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In vitro reprogramming of adult hepatocytes into insulin-producing cells without viral vectors

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ABSTRACT

The pancreas and the liver share the same endodermal origin. We have been studying whether mature hepatocytes can be induced to differentiate into pancreatic β-cells by *in vitro* delivery of transcriptional factors using a non-viral approach. Here we showed that nucleofection allowed suitable transfection of primary hepatocytes employing various non-viral methods. We introduced either pancreatic and duodenal homeobox 1 (Pdx1) or neurogenin 3 (Ngn3), or both, into the mature cells using nucleofection. Co-expression of *pdx1* and *ngn3* using a bicistronic vector activated the transcription of various islet-related genes, and the transfected hepatocytes acquired the ability to synthesize and secrete insulin.

Our results suggest that simultaneous expression of Pdx1 and Ngn3 is an excellent inducer of liver-to-pancreas reprogramming, and that reprogramming will occur even in mature somatic cells without the need for viral vectors. These findings are of considerable significance for further therapeutic development for various intractable diseases including diabetes.

Introduction

In the process of embryogenesis, both the liver and ventral pancreas appear to arise from the same cell population located within the embryonic endoderm [1,2]. Focusing on this embryological homology, several groups have demonstrated that ectopic expression of key transcription factor genes for pancreas development in hepatic cells can induce them to differentiate into β-like cells [3,4]. Pancreatic and duodenal homeobox 1 (Pdx1), a member of the homeodomain-containing transcription factor family, plays an important role in initiating the differentiation of pancreatic endocrine cells. Ferber et al. were the first to report that transient adenovirus-mediated expression of *pdx1* in hepatocytes activated endogenous insulin expression and ameliorated hyperglycemia in mice with streptozotocin (STZ)-induced diabetes [5]. After this initial *in vivo* approach, many other studies [6,7] showed that several regulators of pancreas development, such as v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), neurogenic differentiation 1 (NeuroD1), and neurogenin 3 (Ngn3) were potential inducers of islet cell differentiation in the liver. Although these transcription factors have an ability to induce the development of islet cells, it remains unclear whether the expression of a single gene alone, especially Pdx1, in hepatocytes is sufficient to induce reprogramming. In fact, it has been reported that *in vitro* induction of Pdx1 alone does not induce insulin production by mature hepatocytes, even though this is possible *in vivo* [5]. Kaneto et al. reported that adenoviral ectopic expression of Pdx1/VP16, a constitutively active form of Pdx1, together with Ngn3 or NeuroD, induced transcription of the insulin gene, resulting in amelioration of hyperglycemia in STZ mice, whereas this effect was mild when each gene was delivered independently [6]. These reports established that a suitable combination of transcription factors can activate target genes and act synergistically to induce insulin production in the liver.

On the other hand, several problems have been reported with the delivery of these transcriptional factors into liver cells using adenovirus-mediated strategies. Adenoviral vectors have hepatotoxicity caused by an immunological reaction in the liver [8]. To eliminate this reaction and to allow clinical application for the reprogramming of liver cells into an islet cell fate, a non-viral

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approach for gene delivery is needed. However, no reports have indicated that pancreatic β-cells can be induced from mature hepatocytes *in vitro* by non-viral delivery of transcriptional factors.

In the present study, we demonstrated that nucleofection was effective for achieving transient expression of pancreatic transcriptional factors in mature hepatocytes. Furthermore, we showed that co-expression of *pdx1* and *ngn3* using a bicistronic expression approach activated the transcription of various islet-related genes including Ins1 and Ins2 in primary hepatocytes, and that the transfected cells acquired the ability to synthesize and secrete insulin. These results suggest that, if appropriate transcription factors can be transferred, reprogramming occurs even in mature somatic cells, and that the use of viral vectors is not indispensable for this approach.

Materials and methods

Plasmid construction. We generated three bicistronic plasmids: pCMV-Pdx1-IRES-EGFP (pCPIE), pCMV-Ngn3-IRES-DsRed (pCNID), and pCMV-Pdx1-IRES-Ngn3 (pCPIN) (Figs. 2A and 3A). The *pdx1* and *ngn3* cDNAs were PCR-amplified from the plasmids pZL1-Pdx1 (a generous gift from C. Wright) and pPdx1-IRES-Ngn3 (A. Kubo, unpublished) using restriction site-attached primer sets, and cloned into the pIRES2-EGFP and pIRES2-DsRed (Clontech, Mountain View, CA), respectively. For construction of pCPIN, the Pdx1-IRES-Ngn3 fragment was excised from pPdx1-IRES-Ngn3 and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The details, including the PCR primer sets employed, are described in Supplementary Materials and Methods.

Cell preparation and culture. Hepatocytes were isolated from 6-week-old male C57Bl/6J mice (Clea Japan, Tokyo, Japan) by in situ collagenase perfusion, as described previously [9]. Cell viability measured by the trypan blue exclusion test was more than 95%.

βTC6 (American Type Culture Collection, Manassas, VA), a mouse insulinoma cell line [10], was used as a positive control. The conditions used for culture of these cells are described in Supplementary Materials and Methods.

Transfection with a non-viral procedure. Nucleofection of primary hepatocytes was performed according to the manufacturer's optimized protocol (Amaxa Biosystem, Cologne, Germany). Briefly, approximately 7×10^5 isolated hepatocytes were resuspended in 100 μL of mouse hepatocyte Nucleofector™ Solution, mixed with 6 μg of plasmid DNA, and pulsed with the program T-028. Fifteen minutes later, the cells were seeded at a density of $7 \times 10^4/cm^2$.

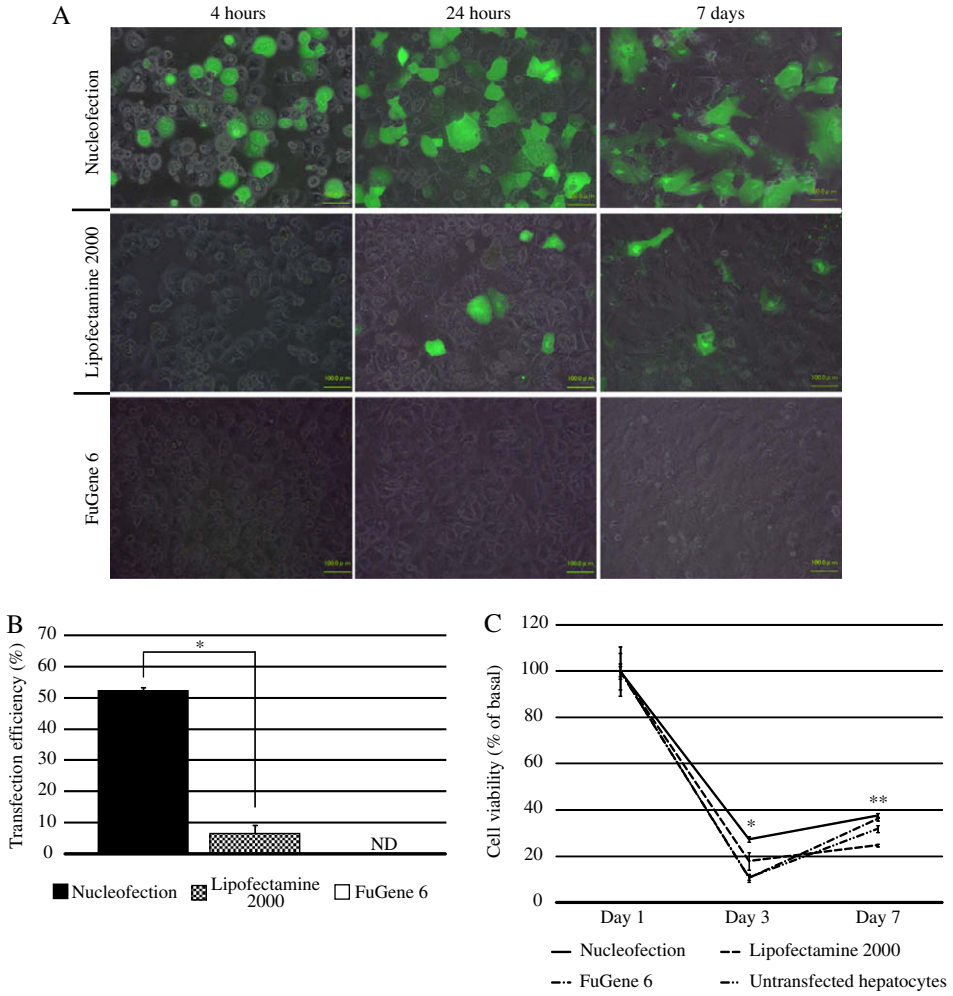

Fig. 1. Optimization of non-viral transfection procedures in primary cultured hepatocytes. (A) GFP expression in transfected hepatocytes. At 4 h after transfection, only the nucleofected hepatocytes expressed GFP. (B) Differential transfection efficiency of primary hepatocytes using three optimized DNA delivery methods. Data represent the mean ± standard deviation (SD) of the proportion of GFP-positive cells to the total number of cells. $^*P < 0.05$. ND, not detected. (C) Time-course analysis of both transfected and untransfected hepatocytes using the MTS assay. We compared the reduction of viability in each group at 3 and 7 days relative to that on day 1. $^*P$ value < 0.05 relative to values for Lipofectamine 2000, FuGene 6 and untransfected hepatocytes ($n = 4$). $^{**}P$ value < 0.05 relative to values for Lipofectamine 2000 and untransfected hepatocytes ($n = 4$).

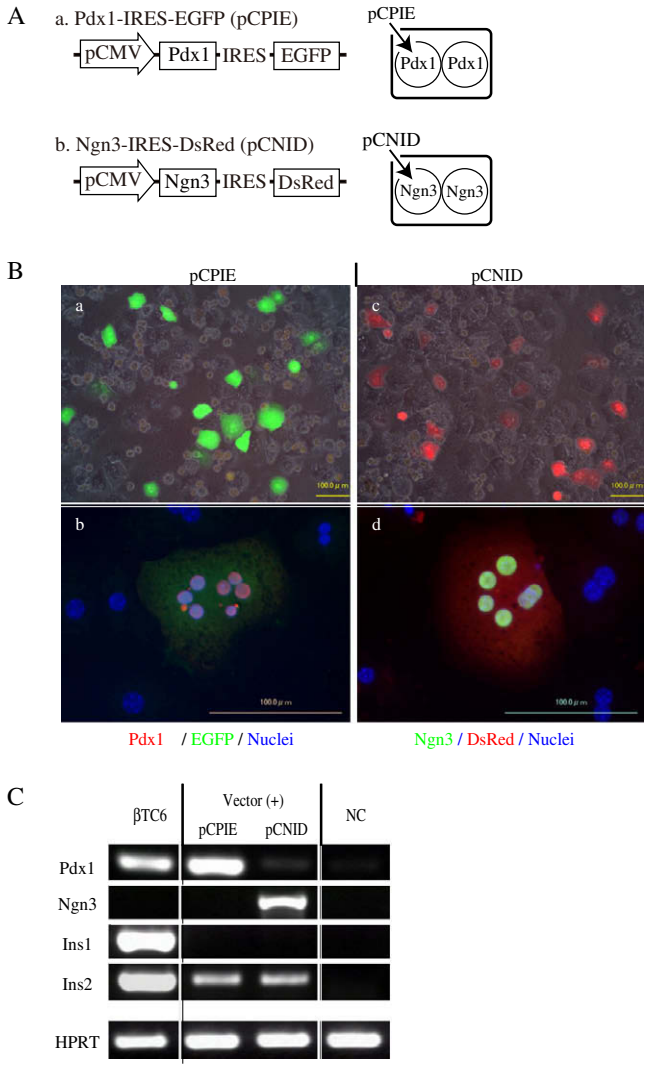

Fig. 2. Influence of ectopic expression of *pdx1* and *ngn3* alone. (A) Diagrams of bicistronic plasmids and schema of the transfection patterns. pCMV, cytomegalovirus promoter. IRES, internal ribosome entry site. (B) Live images and immunofluorescence staining of transfected hepatocytes. pCPIE-transfected hepatocytes expressed EGFP (a) and Pdx1 (b). pCNID-transfected hepatocytes also expressed DsRed (c) and Ngn3 (d). (C) Gene expression profiles of transfected cells. Plasmid-free nucleofected hepatocytes were prepared as a negative control (NC).

We compared the optimized nucleofection method with other non-viral transfection techniques such as Lipofectamine 2000 (Invitrogen) and FuGene 6 (Roche Applied Science, Mannheim, Germany). We performed initial optimization and transfection in accordance with the manufacturer's protocols, and prepared reagent-DNA complexes at ratios of 2.5:1 and 3:1, respectively.

To evaluate the efficiencies of the various techniques, we used the pmaxGFP plasmid, which encodes green fluorescent protein (GFP). The efficiency was determined as the proportion of GFP-positive cells to the total number of cells at 24 h by counting 30 fields at 200× magnification in three independent experiments.

Viability of transfected or untransfected hepatocytes was assessed by using CellTiter®96 AQueous One solution reagent (Promega, Madison, WI) with static incubation at 37 °C for 1 h, as recommended by the manufacturer.

RNA extraction and reverse transcription polymerase chain reaction analysis. Total RNA was extracted from 2- or 5-day-cultured hepatocytes using a Qiagen RNeasy mini kit (Qiagen) and treated with Turbo DNA free (Ambion, Austin, TX) to remove contaminating genomic DNA. Two-microgram aliquots of total RNA were reverse-transcribed to cDNAs using Reverse transcription system (Promega) with an oligo-dT primer. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously [11]. The PCR primers are described in Supplementary Materials and Methods. Gene expression studies were repeated at least three times, and similar results were obtained.

Immunohistochemical analysis. Two-day-cultured hepatocytes were fixed with 4% paraformaldehyde/PBS for 20 min and then permeabilized with 0.1% Triton X/PBS for 10 min at room temperature. The fixed samples were incubated in Protein Block (DAKO, Carpentaria, CA) for 10 min at room temperature. They were then incubated with the primary antibody overnight at 4 °C and with the secondary antibody for 1 h in a humidified chamber. The antibodies used in this study are listed in Supplementary Materials and Methods. For nuclear staining, the cells were incubated for 5 min at room temperature with DAPI (4′, 6-diamidino-2-phenylindole). The samples were then mounted in fluorescent mounting medium (DAKO) and observed using a BZ-8000 microscope (KEYENCE, Osaka, Japan).

Measurement of insulin and C-peptide in the cells and medium. Two-day-cultured hepatocytes were washed four times with PBS and then treated with acid–ethanol (0.18 M hydrochloric acid in 95% ethanol) at 4 °C overnight. The clear supernatants were used to assay the intracellular insulin and C-peptide contents, and the values obtained were normalized relative to the total protein content. The insulin and C-peptide contents were measured by an enzyme-linked immunosorbent assay (Insulin-ELISA kit, C-peptide-ELISA kit, Shibayagi, Gunma, Japan). Total protein content was measured by Protein Assay (Bio-Rad, Hercules, CA). Insulin secretion was measured using static incubation in Krebs-Ringer bicarbonate buffer (KRBB) supplemented with glucose, as described previously [12]. In brief, the cells were washed twice with KRBB containing 0.1% BSA (KRB-BSA). They were then preincubated for 1 h in KRB-BSA at 37 °C, and incubated for 2 h in KRB-BSA supplemented with 5 mM or 25 mM glucose, or without glucose. The concentration of the insulin secreted into the buffer solution was measured using an Insulin-ELISA kit as described above.

Statistical analysis. Values are expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using Student's *t* test or one-way ANOVA (Bartlett's test and Scheffe's test) as necessary. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Nucleofection allows high-efficiency transfection of primary mature hepatocytes

As a first step, we compared the transfection efficiencies of nucleofection with other non-viral transfection techniques, namely Lipofectamine 2000 and FuGene 6, using pmaxGFP. Transfection efficiency was determined as the percentage of GFP-positive cells among all vital cells at 24 h after transfection. Quantitative assessment revealed that transfection using nucleofection, Lipofectamine 2000 and FuGene 6 yielded 52.2 ± 3.7%, 6.3 ± 2.8% and 0% GFP-positive cells, respectively (Fig. 1A and B). GFP expression was maintained for 20 days, with a gradual decrease during that period. Cell viability was measured by MTS assay at 1, 3 and 7 days after transfection (Fig. 1C). The survival curves for each of the groups showed a similar pattern. However, the viability of nucleofected hepatocytes was significantly higher than for the other techniques. These results indicate that nucleofection is an efficient and suitable approach for gene transfer in primary hepatocytes, and accordingly in subsequent experiments we used nucleofection to achieve ectopic pancreatic gene expression in hepatocytes.

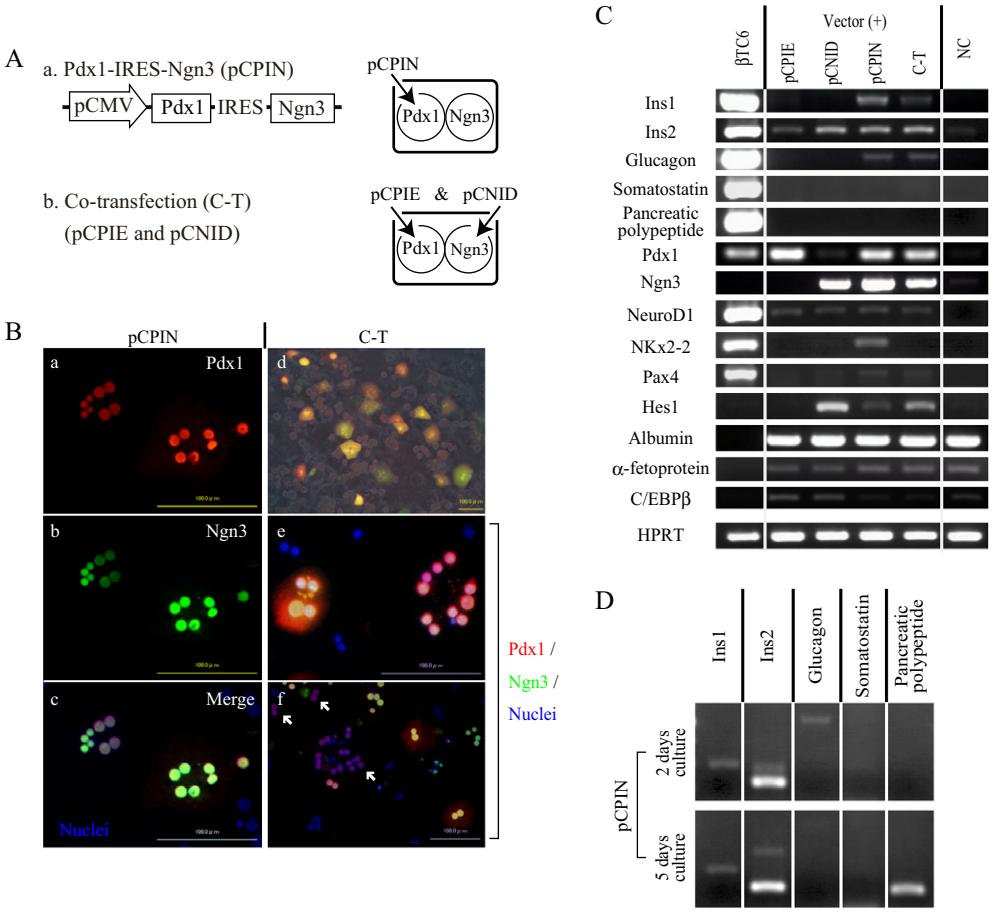

Fig. 3. Effects of co-expression of *pdx1* and *ngn3*. (A) Diagrams of bicistronic plasmids and schema of the transfection patterns. (B) Live images and immunofluorescence staining of transfected hepatocytes. In the C-T group, some of the co-transfected cells failed to coexpress Pdx1 and Ngn3 (arrow) (Fig. 3B-f). (C) Gene expression profiles of transfected hepatocytes. (D) Time-course analysis of gene expression in pCPIN-transfected hepatocytes.

Ecotpic expression of pdx1 or ngn3 alone is insufficient to induce reprogramming of mature hepatocytes

As an inducer of liver-to-pancreas reprogramming, we focused on two transcription factors, Pdx1 and Ngn3. Pdx1 is a master regulator of pancreas development, and Ngn3 is expressed in endocrine progenitors and is recognized as a key transcription factor for islet generation [13]. We constructed two IRES-dependent bicistronic expression plasmids, pCMV-Pdx1-IRES-EGFP (pCPIE) and pCMV-Ngn3-IRES-DsRed (pCNID) (Fig. 2A). When primary hepatocytes were transfected with these vectors, we detected the fluorescences of the corresponding EGFP or DsRed within 20 h after transfection (Fig. 2B-a and c). Immunofluorescence staining also demonstrated the expression of Pdx1 or Ngn3 (Fig. 2B-b and d). Western blotting detected the 47-kDa band of the Pdx1 protein and the 23-kDa band of the Ngn3 protein (data not shown). Transfection efficiencies were almost the same in case of pmaxGFP (45.5 ± 9.1% and 48.6 ± 6.1%, respectively). Next, to analyze the effect of ectopic gene expression, expression of the insulin gene was determined by semi-quantitative RT-PCR (Fig. 2C). In these hepatocytes, expression of insulin II (Ins2) was detected, but not insulin I (Ins1). The Ins2 gene is expressed in the developing brain and yolk sac as well as in pancreatic β-cells [14], whereas Ins1 gene expression is restricted to β-cells, suggesting that the expression of *pdx1* or *ngn3* alone is insufficient to induce specific reprogramming. Therefore we selected another strategy, co-expression of *pdx1* and *ngn3*.

Co-expression of pdx1 and ngn3 efficiently activates liver-to-pancreas reprogramming

To assess the effects of co-expression, we selected two approaches, namely bicistronic expression using IRES (pCPIN, pCMV-Pdx1-IRES-Ngn3) or independent co-transfection (C-T) (Fig. 3A). With IRES-dependent co-expression, the expression of Pdx1 and Ngn3 was mostly visualized in the same nucleus (Fig. 3B-a, b, c). However, in the C-T group, their expressions were observed independently, and co-localization was recognized in about 60.4 ± 16.0% of the transfected cells (Fig. 3B–d, e, f).

We analyzed further the molecular events occurring in nucleofected hepatocytes using semi-quantitive RT-PCR (Fig. 3C). Ins2 was expressed in all transfected hepatocytes, as was the case for sole expression of *pdx1* or *ngn3*. In contrast, expression of Ins1 was observed only in the pCPIN and C-T groups, in which both *pdx1* and *ngn3* were introduced. Glucagon was also expressed only in these groups. Somatostatin and pancreatic polypeptide were not detected in any of the transfected groups. In addition, we analyzed the expression of genes specific to the developing pancreas or liver. NeuroD1 was expressed in all of the transfected groups except negative control. On the other hand, expression of NK2 transcription factor-related locus 2 (Nkx2-2) and paired box gene 4 (Pax4) was restricted to the pCPIN group, in which Pdx1 and Ngn3 were expressed simultaneously. Hairy and enhancer of split 1 (Hes1), a negative regulator of Ngn3, was expressed in *ngn3*-overexpressing cells, and a similar result was obtained in the C-T group. Among li-

ver-specific genes, although expression of albumin and α-fetoprotein was retained in all groups, the expression of CCAAT/enhancer-binding protein β (C/EBPβ), whose suppression indicates hepatic dedifferentiation [15], was diminished only in the co-expressing groups. Furthermore, in the pCPIN group, the expression of Ins1 and Ins2 mRNA was maintained at 5 days after transfection when, interestingly, *de novo* expression of pancreatic polypeptide also appeared (Fig. 3D). These results suggest that nucleofection with the Pdx1 and Ngn3 bicistronic expression vector activated an endocrine reprogram in mature hepatocytes.

The bicistronic expression approach allows reprogramming to β-like cells

In order to confirm the production of endogenous insulin, we measured intracellular C-peptide levels in each of the transfected groups and compared them (Fig. 4A). Cells in which *pdx1* or *ngn3*, or both, had been introduced showed significantly higher levels of C-peptide than plasmid-free transfected hepatocytes used as a negative control. In particular, the level of C-peptide in pCPIN-transfected cells was significantly higher than in other groups. The insulin content of the cells in the pCPIN-nucleofection group was also significantly (8-fold) higher than in the negative control (Fig. 4B). To test the functional ability of pCPIN-transfected hepatocytes, we quantified the glucose dependency of insulin secretion after treatment with 5 mM or 25 mM glucose using an insulin ELISA kit (Fig. 4C). The level of insulin secretion increased along with the glucose dose. These results indicated that hepatocytes simultaneously expressing Pdx1 and Ngn3 acquire characteristics similar to those of pancreatic β-cells.

Discussion

The reprogramming of hepatocytes into pancreatic β-cells would provide a renewable source for cell therapy in diabetic patients. Here we demonstrated that nucleofection is a very efficient system for introduction of foreign DNA into mature hapatocytes. Furthermore, we showed that mature hepatocytes can be reprogrammed to pancreatic fate by transient co-expression of *pdx1* and *ngn3*. Using a bicistronic approach to introduce *pdx1* and *ngn3* into mature hepatocytes, we generated β-like cells that showed elevated cytoplasmic C-peptide content and insulin-secreting ability.

The Nucleofector™ technology is the first highly efficient non-viral gene transfer method that can be used for primary cells and cell lines that are difficult to transfect [16]. With a combination of specialized solutions and specific electrical parameters, this electroporation-based technology has beneficial features for transfection of slowly dividing cells such as primary cells, including hepatocytes. By using this method, we achieved a much higher transfection efficiency compared to other non-viral transfection procedures. Although the cell survival rate after gene delivery appeared to be relatively low, nucleofected hepatocytes showed higher viability in our experiments than untransfected cells (Fig. 1C). These results indicated that nucleofection is a suitable method for *ex vivo* gene transfer of primary hepatocytes, and that pre-transplant *ex vivo* nucleofection is a potentially useful technique for introducing selected transgenes without producing harmful effects.

Many attempts have been made to establish successful induction of islet neogenesis in the liver using viral-mediated delivery

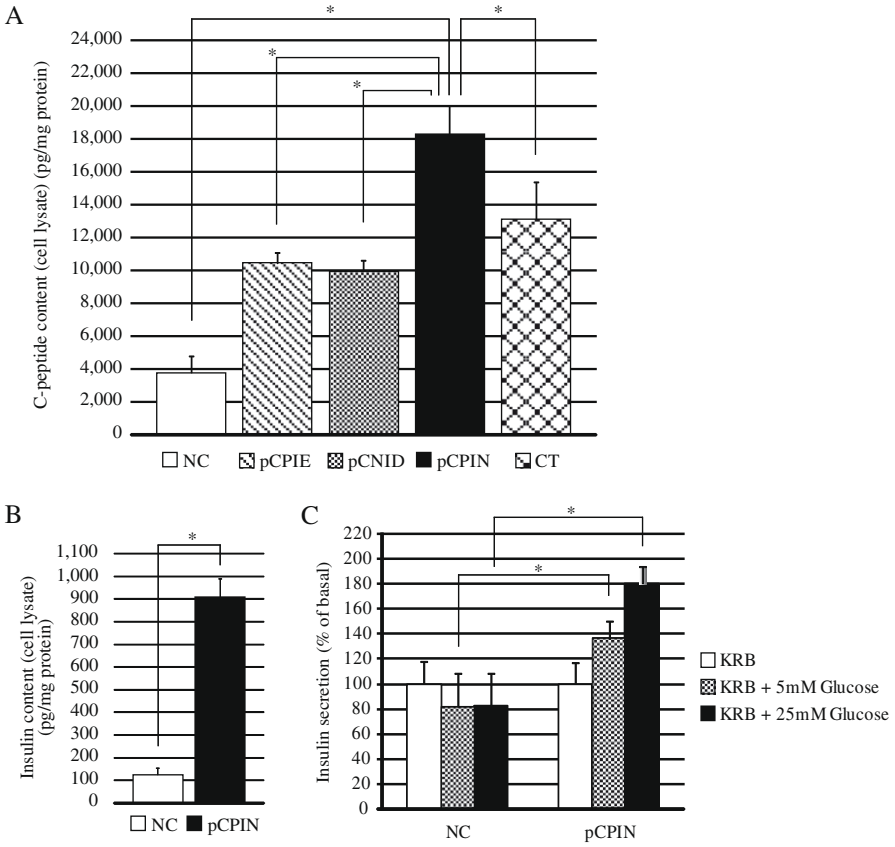

Fig. 4. Analysis of insulin biosynthesis and secretory ability. (A) Intracellular C-peptide content of transfected and untransfected hepatocytes ($n = 3$). The C-peptide levels in the negative control and transfection groups were assessed on day 2 after nucleofection. $^{*}P < 0.05$. (B) Intracellular insulin content in the pCPIN and NC groups at 2 days of culture ($n = 3$). $^{*}P < 0.05$. (C) Measurements of insulin secretion in the pCPIN and NC groups. Data represent the mean ± SD for three different experiments expressed as the percentage increase in insulin release relative to cells incubated in buffer without added glucose. $^{*}P$ value < 0.05 relative to NC incubated with 5 mM or 25 mM glucose.

of pancreatic transcriptional factors. Some studies investigating the effects of exogenous pancreatic transcriptional factors in the liver have demonstrated that Pdx1 alone was able to initiate, but not complete, the conversion process [6,7]. Consistent with those results, our data indicate that simultaneous induction of Pdx1, together with Ngn3, is necessary for reprogramming mature hepatocytes to an islet cell fate. More recently, it was shown that a small number of transcription factors can reprogram cultured adult skin cells into pluripotent stem (iPS) cells. Indeed, recent work on iPS cells suggests that a specific combination of multiple factors, instead of a single factor, might be the most effective way to reprogram adult cells. Melton et al. reported that a specific combination of three transcriptional factors (Ngn3, Pdx1 and MafA) can reprogram differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β-cells using an adenoviral delivery method [17]. This study showed that cellular reprogramming using defined factors might be feasible without reversion to a pluripotent stem cell state. Although it is not clear why these particular combinations are sufficient for adult β-cell reprogramming, we showed that a combination of *pdx1* and *ngn3* was able to directly reprogram hepatocytes into islet cells *in vitro* using non-viral methods. On the light of the results by Melton and colleagues [17], introduction of MafA in our vector might enhance the effect of reprogramming in our experiment. Although we have demonstrated the efficacy of a bicistronic approach using IRES, it has been reported that care should be taken with regard to the decreased capacity of IRES-dependent downstream gene expression [18], and therefore use of another multicistronic expression vector, such as the 2A segment of the foot-and-mouth disease virus [19], might be an interesting approach. Further investigation will be required to clarify the most suitable combinations of transcription factors for reprogramming mature hepatocytes into β-like cells.

In summary, simultaneous ectopic expression of Pdx1 and Ngn3 efficiently induces liver-to-pancreas reprogramming of mature hepatocytes, and the use of viral vectors is not indispensable for induction of such reprogramming in mature somatic cells. Our findings provide valuable information for the development of therapeutic strategies against various intractable diseases, including diabetes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.146.

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