide (IAPP)[16] and somatostatin[17]. PDX-1 knockout mice led to the loss of the whole pancreas[18]. Mice carrying PDX-1 deletion developed diabetes[19]. In humans, patient bearing a mutant PDX-1 gene resulted in agenesis of pancreas at birth[20]. Taking these together, PDX-1 appears to be a major regulator of pancreatic development and determines the function and phenotype of β cell.

Ferber *et al*[21] found that ectopic expression of PDX-1 induced expression of endogenous insulin gene in mouse liver. The same group and another, recently, further demonstrated that PDX-1, as a "master regulator" of directing cell fate, has the sufficient capacity to induce the mature liver cells into pancreatic endocrine cells *in vivo*[22,23]. However, a recent report from Horb *et al*[24] failed to convert liver cells to insulin-producing cells with the transient

expression of wild type PDX-1 alone, but it was achieved when PDX-1 gene was fused with a transcriptional activation domain VP16.

Then the intriguing questions are: if PDX-1 can convert some liver cells to pancreatic cells *in vivo*, what if it is introduced into a hepatocyte-derived or a hepatoma cell line like HepG2 *in vitro*? If transient expression of PDX-1 in HepG2 cannot lead to this kind of conversion, how is it expressed in a constitutive way? What will happen if stably transfected PDX-1$^+$HepG2 cell is further induced by *in vitro* and *in vivo* environment? In order to elucidate these questions, we established a stable PDX-1-expressing HepG2 cell line and transplanted these cells into STZ-induced diabetic nude mice. We found that insulin and other β cell enriched/specific genes were not activated in PDX-1$^+$HepG2 cell line, and no conversion evidence is observed after implantation under renal capsule either.

MATERIALS AND METHODS

Plasmid construction

Human PDX-1 gene coding sequence was amplified by PCR from Human Pancreas Quick-Clone cDNA library (Clontech, Palo Alto, CA) with forward primer 5'-CCATGAACGGCGA GGAGCAGTA and reverse primer 5'-CTGCCTCTCATCGTGGTTCCTG and cloned into pCDNA3 (Invitrogen, Carlsbad, CA), a mammalian expression vector driven by CMV promoter. The construct was designated as pCDNA3-PDX and characterized by restriction analysis and verified by sequencing.

Cell culture and preparation of stable transfectants

HepG2, a human hepatoma-derived cell line, was obtained from ATCC (Rockville, MD) and maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin. To obtain stable transfectants, HepG2 cells were seeded in 24-well plate 24 h before transfection. A total of 0.8 μg of pCDNA3-PDX plasmid DNA was transfected into cells using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommendation. Forty-eight hours after transfection, the cells were diluted and transferred to 10 cm culture plates, cultured with G418-containing medium (500 μg/mL). The selective medium was changed every 4 d. G418-resistant colonies appeared 3-4 wk after transfection. The single colonies were picked out using clone rings and then subjected to proliferate for further analysis.

RNA isolation and RT-PCR analysis

Total RNA was isolated from single clone derived cells directly from culture plate using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples were treated by 10 units of RQ1 Rnase-free Dnase I (Promega, Madison, WI) for 15 min at 37 ℃. Reverse transcription was performed following the manufacturer's instructions (Promega, Madison, WI). Primer sequences and PCR conditions are listed in Table 1. PCR was performed using T-gradient thermocycler (Biometra, Gottingen, Germany) and the product was separated using 1.5% agarose gel and visualized with ethidium bromide. Human fetal pancreas was isolated from a 24-wk gestational age embryo from a natural aborted fetus. Permission to use human embryonic tissues was granted by the Ethics Review Board of Peking University.

Western blot analysis

Expressions of PDX-1 and insulin at protein levels in transfected cells were detected using Western blot analysis as previously

Table 1 **RT-PCR information: Primer sequences and PCR conditions**

Genes	Primer sequences (5′-3′)	Products bp	Annealing		Cycles
			℃	s	
PDX-1	F,GTCCTGGAGGAGCCCAAC	360	58	60	30
	R,GCAGTCCTGCTCAGGCTC				
Insulin	F,GCCTTTGTGAACCAACACCTG	261	62	60	30
	R,GTTGCAGTAGTTCTCCAGCTG				
GLUT-2	F,TGCCACACTCACACAAGAC	260	54	60	30
	R,AGATTGTGGGCAGTTCATC				
Glucokinase	F,CCCGAGGAGAACCACATT	208	56	60	30
	R,GGAACTCTGCCAGGATCT				
IAPP	F,GCTGACATTGAAACATTA	360	56	60	30
	R,TATACAGGAAATCACTAGAA				
Somatostatin	F,TGCGCTGTCCATCGTCCT	258	60	60	30
	R,GCCATAGCCGGGTTTGAGTT				
E47	F,TCAGGCTGGCTTCCTGTCAG	224	62	60	30
	R,CCCTGCCGTATGCCTCACCT				
NeuroD1	F,GCGTTAGCCTTCATGCGTCT	386	60	60	30
	R,GAGGCCCCAGGGTTATGAG				
Isl-1	F,CGGCTTCAGCAAGAACGACT	290	58	60	30
	R,TCTTCTCCGGCTGCTTGTG				
GAPDH	F,GTCAGTGGTGGACCTGACCT	415	55	60	30
	R,AGGGGAGATTCAGTGTGGTG				

PCR conditions: denaturation at 94 ℃ for 30 s; annealing as listed in the table; extension at 72 ℃ for 1 min.

described[25]. The protein concentrations were determined using Bradford assay. Five microgram of cellular lysate were separated by standard SDS-PAGE and then transferred to nitrocellulose membrane. Goat polyclonal anti-PDX-1 or anti-insulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used to probe the blot.

Under-renal-capsule transplantation into STZ-induced diabetic nude mice

Male BALB/c nude mice at age 8-10 wk were used in this study. The animal experiment conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Mice were fasted for 18 h and then treated with STZ (200 mg/kg body weight, i.p., Sigma, St. Louis, MO) freshly dissolved in citrate buffer (pH 4.5). The blood glucose levels were monitored daily by using a Glucotrend® glucose detector (Roche Diagnostics, Mannheim, Germany). Seven days after STZ treatment, the mice with stable hyperglycemia (blood glucose levels >20 mmol/L) were selected for operation. Under pentobarbital sodium (35 mg/kg body weight, i.p.) anesthetization, the left kidney was exposed through a lumbar incision and cells (5×10^6 to 1×10^7) resuspended in PBS were injected into subcapsular cavity by using a 100 μL micro-syringe. The blood glucose level was monitored on 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 18, 26, 30 d after transplantation. Recipient animals were killed by cervical dislocation 30 d after operation. Kidney and pancreatic tissues were removed and fixed with 10% paraformaldehyde in PBS at 4 ℃ for 5 h and embedded in OCT compound for immunohistochemical staining.

Immunocytochemistry and immunohistochemistry

Cells were cultured on non-coated glass coverslips for 24 h before immunostaining. After rinsing with PBS thrice, the cells were fixed with acetone/methanol at 4 ℃ for 15 min, permeabilized with 10 mL/L Triton X-100 in PBS for 10 min, and incubated sequentially with blocking serum, primary antibodies and TRITC or FITC conjugated secondary antibody. The primary antibodies included goat anti-insulin polyclonal IgG (1∶200 dilution, Santa Cruz), goat anti-PDX-1 polyclonal IgG (1∶100 dilution, Santa Cruz); mouse anti-human nuclei monoclonal antibody (1∶200 dilution, Chemicon, Temecula, CA). Fixed tissue sections (8 μm thick) were also stained with antibodies as mentioned above. The cells and tissue sections were examined under a fluorescence microscope (Olympus, Nagano, Japan).

Statistical analysis

Results are given as mean±SD. The one-way ANOVA was performed by SPSS software (SPSS Science, Chicago, Illinois). $P<0.05$ were considered significant.

RESULTS

Establishment of HepG2 cell line stably expressing PDX-1

To test the feasibility of HepG2 cells differentiating into pancreatic endocrine cell by ectopically expressed PDX-1 gene, HepG2 cells were transfected with pCDNA3-PDX construct and stable transfectants were isolated under G418 selection. A total of 13 individual colonies were isolated and subjected to RT-PCR and Western blot analysis. Five PDX-1 positive colonies were identified. The clone 11 was selected to amplify for further experiments according to its higher expression level of PDX-1 than other clones (data not shown).

There was no obvious difference in the appearance of PDX-1 positive HepG2 cells from their parental cells. The results of RT-PCR and Western blot indicated the expressions of PDX-1 at both mRNA and protein levels in PDX-1$^+$HepG2 cells, but not in wild type HepG2 cells (Figure 1). Although less abundant, the expression level of exogenous PDX-1 gene was comparable to that of human fetal pancreas (Figure 1A). Western blot analysis indicated a 46 ku of PDX-1 protein (Figure 1C). The immunocytochemical staining showed that PDX-1 protein was localized mainly in the nuclei of PDX-1$^+$HepG2 cells (Figure 2C). These results indicate that we established the HepG2 cells ectopically express PDX-1 gene in the nucleus as expected.

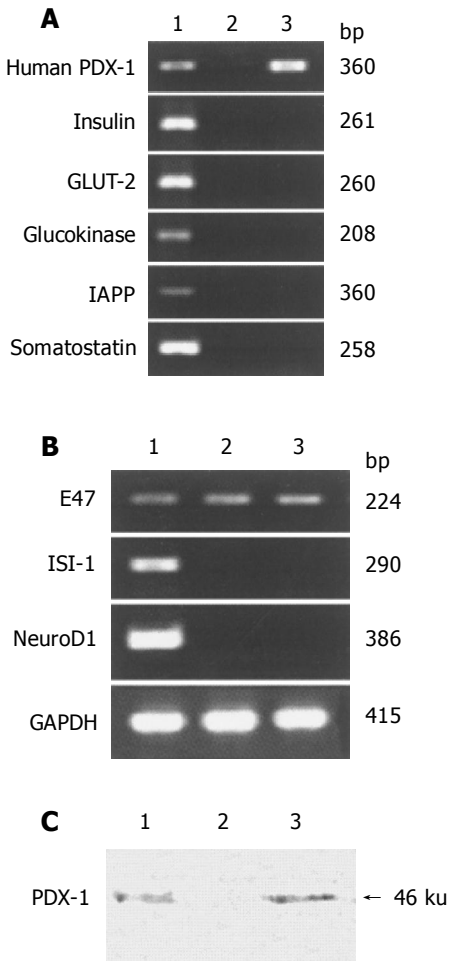

Figure 1 Expression of PDX-1 transgene and other islet-enriched genes in PDX-1$^+$HepG2 cell line. RT-PCR analysis was performed to detect the expression of several islet differentiation markers (**A**) and islet-specific transcriptional factors (**B**). GAPDH mRNA was amplified as an internal control. Western blot analysis was performed to detect the expression of PDX-1 using anti-PDX-1 antibody on total protein lysis (**C**). Lane 1: 24-wk human fetal pancreas (positive control); Lane 2: wild type HepG2 cells (negative control); Lane 3: PDX-1$^+$HepG2 cells. Data are representative of at least three independent experiments.

Examination of islet specific genes' expression in PDX-1⁺HepG2 cells

To examine the possible changes in gene expression patterns caused by exogenous PDX-1 gene in HepG2, we used RT-PCR to detect the presence of mRNA of insulin, glucokinase, GLUT-2, somatostatin and IAPP. None of these genes could be detected in both PDX-1 transfected and wild type HepG2, contrasted to distinct bands from pancreatic tissue (Figure 1A). Absence of insulin was further confirmed by immunocytochemical staining (Figure 2F) and by Western blot analysis (data not shown). To investigate the possible mechanism of silence of these islet marker genes, Isl-1 and neuroD1/beta2, upstream transactivators of insulin gene, were also detected by RT-PCR. They expressed neither in PDX-1⁺HepG2 cells nor in parental HepG2 cells (Figure 1B).

Cell transplantation under renal capsule in diabetic nude mice

Previous studies[26] showed that an *in vivo* microenvironment, such as renal subcapsule, may be advantageous to facilitate the maturation and differentiation of endocrine cells. To examine if PDX-1⁺HepG2 cells could be induced into insulin-producing cells, cell transplantation was performed. BALB/c nude mice were used to exempt implanted cells from immune attack. A total of 5×10^6-1×10^7 PDX-1⁺HepG2 cells or wild type HepG2 cells were implanted to renal subcapsular space of STZ-induced diabetic mice (n = 16 and 7, respectively). The average blood glucose level showed no significant difference between two groups, both at above 20 mmol/L (Figure 3). H&E staining and immunohistochemistry of anti-human nuclei antibody showed that the implanted cells were infiltrated into normal mice nephric tissues (Figure 2), indicating the cell viability and proliferation. The implanted cells displayed stable expression of PDX-1 transgene. No expression of insulin was observed in the kidney sections injected with PDX-1⁺HepG2 cells (Figure 2).

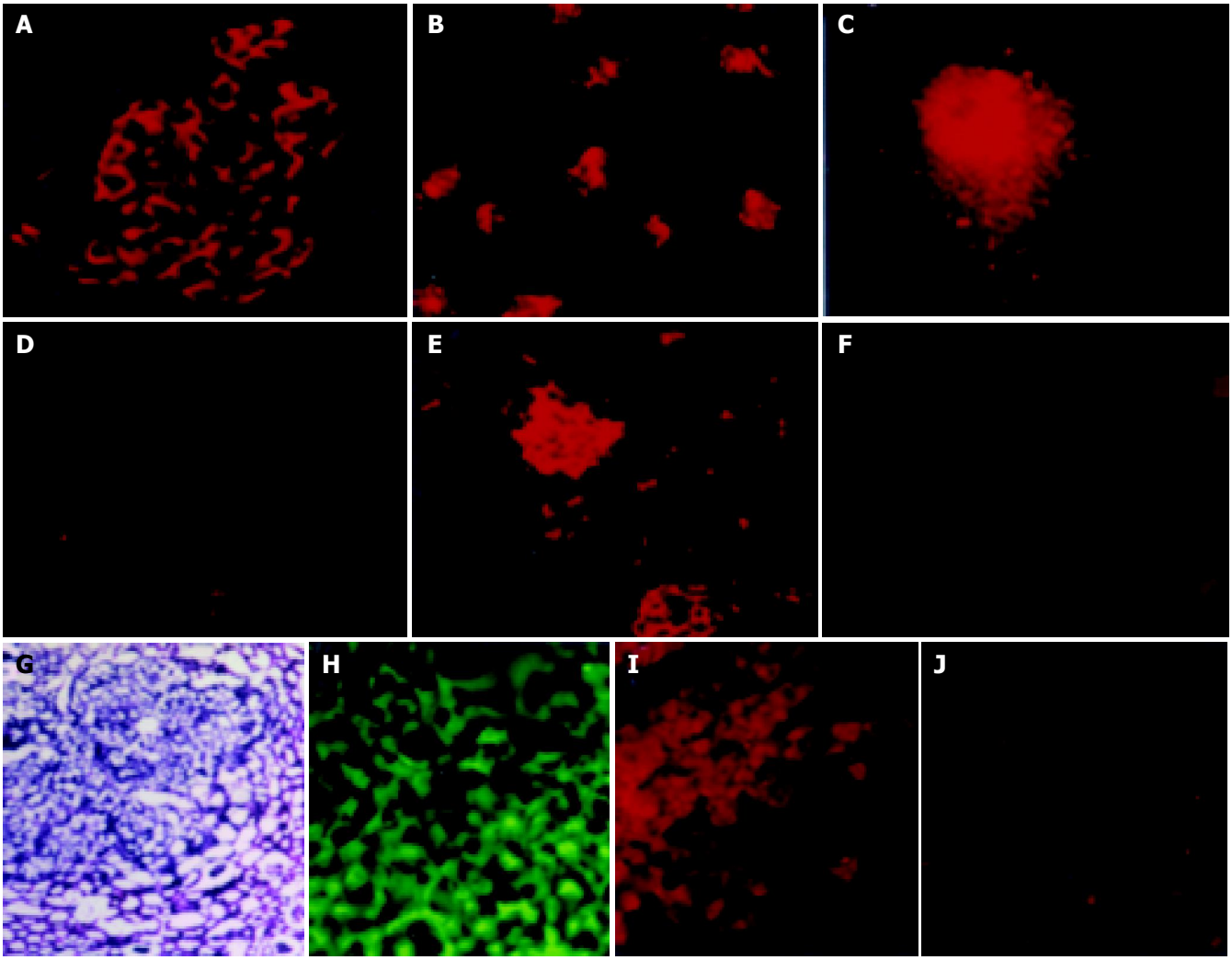

Figure 2 *In situ* immunostaining analysis of PDX-1⁺HepG2 cells in culture and after transplantation into nude mice. Representatives are shown (original magnification 200×, unless otherwise stated). Anti-PDX-1 fluorescence stains cultured PDX-1⁺HepG2 cells (**B**), compared to 24-wk human fetal pancreatic islets (**A**). The ectopically expressed PDX-1 protein locates mainly within nuclear region (**C**, 400×). Parental HepG2 cells serve as a negative control (**D**). Anti-insulin fluorescence immunostaining indicates positive staining in human islet positive control (**E**) but not in PDX-1⁺HepG2 cells (**F**). H&E staining reveals implanted PDX-1⁺HepG2 cells in section of kidney (**G**, 100×), indicating that these cells infiltrated into nephric tissues and underwent proliferation. Anti-human nuclei antibody staining confirmed that these cells were of human origin (**H**, green cells). Anti-PDX-1 staining of mice kidney sections showed consistent expression of PDX-1 transgene in these implanted PDX-1⁺HepG2 cells (**I**), but insulin expression was absent (**J**).

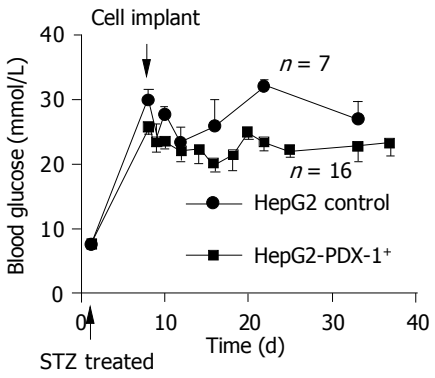

Figure 3 Blood glucose level of STZ-induced diabetic nude mice after implantation of PDX-1$^+$ HepG2 cells under renal capsule. Between 5×10^6 and 1×10^7 PDX-1$^+$HepG2 cells or parental HepG2 cells were injected into subcapsular space. Blood glucose level was measured 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 18, 26, 30 d after operation. Values are expressed as mean±SD for each time point, PDX-1$^+$HepG2 group (n = 16) *vs* HepG2 group (n = 7).

DISCUSSION

Liver cells seem to contain remarkable potential to convert into pancreatic cells. During embryogenesis, pancreas and liver arise from adjacent areas in the anterior endoderm. The default fate of ventral foregut endoderm is to become pancreas, but a FGF-like signal released from the cardiac mesoderm diverts the fate of some cells into liver[12]. This model implied that pancreas and liver, despite distinct phenotype, may share a similar context of gene expression profile but differ in just a few critical "master regulators". Under certain conditions, the interchange of pancreas and liver then could be possible. Increasing experimental models have been reported to validate this potential. Adult rat hepatic oval stem cells[27] can be induced into pancreatic endocrine hormone producing cells in high-glucose environment without genetic modification. Ferber *et al*[21] found and recently confirmed[22] that ectopic expression of PDX-1 induced mouse a pluripotent population of progenitor liver cells to produce biologically mature insulin *in vivo* and ameliorated STZ-induced diabetes. To evaluate whether PDX-1 is able to reprogram a human hepatocyte-derived cell line HepG2 into such pancreatic endocrine cells, we established a PDX-1$^+$HepG2 stable expression cell line. As expected, the expression of PDX-1 transgene in PDX-1$^+$HepG2 was comparable to that of human pancreas islet. PDX-1 protein was properly translocated into cell nuclei, as demonstrated by immunostaining and Western blot. Unexpectedly, the expressions of pancreatic β cell specific genes including insulin, glucokinase, GLUT-2 and IAPP were detected neither in transfected cells nor in mice with cell transplanted under renal capsule.

It is well established that insulin gene transcription in β cells results from the formation of a transcription complex comprising E47, NeuroD1, Isl-1, PDX-1[28], and more recently, Maf A[29]. In PDX-1$^+$HepG2 cell line, E47 was present as a ubiquitous nuclear factor, but neuroD1 and Isl-1 were absent. Kojima *et al*[30] demonstrated that only combined expressions of Isl-1 and PDX-1 could induce immature rat intestinal crypt cell line IEC-6 to produce insulin, which verified the necessity of Isl-1 in insulin transcription. Recently the same group found that without PDX-1, NeuroD1, or a combination of NeuroD1 and betacellulin gene transfer could induce insulin production in mouse liver and partly or completely reversed diabetes of STZ mice[23], indicating that NeuroD1 is another essential factor for the normal function of pancreatic β cells. The absence of Isl-1 and NeuroD1 in HepG2 cells might account for the silence of insulin and other islet specific genes in our model.

Two independent studies[22,23] confirmed that *in vivo* transfer of PDX-1 by adenovirus into mice unambiguously converted some subpopulation of hepatic cells into pancreatic endocrine and exocrine cells. We intend to specify or narrow down our subject to relatively mature hepatocytes. HepG2 cell line shows a large number of properties of differentiated hepatocytes, including expression of albumin, transferrin and transthyretin[24]. However, the data from our *in vitro* and *ex vivo* models ruled out the possibility of PDX-1-mediated conversion from mature hepatocytes to pancreatic tissues.

There are many different subpopulations of liver stem cells, which stand at various stages of developmental process and express more or less different profiles of transcription factors. We believe that this diversity endows every subpopulation different potential of reacting to stimulus, such as PDX-1 gene transfer, inducing developmental shift to pancreas. Our results proved the stubbornness of mature hepatocytes to PDX-1 induction. Kojima *et al*[23] showed that there were definitely some classes of liver cells that could be converted by PDX-1 transfer and diverse plasticity of these cells were observed under the same manipulation. Combining all these data with ours, we propose that more than one type of subpopulations comprising liver may have the potential to incur liver to pancreas conversion, but they require thresholds fundamentally different from each other to initiate this process. Mature hepatocytes, under this consideration, may also be possible to shift to insulin-producing cells under certain but much more complicated or rigorous conditions, which further needs to be defined.

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Science Editor Kumar M and Guo SY **Language Editor** Elsevier HK