J Biomed Sci (2008) 15:487–497
DOI 10.1007/s11373-008-9233-3

ORIGINAL PAPER

Adeno-associated virus-mediated pancreatic and duodenal homeobox gene-1 expression enhanced differentiation of hepatic oval stem cells to insulin-producing cells in diabetic rats

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Received: 13 September 2007 / Accepted: 28 December 2007 / Published online: 6 February 2008

Abstract Inducing autologous liver cells to differentiate into endocrine β cell has been a potential strategy for the treatment of type 1diabetes. However it is still not known which sub-population cells in the liver was responsible for this developmental shift. Pancreatic and duodenal homeobox gene 1 (pdx-1), a crucial transcription factor in pancreatic islet development and differentiation, has attracted much interests in beta cell differentiation experiments. This study was conducted to evaluate whether pdx-1 gene delivered by adeno-associated virus (AAV) could induce autologous liver cells to differentiate into insulin-producing cells and to explore the origin of these cells. Here we used $4 \times 10e^{11}$ AAV to deliver pdx-1 to STZ-induced diabetic rats via the portal vein. Immunofluorescent staining showed more insulin-positive cells, which had similar morphology with hepatic oval stem cells and were positive for hepatic oval stem cell markers, Thy-1 and cytokeratin 19 (ck19). In addition to the expression of pdx-1, insulin1 and insulin2, RT-PCR and quantitative real-time PCR also detected significantly higher levels of other important transcription factors in AAV-pdx-1 treated diabetic rat livers. AAV-pdx-1 treated diabetic rats showed partially ameliorated hyperglycemia, better gain of body weight and improved lipid levels. Our data indicated that rat hepatic oval stem cells were differentiated into bioactive insulin-producing cells by AAV-pdx-1 delivery in diabetic rats, with promoted expression of some transcription factors necessary for beta cell development and function.

Keywords Oval stem cells · pdx-1 · Adeno-associated virus · Differentiation · Insulin

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Introduction

Current therapies for type 1diabetes depend mainly on life-long insulin injections with close monitoring of blood glucose (BG) levels. Although the major diabetic complications can be reduced by intensive insulin therapy, this reduction is achieved at the expense of an increased risk of

severe hypoglycemia and undesirable weight gain, highlighting the limitations of existing insulin therapy. Furthermore, in many patients, glycemic control remains suboptimal despite treatment with insulin. Although islet transplantation as a strategy of cell replacement has been performed clinically, the unavoidable problems of donor shortage and rejection reaction remain to be resolved. As for the use of xenogeneic tissues, graft rejection and the risk of xenobiotic virus infection are two unavoidable impediments.

Cell and gene therapies with stem cells represent paradigmatic therapeutic strategies for diabetes in this new millennium. Embryonic stem (ES) cells have been shown to differentiate into insulin-producing and glucose-responsive cells in vitro by a nestin-selection protocol with addition of differentiation factors [1, 2]. However, ethical debates have arisen with respect to ES, especially those derived from cloned human embryos. Moreover, insulin-producing grafts derived from ES cells had demonstrated a high degree of cellular heterogeneity and tumor-forming potential, with the formation of teratoma [3].

Fetal human progenitor liver cells had been successfully induced into insulin-producing cells after expression of pdx-1 by lentivirus and euglycaemia was maintained for about 2 months when these cells were transplanted into hyperglycemic immunodeficient mice [4]. However, the source of fetal liver cells and immunorejection after transplantation still remain a problem. Adult rat hepatic stem cells were isolated by cell sorting of Thy1-positive cell population and were differentiated into pancreatic endocrine hormone-producing cells in vitro [5]. These differentiated cells formed islet cell-like clusters that expressed pancreatic islet cell differentiation-related transcripts, such as those of pdx-1, insulin-1, insulin-2 and other islet-specific hormones. Reversal of hyperglycemia was observed after transplantation these islet-like clusters into the subrenal capsular space of NOD-scid mice. However, possibly because of immunorejection, the experiment was terminated within 10 days. Ferber et al. [6] showed that delivery of pdx-1 by adenovirus could induce the differentiation of some liver cells into insulin-producing cells in diabetic mice: hepatic and plasma insulin levels were significantly increased and hyperglycemia was ameliorated. The experiment was terminated within 8 days as the gene expression induced by adenovirus was transient as innate immunity was induced by the adenoviral vector, resulting in the elimination of the transduced cells [7]. Nevertheless, this exciting achievement has opened the way to potential clinical use of beta cells formed from the differentiation of autologous hepatic cells, which could obviate the need of life-long immunosuppressive regimens associated with conventional islet transplantation. Kojima et al. also observed islet neogenesis in liver of mice with combined delivery of NeuroD and betacellulin by adenovirus and achieved the reversal of hyperglycemia [8]. Moreover both Ferber et al. [6] and Kojima et al. [8] had ruled out the possibility that ectopic expression of pdx-1 could induce the expression of insulin in hepatocytes and pointed out that additional experiments were needed to define the origin of these insulin-producing cells in livers. However no further studies have been published about the identity of the subpopulation of liver cells which are responsive to this type of differentiation.

Our present study was designed to observe whether AAV-pdx-1 could induce the differentiation of liver cells into insulin-producing cells in diabetic rats and explore which sub-population in the liver was responsible for this developmental shift. AAV was chosen as the vector because its use had been associated with predicted insertion of the transgene, more stable gene expression and less immunological response. On the other hand, pdx-1 has been increasingly recognized as a crucial transcription factor during pancreatic islet development and differentiation, from the early stages [9, 10], and has triggered much interest in differentiation experiments for beta cells [11–13].

Materials and methods

Recombinant adeno-associated virus vectors (rAAV)

The rAAV-pdx-1 construct (Fig. 1) was made by inserting the rat pdx-1 cDNA into the AAV serotype 2 vector (AAV-2) plasmid under control of a 410 bp glucose-responsive fragment of rat insulin I promoter (RIP) [14]. A wood chuck hepatitis B post-transcriptional regulatory element (WPRE) was added after the inserted gene to boost the expression level. The AAV particles were generated by a three-plasmid, helper-virus free, packaging method [15]. The rAAV particles were isolated and purified by heparin affinity column chromatography and the AAV viral genome titer was quantified by real-time PCR using SYBR green (Master Mix kit (Applied Biosystems) [16]. All viral vectors were stored at −80°C before experiments.

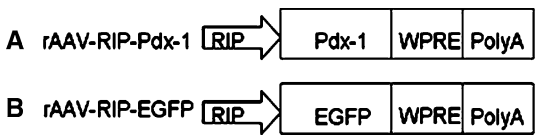

Fig. 1 Vector diagram. RIP: rat insulin I promoter. WPRE: wood chuck hepatitis B post-transcriptional regulatory elements to boost the expression level. EGFP: enhanced green fluorescent protein. AAV-EGFP services as vector control

Experimental animals

Male Sprague–Dawley rats, weighing around 210–240 g, were housed at a constant temperature and 12-h light/dark cycle and supplied with laboratory chow and water ad libitum. All studies were conducted under a research protocol approved by the Committee of the Use of Live Animals in Teaching and Researching in the University of Hong Kong.

Induction of diabetes

Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) at a dosage of 90-mg/kg body weight. Blood glucose was determined after an overnight fast with a glucometer (Elite, Bayer, Elkhart, IN). After 3–4 days, the rats with BG levels of 17–20 mmol/l were selected for study. The AAV-pdx-1 or AAV control vector was injected via the portal vein at the dosage of 4×10^{11} particles/animal to selected diabetic rats or non-diabetic control rats. Fasting BG and body weight were monitored weekly for 6 consecutive weeks after injection.

RNA extraction and RT-PCR analysis

Animals were sacrificed at the end of the study. Blood samples were taken from the inferior vena cava and the liver and pancreas were removed and stored at −80°C prior to analysis. Total RNA was isolated from frozen liver tissues with TRIzol (Life Technologies, Rockville, MD) reagent. Genomic DNA was digested with DNase I incubation. For cDNA synthesis, oligo dT primers were used to prime the RT reactions. The primers were designed to detect the transcripts of pdx-1, Foxα2, NeuroD, Ngn3, Nkx6.1, and beta cell specific gene insulin1 and insulin2 according to published sequences. The primers sequences were as follows: pdx-1: 5′-CAT CTC CCC ATA CGA AGT GC (forward) and 5′-GAG GTT ACG GCA CAA TCC TG (reverse); insulin 1: 5′-CAC CTT TGT GGT CCT CAC CT (forward) and 5′-CCA GTT GGT AGA GGG AGC AG (reverse); insulin2: 5′-CAG CAC CTT TGT GGT TCT CA (forward) and 5′-AGA GCA GAT GCT GGT GCA G (reverse), Foxα2: 5′-GCTCCCTACGCCAA TATGAA (forward) and 5′-CCG GTA GAA AGG GAA GAG GT (reverse), NeuroD: 5′-GGA TGA TCA AAA GCC CAA GA (forward) and 5′-GCA GGG TAC CAC CTT TCT CA (reverse), Ngn3: 5′-CGC TTC GCC TAC-AAC TAC ATC (forward) and reverse: 5′-CTG AAG CCG AGG GAC TAC TG (reverse), Nkx6.1: 5′-ACT TGG CAG GAC CAG AGA GA (forward) and 5′-GGG CTT GTT GTA ATC GTC GT (reverse). The PCR reactions were performed for 35 cycles as follows: 60 s at 94°C for denaturation, 30 s at 56°C for annealing, and 30 s at 72°C for extension. The PCR products were separated on 1.8% agarose gel electrophoresis.

Real-time quantitative PCR

The real-time quantitative PCR was conducted with cDNA as template, using SYBR green (Master Mix kit (Applied Biosystems) in ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). All primers for GAPDH, pdx-1, insulin1, insulin2 and transcription factors Ngn3, NeuroD, Nkx6.1, Foxα2 were designed and optimized according to the SYBR green Master Mix and RT-PCR protocol (Applied Biosystems, www.appliedbiosystems.com). The thermal cycling parameters were 50°C for 2 min followed by 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min repeat. The mRNA transcripts levels were determined using the ΔΔCT method based on the measurements of threshold cycle (CT) by the real time PCR [17]. Briefly, CT indicates the lowest cycle number at which the amount of amplified target reaches a fixed threshold. ΔCT was calculated for each sample by subtracting the CT value of internal control GAPDH from the CT value of target gene. ΔΔCT value was then calculated by subtracting the ΔCT of the target gene of the non-diabetic pancreas control, which serves as the calibrator, from each ΔCT of the target gene of treatment group. Finally, the amount of mRNA level of target gene, normalized to an internal control GAPDH and then relative to a calibrator, is given by: $2^{-\Delta\Delta CT}$, indicating an relative mRNA transcripts levels to the pancreas of the non-diabetic control group.

Immunofluorescence

At the end of the study, livers were removed and kept in 4% paraformaldehyde (PFA) at 4°C overnight after being perfused with 1× PBS and 4% PFA. Routine paraffine embedding was conducted and livers were sectioned at the thickness of 6 μm. Immunostaining was performed to detect the expression of pdx-1, insulin, ck19 and Thy-1 in liver sections using polyclonal rabbit anti-pdx-1 antibody (1:200), monoclonal mouse anti-insulin antibody (1:150, Neomarkers, USA), monoclonal mouse anti-ck19 antibody (1:150, Oncogene, USA), and monoclonal mouse anti-Thy-1 antibody (1:100, Santa Cruz Biotechnology, USA), respectively. Sections were incubated overnight at 4°C with primary antibodies. The primary antibodies were localized by anti-mouse FITC (1:150, Sigma) for insulin or ck19, anti-mouse cy3 (1:150, Sigma) for ck19 or Thy-1,

and anti-rabbit cy3 (1:150, Sigma) for pdx-1. Propidium iodide (PI) or 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI) was used for counterstaining the nucleus. Sections were observed under a fluorescence microscope (Olympus TH4-200, Olympus Optical Co. LTD, Tokyo, Japan).

Analysis of pdx-1 gene expression in livers

Liver extracts were obtained by homogenization in protein lysis buffer. Protein content was determined with BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). Tissue pdx-1 levels were measured with Western blot analysis and enzyme-linked immunosorbent assay (ELISA) as previously described [18]. The antibodies used were polyclonal rabbit anti-rat pdx-1 (1:2,000) and HRP-conjugated secondary antibody (1:2,000).

Measurements of insulin content, lipid metabolism and liver enzymes

Insulin levels in liver extracts were detected with a rat insulin ELISA kit (Mercodia, Sweden). Serum triglyceride and cholesterol were determined with enzyme-coupled rate colorimetric reaction on the Hitachi-912 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed by the standard rate colorimetric method (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

Data were expressed as the mean ± SE. Statistical analyses of data were performed using one-way ANOVA followed by Tukey's post test. The P value < 0.05 was considered to be statistically significant.

Results

Gene expression in the liver after rAAV-pdx-1 delivery

RT-PCR revealed that the gene expression of transcription factors, pdx-1, NeuroD, Ngn3, Nkx6.1 and beta cell specific gene insulin1 and insulin2 increased markedly, while AAV diabetic control group expressed undetectable or very weakly positive transcripts (Fig. 2). Real-time quantitative PCR further confirmed a significant higher mRNA transcripts level relative to non-diabetic pancreas for Ngn3, NeuroD, pdx-1, Nkx6.1, insulin1 and insulin2 in the AAV-pdx-1treated diabetic group, when compared to the AAV diabetic control group (Fig. 3). Nevertheless, the expression of all these transcripts in the livers of AAV-pdx-1 treated diabetic group was still significantly lower when compared to those in the normal pancreas of non-diabetic control group.

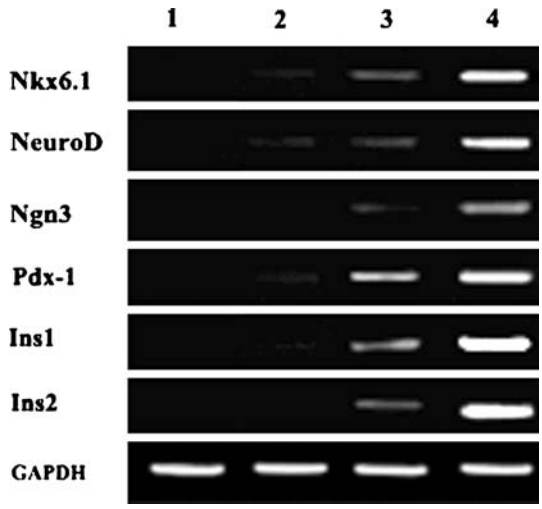

Fig. 2 RT-PCR analysis of gene expression of beta cell related genes with GAPDH as internal standard at 6 weeks after portal vein delivery of AAV vectors or AAV-pdx-1 (4×10^{11} particles/animal). Lane 1, liver of the non-diabetic control group, lane 2, liver of the AAV control diabetic group, lane 3, liver of the AAV-pdx-1-treated diabetic group, lane 4, pancreas of non-diabetic controls. Ins1: Insulin1, Ins2: insulin2, Neurogenin 3: Ngn3

Identification of insulin-producing cells in livers of experimental rats by immunofluorescence

The AAV control diabetic group showed the presence of fewer insulin-positive cells in the livers, and these cells appeared as single cells only (Fig. 4a). In contrast, the AAV-pdx-1 group showed much more insulin-positive cells in liver sections, which occurred mainly as small clusters (Fig. 4b). They were located very close to the portal triad or between the hepatic plates, and had a larger nucleus-to-cytoplasm ratio, but smaller cell size and nucleus size compared to the adjacent hepatocytes. These insulin-positive cells in both groups were also positive for islet hormone, glucagons and somatostatin (Fig. 4c, d), consistent with the published data [8], and also positive for hepatic stem cell markers, ck19 and Thy-1 (Fig. 4e, f). Further double staining showed that Thy-1-positive cells were also positive to ck19 (Fig. 4g), indicating that these cells had originated from hepatic oval stem cells. Double immunostaining with insulin and pdx-1 antibodies clearly demonstrated that both insulin and pdx-1 immuno-reactive positive cells were present in small clusters (Fig. 5),

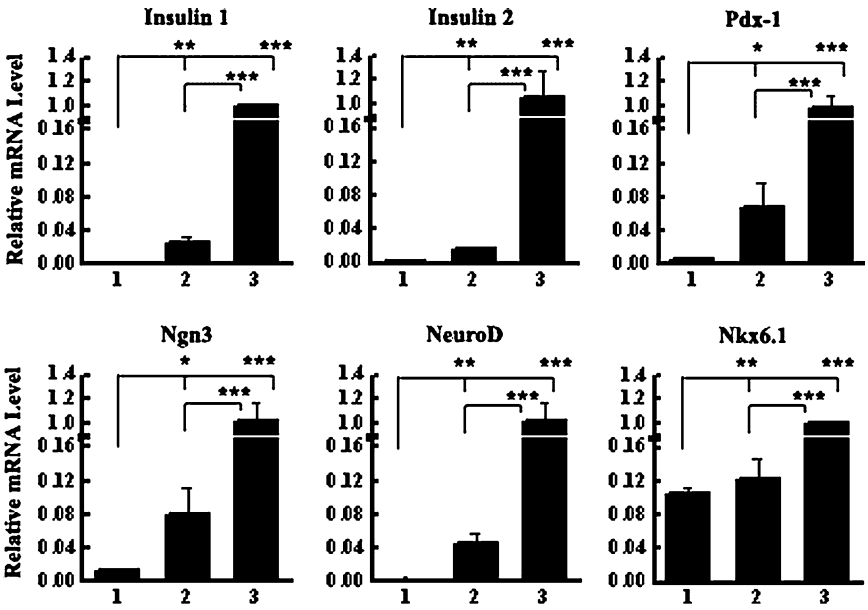

Fig. 3 Relative mRNA level of beta cell related genes at 6 weeks after portal vein delivery of AAV vectors or AAV-pdx-1 (4×10^{11} particles/animal). Lane 1: liver of AAV control diabetic group; lane 2: liver of AAV-pdx-1 diabetic group, lane 3, pancreas of non-diabetic control group. Values of mRNA transcripts were expressed as a relative to the pancreas of the non-diabetic control group ($n = 3$). Statistical analyses of data were performed using one-way ANOVA followed by Tukey's post test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

suggesting AAV-mediated pdx-1 delivery induced these insulin-producing cells. No insulin-positive cells were detected in normal control liver sections.

Expression of liver pdx-1 by western blot and ELISA

As shown in Fig. 6, the AAV-pdx-1 diabetic group showed significantly higher expression of pdx-1 protein in a predominantly active form (~46 kDa), and the inactive form (~31 kDa) than the AAV diabetic control group by Western blot. No pdx-1 expression was detected in the non-diabetic control group. ELISA analysis also indicated a significantly higher expression of pdx-1 in the AAV-pdx-1 diabetic group than that of the AAV control diabetic group.

Effects on liver insulin level

Insulin levels in liver extracts of the AAV-pdx-1treated diabetic group were significantly higher than those in the AAV control diabetic group (3.1 ± 0.37 ng/g protein versus 0.8 ± 0.20 ng/g protein, $P < 0.01$), whereas there was significantly much lower levels of insulin in the non-diabetic control group (0.03 ± 0.01 ng/g protein) (Fig. 7).

Effects on hyperglycemia and body weight

As shown in Fig. 8, BG levels were measured every week for 6 consecutive weeks. The AAV control diabetic group showed significantly elevated BG levels. Although the AAV-pdx-1 treated diabetic group showed persistently elevated BG, the hyperglycemia was partially ameliorated when compared to the AAV control diabetic group. As a consequence, the AAV diabetic control group showed a gradual decrease in body weight, while the AAV-pdx-1 treated diabetic group showed a significantly better gain of body weight during the whole experiment period.

Effects on lipid levels and liver enzymes

To examine the effect of AAV-pdx-1 treatment on lipid levels, serum triglyceride and total cholesterol levels were measured in the different groups (Fig. 9a, b). In contrast to the non-diabetic control, serum total cholesterol and triglyceride levels were significantly elevated in the AAV control diabetic group. These abnormalities in lipid metabolism were partially corrected by the treatment with AAV-pdx-1. To examine whether the AAV-pdx-1 treatment could cause hepatotoxicity, serum levels of liver enzymes, AST and ALT were measured. As shown in Fig. 9c and d, serum AST and ALT levels were significantly elevated in the AAV control diabetic group compared with the non-diabetic control, whereas AAV-pdx-1 treatment led to significantly decreased levels of both AST and ALT compared with the AAV diabetic control.

Discussion

Autologous liver cells have been reported to be induced into insulin-producing cells with correction of hyperglycemia, which will be a novel promising approach since

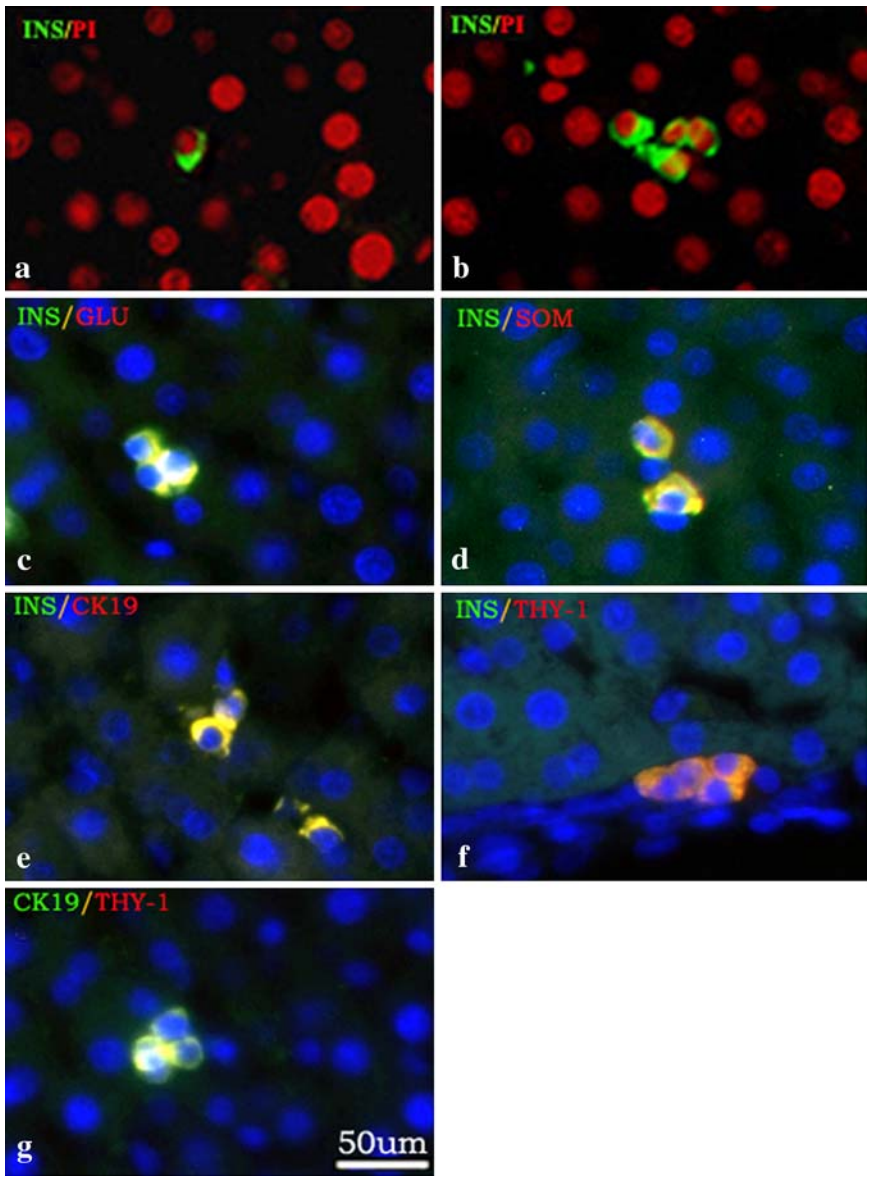

Fig. 4 Immunofluorescence staining of insulin-positive cells in livers of experimental rats at 6 weeks after portal vein delivery of AAV vectors or AAV-pdx-1 (4×10^{11} particles/animal). (**a**) AAV control diabetic group; (**b**) AAV-pdx-1-treated diabetic group; (**c**–**g**) AAV-pdx-1-treated diabetic group. INS, insulin; GLU: glucagon; SOM: somatostatin; ck19: cytokeratin 19. PI (propidium iodide) or DAPI (4′, 6-diamidine-2′-phenylindole dihydrochloride) was used for nucleus counterstaining

there is no need of life-long immunosuppressive therapy associated with conventional islet transplantation. Further work is required to explore which sub-population cells in the liver are responsible for this developmental shift. Our study has provided data suggesting the hepatic oval stem cells to be the source of these transdifferentiated insulin-producing cells.

The liver has a tremendous regenerative capacity and two epithelial cell types are known to be possible hepatic stem cells: the hepatocytes and the oval cells, especially the latter [19–22]. Oval cells are small in size (approximately 10 μm), with a large nucleus-to-cytoplasm ratio and an oval-shaped nucleus. They are pluripotent and retain the ability of differentiation into hepatocytes, bile duct epithelium, intestinal acinar epithelium or the endocrine pancreas. In the adult, oval cells are located in the canals of Hering, which are more elaborate than previously thought and extend far into the hepatic parenchyma. Petersen et al. [23] showed that rat hepatic oval stem cells expressed high levels of Thy-1 and Thy-1 positive cells were indeed oval stem cells, because they also expressed a-fetoprotein (AFP), g-glutamyl transpeptidase (GGT), cytokeratin 19 (ck19) and cytokeratin 14 (ck14), all known markers for oval cell identification. Using Thy-1 antibody as the marker for the identification of oval cells, they isolated more than 95% pure oval cell population by flow cytometric methods. Later, they counted percentage of hepatic oval stem cells in mice livers according to Thy-1 positive signals [24]. Yang et al. [5] isolated and purified rat hepatic oval stem cells also by Thy-1 positive signals using flow cytometry and resulted in hepatic oval cell populations with a purity >95%. To date, this is the highest reported purity for

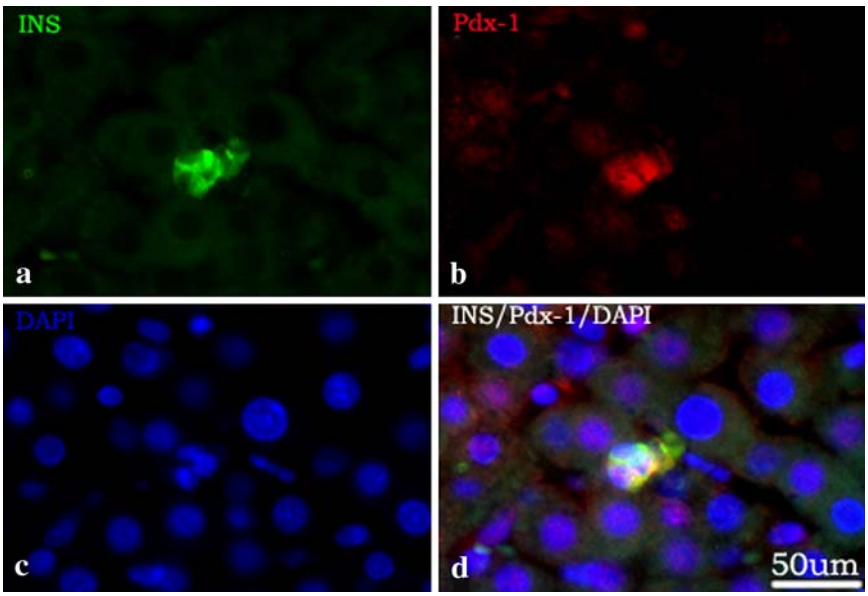

Fig. 5 Double staining of pdx-1 and insulin in the livers of experimental rats at 6 weeks after portal vein delivery of AAV-pdx-1 (4×10^{11} particles/animal). INS: insulin; Pdx-1: pancreatic and duodenal homeobox gene 1. DAPI: 4′, 6-diamidine-2′-phenylindole dihydrochloride, was used for nucleus counterstaining

isolated oval cells, indicating that Thy-1 is the most suitable marker for hepatic oval stem cells.

Petersen et al. [24] showed that some of the hepatic oval stem cells originated from bone marrow in an oval cell activation model by an acute liver injury method after bone marrow transplantation. But subsequent researches led to different conclusion that bone marrow stem cells were previously showed to differentiate into hepatocytes and cholangiocytes and to yield engraftment of injured liver, however it appears now that this phenomenon is minimal or even absent in physiological and usual pathological conditions. Hepatic oval stem cells are of intrinsic hepatic origin [25–29].

In our present study AAV-2 vector and pdx-1 gene were chosen to differentiate liver cells into insulin-producing cells and to explore which sub-population in the liver was responsible for this developmental shift. The AAV is a well-accepted viral vector as it has several major advantages, such as much less immunological response, persistent gene expression, and the ability to infect both dividing and non-dividing cells. The AAV-2 is the most extensively studied serotype and also the most commonly used. Moreover, AAV-2 preferentially transduced liver cells when administered via tail vein and portal vein, and portal vein injection resulted in about 10 folds expression than tail vein delivery [30–33]. The Pdx-1 has been accepted as an essential and crucial transcription factor from the earliest stages of pancreatic islet development and differentiation. Knockout of pdx1 gene results in an arrest of pancreatic development at very early stage, just after the formation of the initial pancreatic bud, and gives rise to an animal with no pancreas [9, 10]. In terminally differentiated beta cells, pdx-1 plays a critical role in the maintenance of normal function as a glucose-responsive regulator of insulin gene expression [9, 10, 34, 35]. Therefore pdx-1 has attracted much interest in cell and gene therapy research for diabetes [6, 12, 13, 36].

Our present study indicated a significant increase of insulin content in the liver extracts of AAV-pdx-1 treated diabetic rats compared with AAV diabetic control. Immuno-fluorescent staining showed significantly more insulin-positive cells in AAV-pdx-1 treated diabetic rat livers, occurring mainly as small clusters, and also as individual cells. Unexpectedly, single insulin-positive cells, much fewer in number, were also present in livers of AAV diabetic controls, but not in livers of non-diabetic control rats. These insulin-positive cells were located between the hepatic plates with a larger nucleus-to-cytoplasm ratio but smaller cell size and nucleus size, compared to the adjacent hepatocytes. Moreover, these cells were also positive for hepatic oval stem cell markers, Thy-1 and ck19. Our findings on morphology, location and biomarkers of insulin-producing cells suggested that these cells originated from hepatic oval stem cells and the transduction of AAV-pdx-1 into the diabetic livers could significantly enhance such a developmental shift from hepatic oval stem cells to insulin-producing cells.

Further supporting evidence came from real-time PCR data that clearly indicated significant increases in some key factors in pancreatic development and formation, following AAV-pdx-1 delivery. During the development and formation of the pancreas a large number of genes work in coordination and a unique set of transcription factors control these gene expression events. Ngn3, one of the basic helix-loop-helix (bHLH) factors, expressed mainly in pancreatic endocrine precursors, has been generally

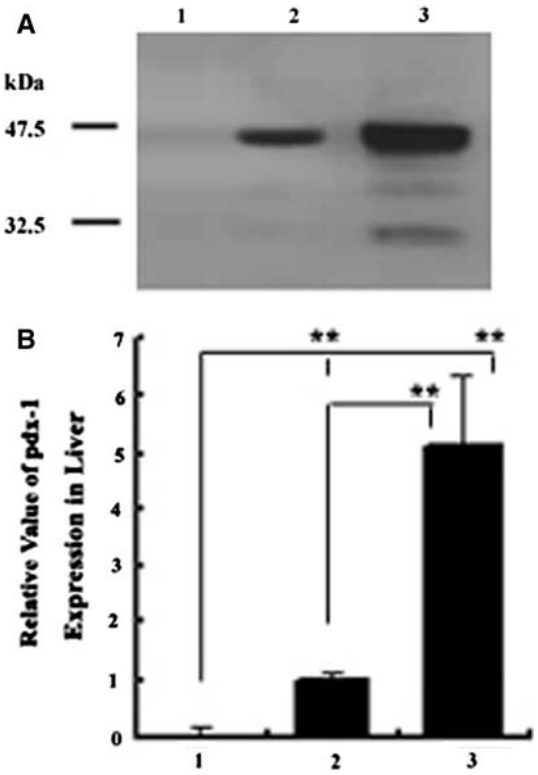

Fig. 6 Pdx-1 expression in livers of experimental rats by Western blot (**a**) and ELISA (**b**) at 6 weeks after portal vein delivery of AAV control vectors or AAV-pdx-1 (4×10^{11} particles/animal). Liver extracts were analyzed for pdx-1 protein expression with rabbit anti-rat pdx-1antibody (1:2,000). Lane 1: non-diabetic control; lane 2: AAV control diabetic group; lane 3: AAV-pdx-1 diabetic group. Values of pdx-1 expression by ELISA were presented as the ratio to those of AAV diabetic control group ($n = 4$). Statistical analyses of data were performed using one-way ANOVA followed by Tukey's post test. $^{**}P < 0.01$

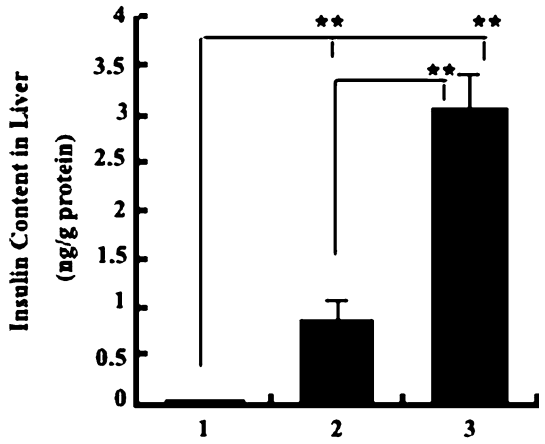

Fig. 7 Insulin expression in livers of experimental rats at 6 weeks after portal vein delivery of AAV vectors or AAV-pdx-1 (4×10^{11} particles/animal) Insulin levels in liver extracts were detected with a rat insulin ELISA kit. Lane 1: non-diabetic control; lane 2: AAV control diabetic group; lane 3: AAV-pdx-1 diabetic group ($n = 4$). Statistical analyses of data were performed using one-way ANOVA followed by Tukey's post test. $^{**}P < 0.01$

accepted as the triggering factor for the endocrine cell lineage in pluripotent pancreatic progenitors, especially in the differentiation of beta cells [37, 38]. NeuroD (Beta 2), also one of bHLH transcription factor and a component of native insulin E box binding complex, is required for islet growth and proliferation and is important for insulin gene activation in mature beta cells [39, 40]. NeuroD appears to lie immediately downstream of Ngn3 [38, 41]. Nkx6.1 expressed in both the developing and mature pancreas [42, 43]. Disruption of the Nkx6.1 gene leads to selective reduction of beta cells with a normal complement of other endocrine cell types, indicating a specific role of Nkx6.1 in the development of beta cells [44]. The increase in these transcription factors after rAAV-pdx-1 delivery in our diabetic rats suggested that pdx-1 may promote the expression of some transcription factors necessary for beta cell development and function, and that these transcription factors may act as a network to direct hepatic oval stem cells to the beta cell lineage. Therefore, hepatic oval stem cells may provide a potentially unlimited source of β-cells through appropriately induced trans-differentiation.

To investigate whether this ectopic expression of insulin in the liver had any biological relevance, we measured fasting BG and body weight weekly after the injection of AAV-PDX-1. The AAV-pdx-1 treated diabetic rats showed an amelioration of hyperglycemia with better gain of body weight compared to the AAV control diabetic rats for up to six weeks. Moreover, they showed an improvement in lipid metabolism and liver function, as reflected by the lowering of serum levels of TC, TG, and ALT, AST, suggesting the bioactivity of these ectopic insulin-producing cells.

Kojima et al. [8] reported severe liver toxicity following the ectopic expression of pdx-1 by adenovirus in mice. It may be because they used a much higher dosage of pdx-1 gene delivered by adenovirus under the control of a potent elongation factor-1α promoter, resulting in a constant expression of pdx-1 at high levels, leading to the increased expression of exocrine pancreatic genes, and subsequent hepatic damage by the exocrine secretion. However, proenzymes contained in the zymogen granules secreted by exocrine pancreatic become activated by limited proteolysis only after entering the duodenum [45]. Moreover, in our experiment, in order to prevent similar liver injury and to explore the origin of the insulin-producing cells in liver, we used lower dosage of AAV-pdx-1 and a regulating promoter, the glucose-responsive fragment of rat insulin I promoter (RIP), which has glucose-dependent activity. It was used to turn on the expression of pdx-1 in high glucose

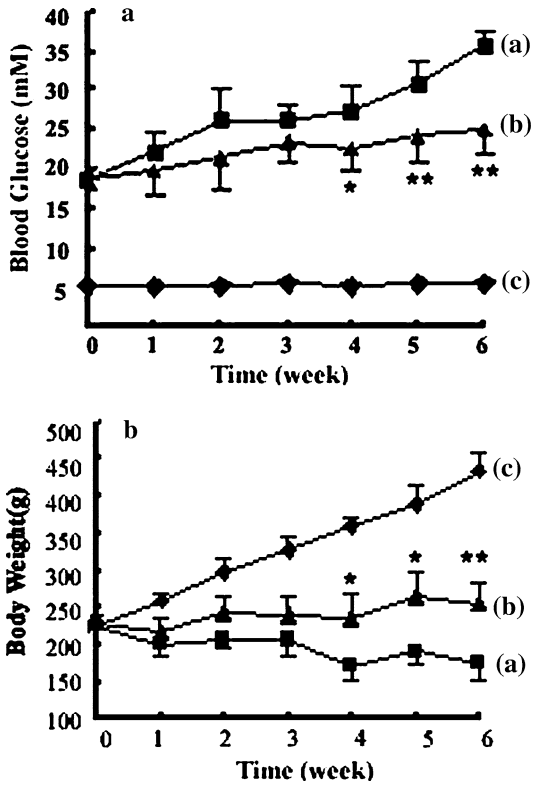

Fig. 8 Ectopic expression of AAV-pdx-1 in liver partially ameliorates STZ-induced hyperglycemia. (**a**) Blood glucose (mM); (**b**) Body weight (g). The AAV-pdx-1 or AAV control vector was injected via the portal vein at the dosage of 4×10^{11} particles/animal. Fasting BG and body weight were monitored weekly for 6 consecutive weeks after injection. (a) AAV control diabetic group, (b) AAV-pdx-1-treated diabetic group, (c) non-diabetic control group ($n = 8$). Tukey's post test was used to analyzed the difference between groups at the same time point after univariate analysis of General linear model for whole groups. $^{*}P < 0.05$ versus the AAV-treated diabetic control group, $^{**}P < 0.01$, versus the AAV-treated diabetic control group

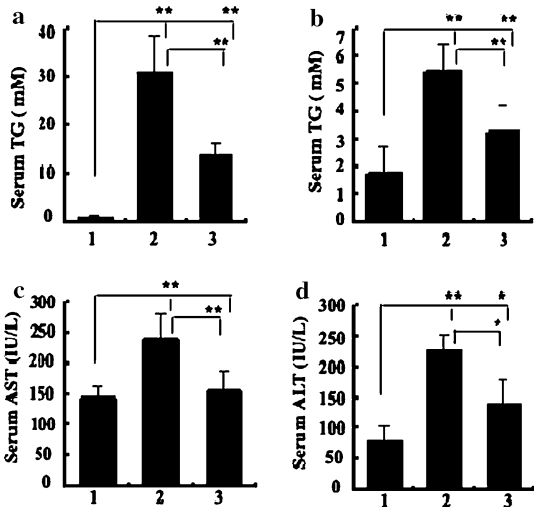

Fig. 9 Levels of lipid and liver enzyme in the serum of experimental rats at 6 weeks after treatments with AAV vectors or AAV-pdx-1 (4×10^{11} particles/animal). Serum triglyceride (TG) and total cholesterol (TC) were determined with enzyme-coupled rate colorimetric reaction. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed by the standard rate colorimetric method. Lane 1: the non-diabetic control; lane 2: the AAV control diabetic group; lane 3: the AAV-pdx-1 diabetic group ($n = 6$). Statistical analyses of data were performed using one-way ANOVA followed by Tukey's post test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the AAV-treated diabetic control group

environment in diabetic rats and to turn off the expression of pdx-1 to stop further differentiation when hyperglycemia was corrected by enough differentiated insulin-producing cells. We did not observe any liver toxicity in our system, but an improvement of liver function with lowered level of ALT and AST compared to the AAV diabetic control group, possibly secondary to a reduction in hyperlipidemia and fatty liver changes. Kay et al. delivered Pdx-1 with AAV-8 vector to the livers of diabetic mice and showed partial amelioration of hyperglycemia, which is consistent with our findings with AAV-2 vector, although they didn't observe the correction of hyperglycemia [46]. However, they found the correction of hyperglycemia by the delivery of Pdx-1 or Ngn-3 in the liver combined with irrelevant adenoviral vector and draw the conclusion by further studies that an antigen-dependent immune response elicited by the adenoviral capsid was required for the correction of diabetes. It is possibly because the strong host immune response to adenovirus triggered certain immune molecules to involve in the differentiation pathway or stimulated the neogenesis or differentiation of hepatic oval stem cells because of the destruction of hepatocytes by the strong host immune response.

In summary, our findings suggest that the rat hepatic oval stem cells were differentiated into bioactive-insulin-producing cells in vivo, under the influence of hyperglycemia and Pdx-1. Despite the modest effects of our treatment strategy on ambient hyperglycemia in the diabetic rats, our study had provided further support for the potential application of cell and gene therapy-based treatment of type 1 diabetes, via the generation of insulin-producing cells differentiated from autologous liver cells. With the rapid development in biomedical technology, further improvements on the treatment system, such as the use of a more efficient and safer delivery vector like the AAV 8 [47, 48], a regulating promoter leading to a conditional differentiation, and/or the combined delivery of therapeutic genes or combined delivery of adenovirus vector [46], may lead ultimately to the clinical application of such treatment strategy.

Acknowledgments The authors are grateful to Drs. I. Rofiq and H. Watada for gifts of pdx-1 construct and Professor Christopher V. E. Wright for gifts of polyclonal anti-rabbit pdx-1 antibody. This study was supported by the RGC Grant HKU 7368/02M to R. Xu and K. S. Lam.

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