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Original Article

Pancreatic and duodenal homeobox gene 1 (*Pdx1*) down-regulates hepatic transcription factor 1 alpha (*HNF1α*) expression during reprogramming of human hepatic cells into insulin-producing cells

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Abstract: Ectopic expression of Pdx1 triggers rapid hepatocyte dedifferentiation by down-regulating liver-enriched transcription factors and liver-specific functional genes such as hepatic nuclear factor-1α (HNF1α), albumin, and AAT. However, the links between Pdx1 over-expression and hepatic gene down-regulation are incompletely understood. HNF1α and HNF4α are important transcription factors that establish and maintain the hepatocyte phenotype. The human HNF4α gene contains two promoters (P1 and P2) that drive expression of P1-(HNF4α 1-6) or P2-(HNF4α 7-9)-derived isoforms, which are used in different tissues and at different times during development. We hypothesized that the relative expression of HNF1α and HNF4α following ectopic Pdx1 expression may promote hepatic cell dedifferentiation and transdifferentiation toward pancreatic beta-cells. We produced lentiviruses expressing Pdx1, Pdx1-VP16, and Ngn3, along with dual-color reporter genes to indicate hepatic and pancreatic beta-cell phenotype changes. Using these PTF alone or in combinations, we demonstrated that Pdx1 not only activates specific beta-cell genes but down-regulates HNF1α. Pdx1-mediated reduction of HNF1α is accompanied by altered expression of its major activator, HNF4α isoforms, down-regulating hepatic genes ALB and AAT. Pdx1 up-regulates HNF4α via the P2 promoter. These P2-driven isoforms compete with P1-driven isoforms to suppress target gene transcription. In Huh7 cells, the AF-1 activation domain is more important for transactivation, whereas in INS1 cells, the F inhibitory domain is more important. The loss and gain of functional activity strongly suggests that Pdx1 plays a central role in reprogramming hepatocytes into beta-cells by suppressing the hepatic phenotype.

Keywords: Pdx1, Ngn3, Pdx1-VP16, reprogramming, transdifferentiation, human hepatic cells, Huh7 cells, insulin-producing cells (IPCs), HNF1α, HNF4α

Introduction

One approach for safe long-term control of blood glucose in patients with type 1 diabetes (T1D) is to establish an effective source of patient-specific autologous insulin-producing cells (IPCs) for transplantation. However, differentiated or mature beta cells cannot be efficiently expanded *in vitro* [1]. One of the most successful approaches for long-term treatment is islet cell transplant therapy, but its application is severely limited by the lack of donor tissue and the requirement of toxic immunosuppressant [2-7]. Cellular reprogramming has the potential to avoid these limitations by converting and expanding patients' own tissues *in vitro* into the needed functional tissues [2-7]. Reprogramming studies for treatment of diabetes have mainly focused on using the liver [8-19] as a tissue source due to its high level of regenerative capacity [20] and plasticity [8, 10, 16], and common developmental kinship with the pancreas [21]. The liver and pancreas share a strikingly similar gene expression profile including expression of many specific transcription factors [22, 23] and both tissues are responsive to changes of blood glucose [24, 25]. Most studies by forced expression of key

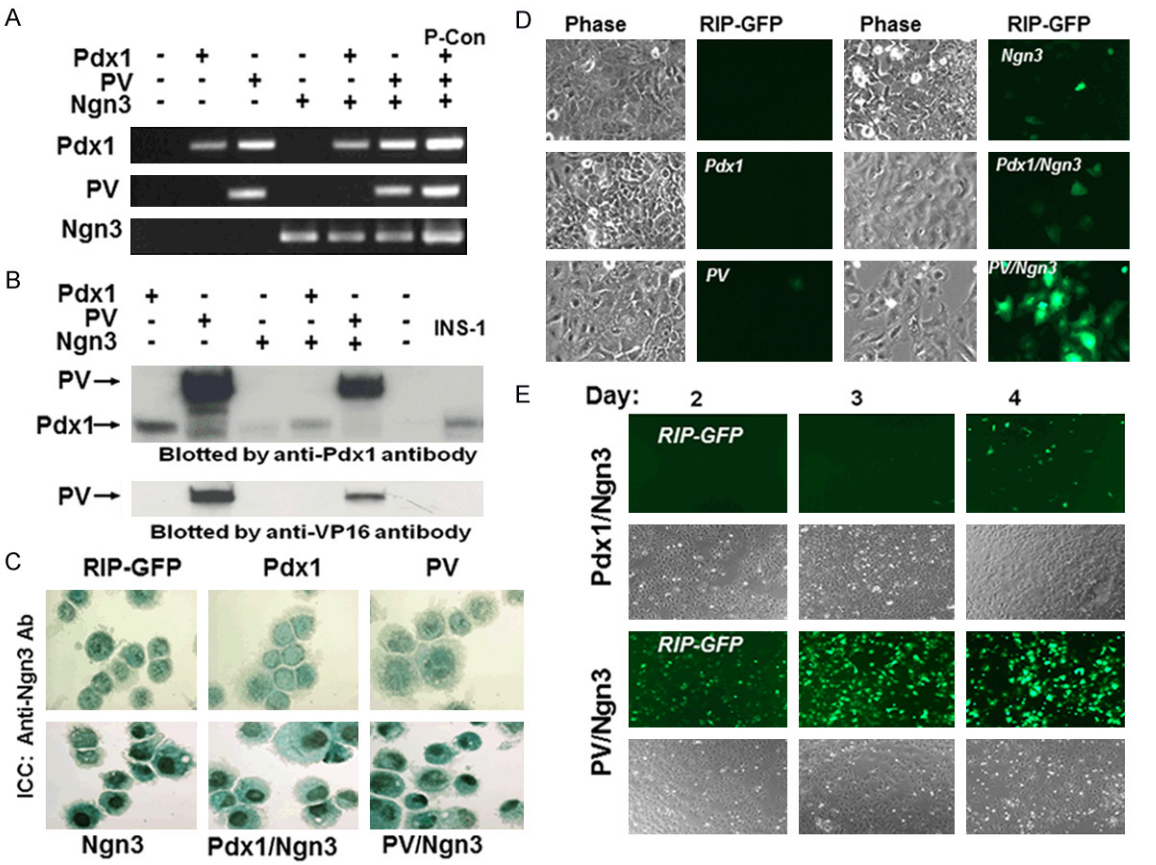

Figure 1. Confirmation of LV-PTF gene expression by RT-PCR (A), Western blotting (B), and immunocytochemistry (C) and activation of rat insulin I gene in Huh7 cells (D&E). (A) Total RNA was collected from Huh7 cells following transduction with LV expression vectors for *Pdx1, Pdx1-VP16, Ngn3,* or combinations as indicated. Indicated gene expression was measured by RT-PCR. (B) Huh7 cells were transduced with expression vectors for *Pdx1, Pdx1-VP16, Ngn3,* or combinations as indicated. Following transduction, Huh7 cells were scraped off and lysed in RIPA buffer. Equal amounts of cell lysate were separated by SDS-PAGE and immunoblotted with polyclonal antibodies against *Pdx1 or VP16* as indicated. INS-1 cells were used as a control for *Pdx1*. (C) Huh7 cells were transduced with expression vectors for *Pdx1, Pdx1-VP16, Ngn3,* or combinations as indicated. Following transduction, Huh7 cells on cytospin slides were fixed and stained with Anti-*Ngn3* antibody by ICC. (D) Huh7 cells were transduced with LV-*RIP-GFP* reporter alone or with *Pdx1, Pdx1-VP16, Ngn3, Pdx1/Ngn3, or Pdx1-VP16/Ngn3* expression LVs for 4 days. Expression of RIP-*GFP* was observed and photographed at the day 4 or (E) at different days (2, 3 and 4) following Huh7 cells transduced with the combination either LV-*Pdx1/Ngn3* or LV-*Pdx1-VP16/Ngn3* under fluorescence microscopy.

pancreatic transcription factors (PTFs), either alone or in combination, delivered into hepatic cells by a variety of means in addition to external factors such as glucose and nicotinamide have demonstrated PTF-directed hepatic progenitors to differentiate into IPCs [14, 16, 17].

The wide variety of differentiated tissues that arise in a developing organism are due to selective expression and/or suppression of certain sets of transcription factors that regulate the downstream gene expression in a given cell type [26-28]. Much focus has been placed on activation of the PTF to obtain beta cell phenotype that is necessary to generate functional IPCs. However, how the PTFs-mediated inactivation of the host hepatic gene program has not been well studied and is arguably of equal importance. The liver plays a major role in metabolism, glycogen storage, plasma protein synthesis, hormone production, and detoxification. Ectopic expression of *Pdx1* triggers rapid hepatocyte dedifferentiation by down-regulating several liver-enriched transcription factors and liver-specific functional genes such as hepatic nuclear factor-1α (*HNF1α*), albumin (*ALB*), alcohol dehydrogenase-1β (*ADH1β*), glucose 6-phosphatase (*G6PC*), glutamate synthase (*GLUL*), α-1-antitrypsin (*AAT*), and hexokinase 2 (*Hxk 2*) at the mRNA and protein levels

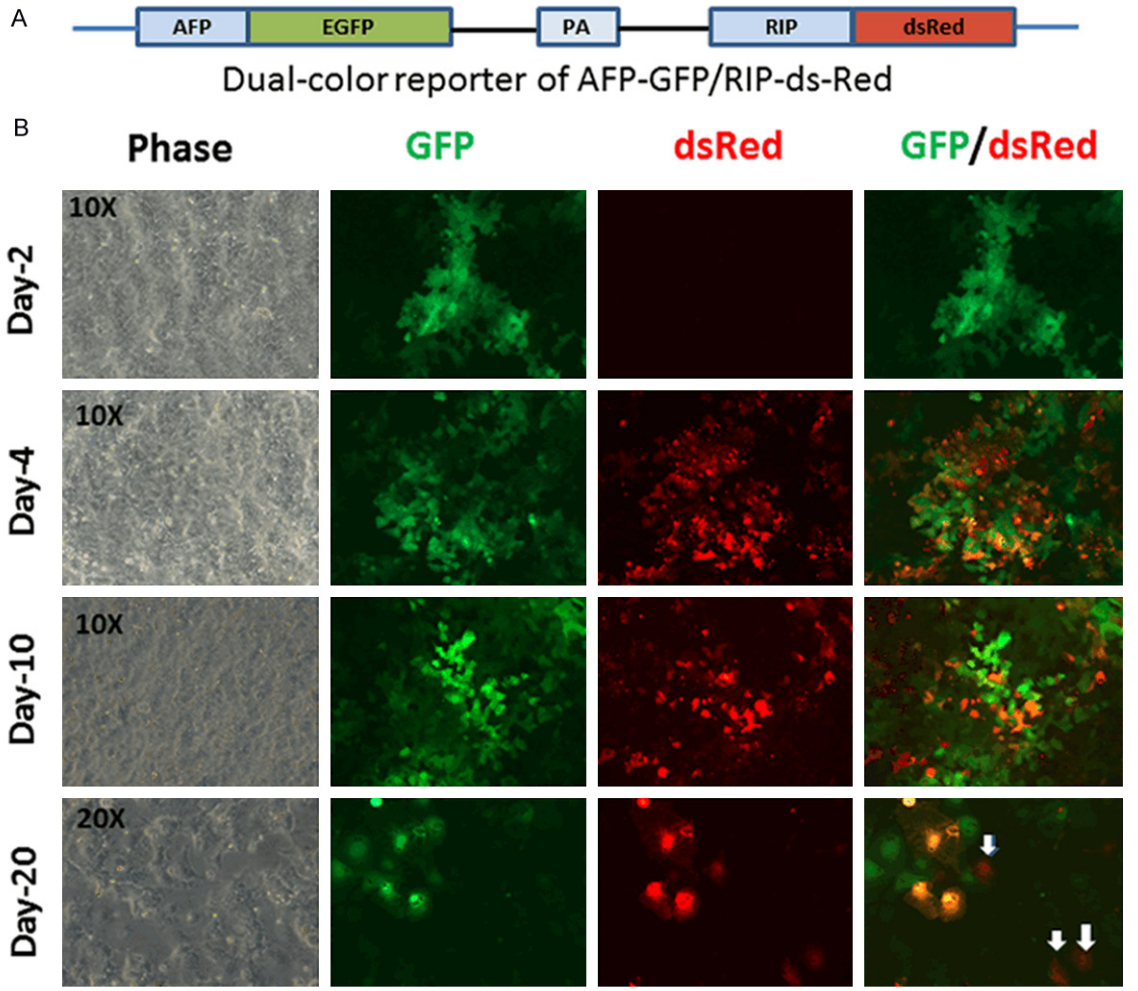

Figure 2. Identification of insulin producing cells by using a dual-color reporter system. A. Schematic picture of the dual-color reporter system that contains a human alpha fetal protein promoter (AFP) driving expression GFP and a rat insulin promoter (RIP) driving expression of dsRed (*AFP-GFP/RIP-dsRed*). B. Time-dependent activation of *AFP-GFP/RIP-dsRed* reporter genes following Huh7 cells transduced by lentiviruses encoding *Pdx1, Ngn3* and the dual-color reporter. Cells of red fluorescence only as indicated by white arrows at 20 days are fully transdifferentiated insulin-producing cells.

[29, 30]. However, the molecular links between *Pdx1* over-expression and down-regulation hepatic genes are not entirely understood.

HNF1α and *HNF4α* are important liver-enriched transcription factors with an important role in establishing and maintaining the hepatocyte phenotype [22, 23, 31]. *HNF1α* and *HNF4α* regulate the expression of hundreds of downstream target genes in both hepatocytes and beta cells [22] and the expression of specific isoforms, regulated by alternative splicing, differs significantly between hepatocytes and beta cells [22, 23, 32]. The human *HNF4α* gene contains two promoters (P1 and P2) that drive the expression of P1-derived isoforms (*HNF4α* 1-6) or P2-derived isoforms (*HNF4α* 7-9) by alternative splicing and usage of different promoters [33], which are used in different tissues and at different times during development. *HNF4α* P2-isoforms are exclusively detected in adult pancreatic islets and positively regulated by *Pdx1*, *HNF1β* and *HNF1α* [32-34]. In contrast, P1-derived isoforms are most abundant in adult hepatic tissues with relatively low levels of P2 isoforms [32, 34, 35]. Due to the tissue-specific expression of *HNF4α* in hepatocytes, *HNF1α* is regulated mainly by *HNF4α* P1 isoforms; in contrast it is regulated by P2 isoforms in beta cells. Previous studies indicate

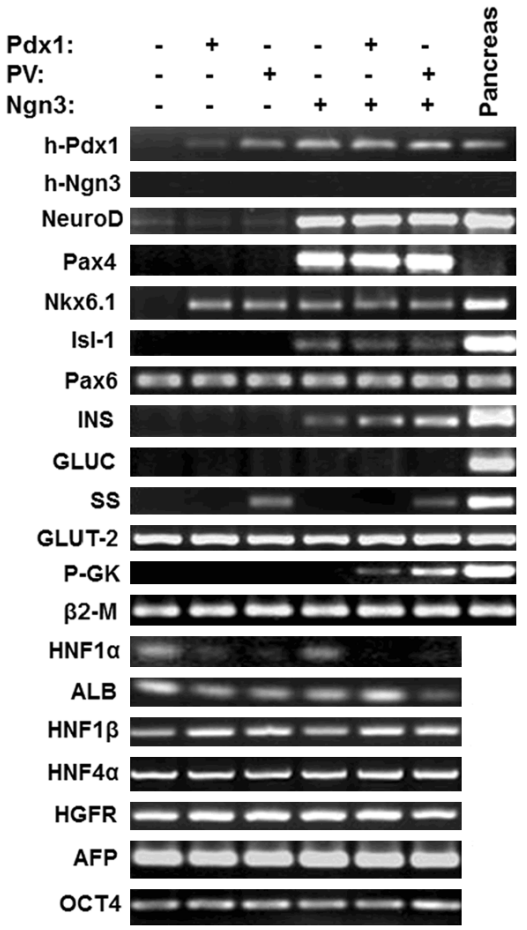

Figure 3. RT-PCR analysis of PTFs and liver specific gene expression induced by various LV-PTF treatments. Huh7 cells were treated with LV-PTFs as indicated for 96h. The total RNA was isolated and reverse transcribed into cDNA. Equal amounts of each cDNA sample were used for RT-PCR analysis with gene specific primers.

that P2 isoforms transactivate *HNF1α* more weakly than P1 isoforms [36, 37]. Differences in the relative expression of the *HNF1α* and *HNF4α* isoforms following ectopic expression of *Pdx1* may be responsible for hepatic cell dedifferentiation and transdifferentiation toward pancreatic beta cells.

To successfully create lasting beta cell surrogate applicable to the treatment of patients with T1D, the key events involved in the beta cell reprogramming process, especially the under-studied parallel process-how to suppress the host cell phenotype need to be understood. In this study, we specifically focused on studying the role of *Pdx1* over-expression in human hepatocellular carcinoma cells (Huh7) in the activation of endocrine pancreatic genes and, at same time, the effects on shutting down the hepatic genes during hepatic cell reprogramming into IPCs. This work sheds insight into the mechanism of reprogramming from hepatic cells into pancreatic endocrine IPCs. Understanding the molecular and cellular events in cell type conversions may help to explain the mechanisms underlying tissue regeneration and plasticity.

Methods

Cell lines and cell culture

3T3 mouse fibroblast cells, 293 human embryonic kidney cells (ATCC cat#CRL-32610), Huh7 (JHSRRB, cat#JCRB0404, Japan), and HepG2 (Sigma, cat#85011430) human hepatocellular carcinoma cell lines were cultured in DMEM supplemented with 10% FBS, and 1% Penicillin/Streptomycin in a 37°C incubator with 100% humidity and 5% CO_2. Huh7 is a well differentiated hepatocyte-derived cellular carcinoma cell line and is commonly used studying liver cell biology, cancer and its potential therapies. In addition, Huh7 cells are easily transduced and exhibit some characteristics of hepatic stem cell properties. Therefore, here we chose Huh7 cells as our model cell line for this study. Rat insulinoma (INS1) cells were cultured in RMPI 1640 supplemented with 200 mM L-glutamine, 100 mM sodium pyruvate, 2-mercaptoethanol, and 1% Penicillin/Streptomycin in the same incubator.

Construction of plasmids

The human *Pdx1* expression plasmid (pCMV6-XL5) was purchased from Origene. The mouse *Pdx1* expression plasmid was constructed by insertion of mouse *Pdx1* cDNA into the *BamHI/XbaI* site of the *pCDNA3* vector (Invitrogen). The truncated mouse *Pdx1* expression vectors were constructed by introduction of a stop codon at the amino acid positions of 121, 161, and 201, using the standard site-directed mutagenesis method. The *HNF4α2* expression plasmid (pCMV-Sport6) was purchased from Open Biosystems. Exon 1A was removed from this plasmid and it was replaced with exon 1D in order to construct the *HNF4α8* expression plasmid. The *HNF4α3* and *HNF4α9* expression plasmids were constructed by modifying the

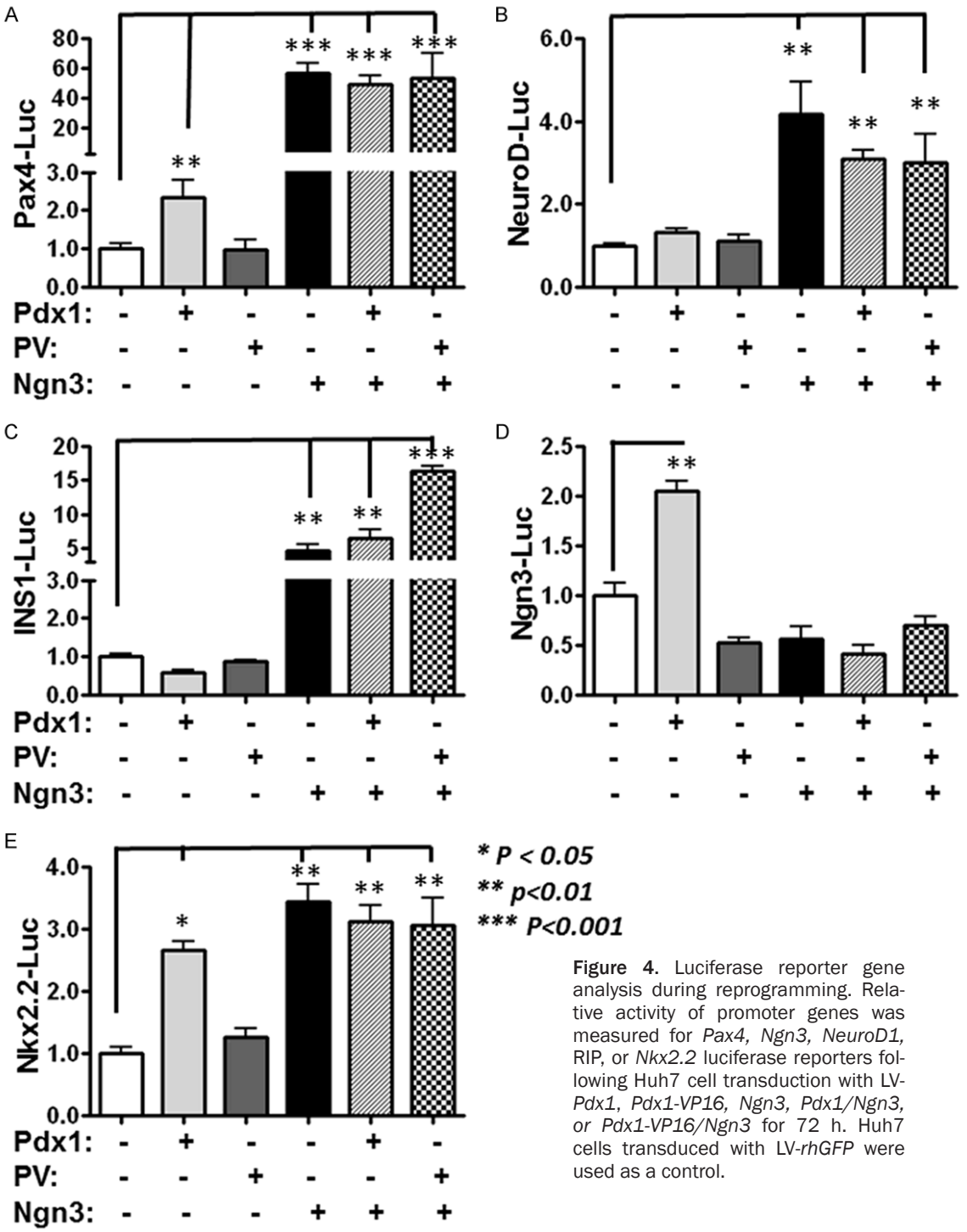

Figure 4. Luciferase reporter gene analysis during reprogramming. Relative activity of promoter genes was measured for *Pax4, Ngn3, NeuroD1,* RIP, or *Nkx2.2* luciferase reporters following Huh7 cell transduction with LV-*Pdx1*, *Pdx1-VP16, Ngn3, Pdx1/Ngn3, or Pdx1-VP16/Ngn3* for 72 h. Huh7 cells transduced with LV-*rhGFP* were used as a control.

HNF4α2 and HNF4α8 plasmids respectively by introducing a stop codon at the 3' end of exon 8 (note: The *HNF4α3* and *HNF4α9* expression plasmids code for proteins lacking the 41 amino acids from the extended exon 8). The pRL-TK expression vector was purchased from Promega. The mouse *HNF1α* luciferase reporter was constructed as previously described) [38]. The rat insulin I promoter (RIP) luciferase reporter was constructed by removing the TK promoter from the pRL-TK plasmid and cloning the RIP promoter into this site using the *BglII/*

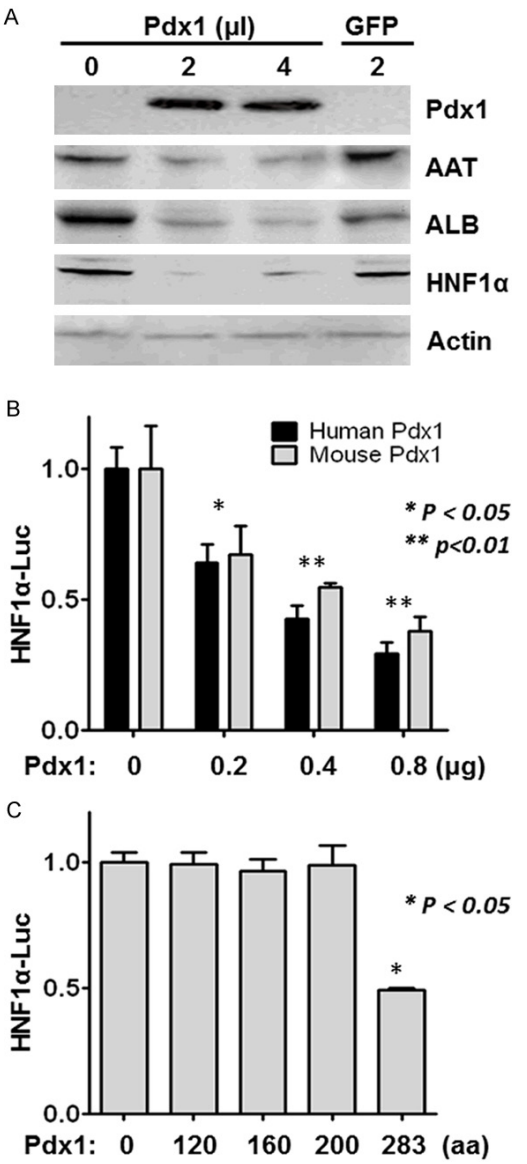

Figure 5. Down-regulation of *HNF1α, ALB,* and *AAT* in Huh7 cells over-expressing *Pdx1*. A. Huh7 cells were transduced with or without LV-*Pdx1* or LV-*rhGFP* for 48 h. Equal amounts of Huh7 cell lysate were separated by SDS-PAGE and immunoblotted with polyclonal antibody against *Pdx1, HNF1α*, *ALB, AAT,* or β-actin. B. Huh7 cells were transfected with 1.0 μg of *HNF1α*-luciferase reporter plasmid and 0.2-0.8 μg *Pdx1* expression plasmid as indicated, with pcDNA3 used as a DNA quantity control. C. Huh7 cells were transfected with 1.0 μg of *HNF1α*-luciferase reporter plasmid and 0.8 μg of each truncated *Pdx1* (120, 160, or 200 amino acids) or full length (283 amino acids), along with *pcDNA3* as a DNA quantity control. All experiments were done in triplicate for three times.

HindIII restriction sites. The *Pax4, Ngn3, NeuroD1, and Nkx2.2* luciferase reporters (pFOXluc) were generous gifts from Dr. Michael German, the University of California, San Francisco, CA.

Lentiviral vector preparation, titration, and transduction

The insulin I promoter driving the green fluorescence protein (*RIP-GFP*) reporter was constructed as previously described [17]. The lentiviral vector (LV) containing the mouse *Pdx1-VP16* fusion gene was constructed as previously described [17, 39]. The LV containing *rhGFP,* and mouse *Pdx1,* were constructed as previously described [14]. The LV containing mouse *Ngn3* was constructed by inserting the cDNA of mouse *Ngn3* into the pTYF vector cassette under the control of the elongation factor-1 alpha (eEF-1α) promoter. The LV containing dual promotors of AFP-GFP/RIP-DsRed were constructed with similar approaches [17]. LVs were generated and titrated as previously described [40-42]. For lentiviral transduction, Huh7 cells were transduced with different virus as indicated at a multiplicity of infection (MOI) of 10 in the presence of 10 μg/ml polybrene for 24 hours. The transduction efficiency was monitored by transducing Huh7 cells with LV encoding *rhGFP*. The insulin promoter activity in Huh7 cells was detected by observing *RIP-GF*P expression under fluorescence microscopy.

Western blotting and immunocytochemistry (ICC)

Equal amount of cell lysates was loaded on 10% SDS-PAGE and the proteins were transferred to nitrocellulose membrane. Western blotting was performed using primary antibodies to either PV or *Pdx1* as previously described [14]. For ICC, cultured cells were released by trypsin digestion and 10,000 cells/cytospin slides were prepared by centrifugation. Cells were stained with rabbit polyclonal antibodies to *Ngn3* at a dilution of 1:1000 for 2 hours at room temperature (generous gift from Dr. Michael German) and followed by goat-anti-rabbit secondary antibody linked with HRP and visualized with DAB.

RT-PCR and real time RT-PCR analysis

Total RNA was extracted from Huh7 cells transduced with different combinations of PTFs or from human islets (generous gift from Dr. Xiaoping Deng at The University of Pennsylvania) using Trizol according to the manufacturer's

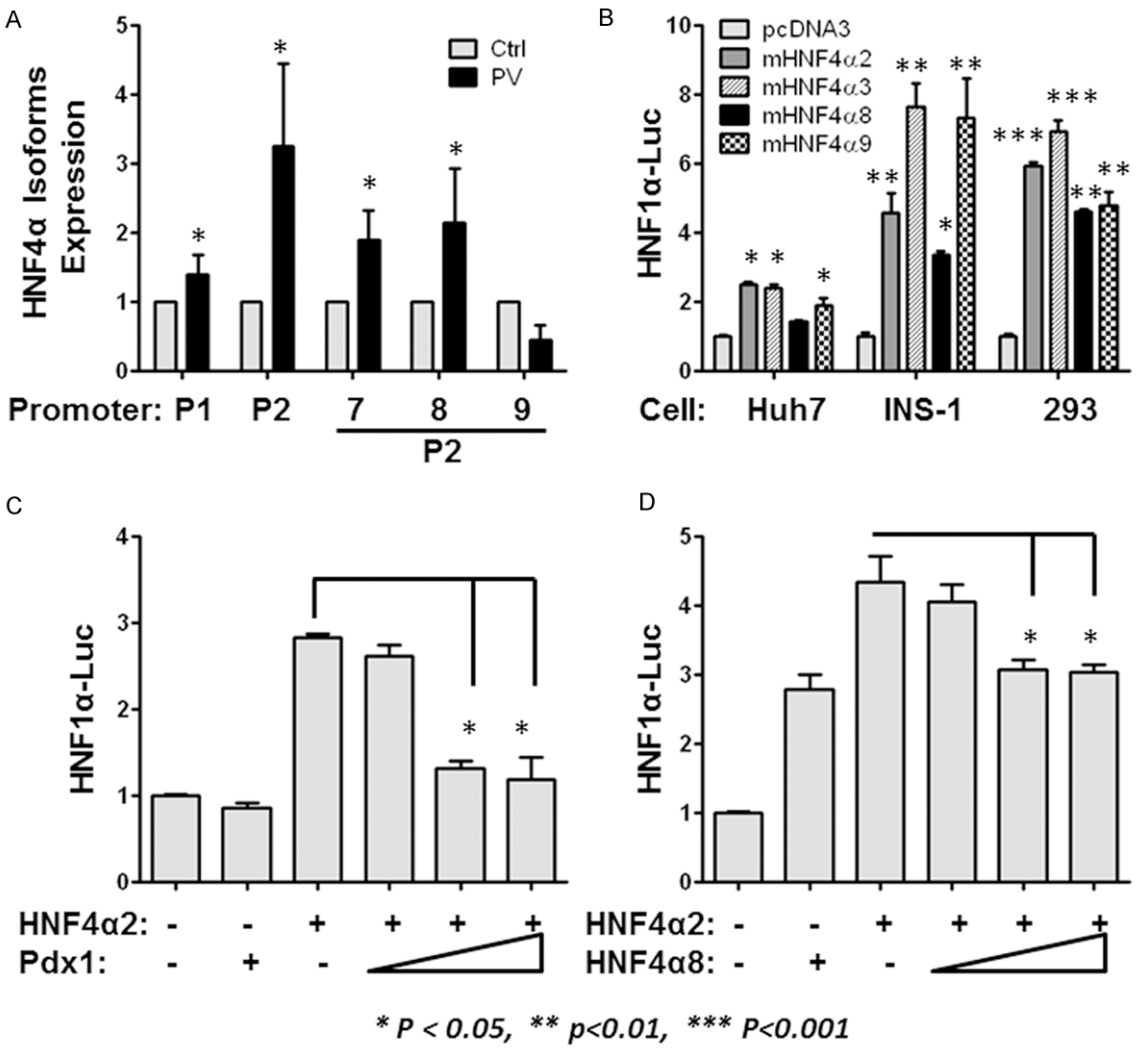

Figure 6. *Pdx1* increases expression of *HNF4α* P2 transcripts that compete with P1 transcripts. A. Total RNA was collected from Huh7 cells following transduction with LV-*Pdx1-VP16* or control (LV-*GFP*). Indicated gene expression was measured by real time RT-PCR. Primers were designed to detect general products of *HNF4α* from the P1 promoter (Exon 1A) or the P2 promoter (Exon 1D) as well as the three *HNF4α* P2 transcripts (7, 8, & 9). B. Relative activity was measured for the mouse *HNF1α*-luciferase reporter (1 μg/μl/well) following transfection of various *HNF4α* isoforms (1 μg/μl/well) in Huh7, INS1, and 293 cells. Activity was normalized in each cell type to the *pcDNA3* control. C. Relative activity was measured for the mouse *HNF1α*-luciferase reporter following transduction of *HNF4α2* and *Pdx1* alone, and in combination. D. Relative activity was measured for the mouse *HNF1α*-luciferase reporter following transfection of *HNF4α2* and *HNF4α8* alone, and in combination. + indicates 1 μg/μl/well and the concentration gradient shows three concentrations (0.1 μg/μl, 0.5 μg/μl, and 1.0 μg/μl)/well.

protocol. Gene expression was detected by RT-PCR. The forward and reverse PCR primers were designed (IDT Technologies) to be located in different exons. Amplification was performed for 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and 72°C for 7 min. The PCR products were separated in 2% agarose gels by electrophoresis in TAE buffer. Digital images were captured and analyzed with a Quantity One Imager (BioRad). All of the PCR products were confirmed by Big-Dye DNA sequence analysis in an ABI 377 sequencer (Global Medical Instrumentation, Inc.) following the manufacturer's protocol. Real-time RT-PCR was performed on selected samples, collected as described above, using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Primers were designed to detect *HNF4α* P1 products (exon 1A) or *HNF4α* P2 products (exon 1D) as well as

the C-terminal modifications by detecting exon 9, exon 9+, or exon 8+. Primer sequences are available upon request.

Transfections and luciferase assay

Cells were cultured as previously indicated in 12-well plates and transfected with 0.1-1.0 μg/well DNA (as indicated) using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. 0.02 μg/well TK-Luc plasmid was used as a transfection control in all experiments. Cell lysates were harvested and measured 24 hours post transfection using the Dual Luciferase Reporter Kit (Promega) according to manufacturer's protocol except that only 50 ul of each substrate reagent was used (as optimized by our lab) to read samples using a Lumat LB 9507 Luminometer (Berthold Technologies). All luciferase assays were done in triplicate.

Statistical analyses

Statistical analyses were performed with a 2-sample Student's test assuming equal variances. Statistical significance was determined by requiring a *P* value of less than 0.05 for the data to be considered statistically significant.

Results

Pdx1-VP16 and Ngn3 together strongly induced insulin promoter activity

We first assessed if our LVs designed to express PTFs were functional. Transgene expressions at gene and protein levels were confirmed by RT-PCR, Western blotting, and ICC (**Figure 1A-C**). In order to online monitor the activation of the insulin gene during the reprogramming of hepatic cells towards IPCs, a LV-*RIP-GFP* was used as indicator. The LV transduction efficiency of Huh7 cells was determined by LV-mediated expressing *hGFP* driven by the *EF1α* promoter, a constitutive promoter without cell specificity. We observed more than 99% of transduced Huh7 cell expressing *GFP* at day 2 (data not shown). *RIP-GFP* was transduced into Huh7 cells, along with *Pdx1*, *Pdx1-VP16*, *Ngn3*, or with combinations of *Pdx1/Ngn3* or *Pdx1-VP16/Ngn3* as indicated. At 96 hours of transduction, expression of *GFP* was examined (**Figure 1D**). Green fluorescence was observed in less than 2% of Huh7 cells transduced with *RIP-GFP* only; indicating that insulin was not expressed in the Huh7 cell line. Green fluorescence was observed in rare Huh7 cells transduced with single gene *Pdx1, Pdx1-VP16,* or *Ngn3.* Therefore, *Pdx1, Pdx1-VP16,* or *Ngn3* alone was not sufficient to activate the insulin gene at day 4 post-transduction. Green fluorescence was observed in nearly 50% of Huh7 cells transduced with *Pdx1/Ngn3* and in more than 90% of Huh7 cells transduced with *Pdx1-VP16/Ngn3.* This result is consistent with our previous studies that the insulin promoter can be more effectively activated by *Pdx1-VP16*, a super active form of *Pdx1* [14, 17].

To further compare the efficiency of *Pdx1* and *Pdx1-VP16*-mediated activation of insulin gene, *GFP* expression was observed at 48, 72, and 96 hours after Huh7 cells were transduced with *Pdx1/Ngn3* or *Pdx1-VP16/Ngn3* (**Figure 1E**). The results showed that *Pdx1-VP16* was much more effective than *Pdx1* in activating *RIP-GFP*. Green fluorescence was observed in 50% of Huh7 cells transduced with *Pdx1-VP16/Ngn3* at day 2 compared with only 2% of cells treated with *Pdx1/Ngn3*.

Examination of the Huh-7 cell transdifferentiation progress by using a dual-color reporter system AFP-GFP/RIP-dsRed

Cell transdifferentiation means that the original cell genes have to be gradually shut-down along with the new-type of cell marker genes turned-on. To observe the progress of transdifferentiation from liver cell to IPC, we constructed plasmid of a dual-color reporter system by introducing the *GFP* reporter under control of α-fetal protein promoter and dsRed under control of rat insulin-1 gene promoter (*AFP-GFP/RIP-dsRed*) (**Figure 2A**) and produced its lentivirus that was used for simultaneously observing the liver-to-IPC transdifferentiation process. Using this dual-color reporter, liver cells would exhibit only green fluorescence, whereas IPCs would exhibit red fluorescence, and the intermediate phase cells would exhibit both red and green (yellow) fluorescence. When Huh-7 cells were transduced *with LV-Pdx1-VP16* and *LV-Ngn3,* along with the dual-color reporter, at day 2 the cells only displayed green fluorescence. At day 4 to 10, approximately half of transduced cells (green cells) produced red fluorescent protein and the other cells remained

green color (**Figure 2B**), indicating that the activation of the insulin gene was not equivalent to a complete cell transdifferentiation. After 20 days, the cell morphology started to change into smaller size. In the transduced cells, 45-50% of cells remained as liver cells (green cells), 25-30% of the cells displayed yellow cells, and 20-25% of the cells displayed only red cells. Therefore, this dual-color reporter system allowed us to observe a dynamic progression of transdifferentiation of hepatic cells into pancreatic IPCs, as it occurred as early as day 4, and became more dominant in one or the other phenotype after 20 days post transgene expression, with an estimated transdifferentiation efficiency of 20-25%. However, we cannot exclude that the low efficiency was caused by unequal transduction of the three lentiviruses. Compared to Huh-7 cells, the ability of transdifferentiation of HepG2 cells was much lower (Figure S1), likely due to low efficiency of cell transduction by lentivirus.

Pdx1 and Ngn3 induced gene expression related to endocrine pancreas

Given the limitations imposed by the chromatin structure on endogenous genes, next we questioned whether the activation of the *RIP-GFP* reporter might be applicable to the endogenous human insulin gene. As shown in **Figure 1E**, although LV transduction with *Pdx1-VP16/Ngn3* led to activation of the ectopic promoter *RIP-GFP* reporter as early as 24 hours, the activation of the endogenous human insulin gene could only be detected at 96 hours by RT-PCR (**Figure 3**). Notably, the level of activation of the human insulin gene in the cells still remained low, implying that limitations may be due to the chromatin structure of the endogenous gene or the different regulation requirements of the human and rat insulin genes.

To investigate the gene expression profile of Huh7 cells with different treatment after 96 hours, cellular RNA samples were collected and RT-PCR analysis was performed (**Figure 3**). Several endocrine pancreas functional genes were screened and it was found that endogenous expression of h-*Pdx1* and *Nkx6.1* could be activated by all treatments. *Ngn3, Pdx1/Ngn3*, and *Pdx1-VP16*/Ngn3 activated *Pax4*, *NeuroD1,* and *Isl1*, which are involved in endocrine pancreas development, consistent with previous findings that these genes are direct targets of *Ngn3* [43]. *Pdx1-VP16* alone or combined with *Ngn3* can induce expression of *somatostatin (SS).* Pancreatic type glucokinase (*GK*) was only expressed in cells transduced with *Pdx1/Ngn3* or *Pdx1-VP16/Ngn*3. No glucagon or pancreatic polypeptide was detected in any sample. Huh7 cells did have basal expression of *Pax6* and *GLUT-2,* and continued expressing hepatic marker genes such as *HNF1β, HNF4α, HGFR, AFP* and *OCT4* following all treatments. Expression of *AFP* and *OCT4* genes are consistent with Huh7 cells possessing some properties of hepatic stem cells.

Quantitative analysis of PTF promoter activity and the relationship between upstream and downstream PTFs induced by Pdx1 or Pdx1-VP16

Using the dual-luciferase assay we quantitatively analyzed the promoter activity of PTFs in the reprogrammed Huh7 cells. Huh7 cells were transduced with LV carrying *Pdx1, Pdx1-VP16, Ngn3, Pdx1/Ngn3,* or *Pdx1-VP16/Ngn3* for 72 hours and then separately transfected with PTF-luciferase reporter genes (*Pax4, Ngn3, NeuroD1, RIP,* or *Nkx2.2)*. The relative luciferase activities are shown in **Figure 4**. *Ngn3* alone can directly activate *Pax4, NeuroD1, INS1,* and *Nkx2.2* promoters. However, *Ngn3* alone failed to activate its own promoter, which seems inconsistent with the observation that *Ngn3* autoactivates its own expression via cooperation with Foxa2 *in vitro* [44]. *Ngn3* combined with *Pdx1* or *Pdx1-VP16* did not further increase *Ngn3* activation, indicating that both *Pdx1* and *Pdx1-VP16* have no synergistic effect on the activation of *Pax4*, *NeuroD1,* and *Nkx2.2* genes. *Pdx1* can activate *Ngn3* promoter, consistent with *Pdx1* as upstream factor controlling *Ngn3* expression. Previous studies have shown that a modified form of *Pdx1* carrying the *VP16* transcriptional activation domain from the herpes simplex virus more efficiently induces insulin gene expression in the human HepG2 cell line and the rat WB cell line [14, 17], but whether this attribute is a general characteristic of other PTF genes or limited only to the insulin gene was not explored. Our results showed that insulin promoter activity was about 3-fold higher in cells transduced with *LV-Pdx1-VP16/Ngn3* than in those treated with *LV-Pdx1/Ngn3.* However, the *Pdx1* modified with *VP16*

showed lower activation of *Pax4*, *Ngn3,* and *Nkx2.2* promoters when compared to *Pdx1* alone. Therefore, the addition of *VP16* restricts *Pdx1* transactivation in most cases, indicating the mechanism of activation of insulin by *Pdx1* different from activation of the *Pax4, Ngn3,* and *Nkx2.2* promoters.

Effect of Pdx1 on HNF1α promoter activity in hepatic cells

Previous studies have shown that several hepatic genes are down-regulated following over-expression of *Pdx1* in hepatic cells [29, 30]. In the current study, we have shown a down-regulation of endogenous *HNF1α* and its target genes of *ALB* and *AAT* in Huh7 cells and HepG2 cells following treatment with *Pdx1* or *Pdx1-VP16* by RT-PCR (**Figure 3** and S2) and Western blot analysis (**Figure 5A**). This prompted us to evaluate the effect of *Pdx1* on expression of *HNF1α* in hepatic cells. Huh7 cells were co-transfected with the *HNF1α* -luciferase reporter and various concentrations of human *Pdx1* or mouse *Pdx1* expression plasmid as indicated for 24 hours. Transfection of both *Pdx1* plasmids decreased *HNF1α* promoter activity in a concentration dependent manner (**Figure 5B**). With the 0.8 ug *Pdx1* expression plasmid, approximately 60% of the activity of the *HNF1α* promoter was lost. The specificity of inhibition of *HNF1α* promoter activity by *Pdx1* was supported by the fact that C-terminal truncated *Pdx1* constructs did not show any inhibitory activity (**Figure 5C**).

Pdx1 increases HNF4α P2 transcripts that compete with P1 transcripts

The major regulator of *HNF1α* is *HNF4α* [45]. *HNF4α* is a complex gene regulated by two distinct promoters (P1 and P2) and alternative splicing [33, 46-48] that shows tissue specific expression [32, 34, 35]. In the adult human liver, *HNF4α* isoforms are primarily expressed from the P1 promoter whereas, in the adult human pancreas and islets, P2 driven isoforms are predominant [32, 34, 35]. In addition, *Pdx1* is a known regulator of *HNF4α* P2 driven isoforms and has a binding site in the P2 proximal promoter [33]. Thus, real time RT-PCR was performed in Huh7 cells and HepG2 cells following treatment with LV-*Pdx1-VP16* to examine the effects on the *HNF4α* promoter and isoform expression. As expected, a pronounced increase in *HNF4α* P2 transcripts was observed compared to P1 transcripts (**Figures 6A** and S3). Using primers to identify specific *HNF4α* P2 driven isoforms, an increase in *HNF4α7* and *HNF4α8*, and a decrease in *HNF4α9* were observed (**Figure 6A**).

HNF4α P2 driven isoforms were previously reported to be weaker transactivators of their target genes [36, 37]. We therefore investigated the transactivation potential of various *HNF4α* isoforms from both the P1 and P2 promoters in Huh7, INS1, and 293 cells using the *HNF1α*-luciferase reporter (**Figure 6B**). In all of these cell lines it is clear that *HNF4α* P1 driven isoforms are stronger activators than their corresponding (same c-terminal domain) P2 driven isoforms. However, *HNF4α* mediated regulation of *HNF1α* is different between Huh7 and INS1 cells. In Huh7 cells, both *HNF4α* P1 isoforms function as stronger activators whereas in INS1 cells, the strongest activators are the isoforms containing the same truncated C-terminal end (*HNF4α3* and *HNF4α9*).

To further investigate the mechanism by which *Pdx1* influences down-regulation of *HNF1α*, we set up competition assays in 3T3 cells because they do not express any transcription factors related to our system (data not shown). It was demonstrated that *Pdx1* has no direct effect on the *HNF1α*-luciferase reporter (**Figure 6C**). We then activated this reporter using the liver specific *HNF4α* isoform *HNF4α2* and demonstrated that *Pdx1* can then suppress this activation (**Figure 6C**). Since *Pdx1* does not directly interact with the *HNF1α* promoter, it was hypothesized that it may function indirectly by altering the regulation of its major activator *HNF4α*. Since *Pdx1* can increase expression of *HNF4α* P2 driven isoforms, we set up a competition assay in 3T3 cells to examine the effect of expressing multiple *HNF4α* isoforms (**Figure 6D**). In this case, the *HNF1α*-luciferase reporter was activated using the liver specific *HNF4α* isoform *HNF4α2* and then the effect of competition with the corresponding beta cell specific isoform *HNF4α8* was examined (both have the same C-terminal modification). As expected and similar to the effect of *Pdx1*, *HNF4α8* can competed with *HNF4α2* and significantly reduced the activity of the *HNF1α*-luciferase reporter. With a 1:1 ratio of *HNF4α2/HNF4α8*, the activity of the *HNF1α*-luciferase reporter is

almost the same as the *HNF4α8* base level. Therefore, it appears that *HNF4α* P2 driven isoforms are able to compete with P1 driven isoforms to reduce the activation of target genes.

Discussion

In the present study, we established an effective model by LV expression of *Pdx1*, *Pdx1-VP16*, *Ngn3*, and combinations of *Pdx1/Ngn3* or *Pdx1-VP16/Ngn3* in Huh7 cells in which to study the process of *Pdx1*-mediated reprogramming of hepatic cells into IPCs. We have demonstrated that co-expression of *Ngn3* with *Pdx1* is important for the activation of several endocrine pancreas genes such as *Pax4, NeuroD,* Isl-1, and *Nkx2.2*. We also examined the role of a *Pdx1* fusion protein (*Pdx1-VP16*) that strongly activates insulin gene expression and increases the efficiency of hepatic to pancreatic reprogramming [8, 14, 17]. In the present study we further investigated the effects of *Pdx1-VP16* on other pancreatic endocrine genes, in addition to the insulin gene, in order to determine if this fusion gene has the same synergistic potential on the transactivation of other PTF genes. It was shown that while *Pdx1-VP16* synergistically increased insulin gene expression as previously demonstrated, it failed to have the same effect on several other key genes that are essential to the pancreatic endocrine phenotype. The results show that the *Pdx1-VP16* fusion gene only increases transactivation of the insulin gene and suggests that the insulin gene may use a different mechanism of transcription than other pancreatic endocrine genes.

The liver is largely composed of hepatocytes, which occupy 78% of parenchymal liver volume [49, 50]. Hepatocytes carry out the primary functions of the liver such as metabolism, detoxification, and protein synthesis of several essential compounds including serum albumin, fibrinogen, and transferrin. It has been suggested that the dominant mechanism for controlling expression of hepatocyte specific genes is at the transcriptional level [51], however, the molecular mechanism by which *Pdx1* regulates expression of these hepatic genes is not well established. Using our reprogramming model, it was demonstrated that over-expression of *Pdx1* can down-regulate expression of *HNF1α* in hepatic cells. Because *HNF1α* is very important in the regulation of a wide array of hepatic genes [22], it may play a fundamental role in the process of dedifferentiation of the hepatic cell phenotype during the reprogramming into IPCs. The RT-PCR and Western blotting data showed that down-regulation of endogenous *HNF1α* resulted in decreased expression of downstream target genes *ALB* and *AAT* during reprogramming. Our findings are consistent with a previous study [29], where adenovirus-mediated expression of *Pdx1* led to down-regulation of several mature hepatocyte-specific genes including *ALB, ADH1B, G6PC, GLUL*, and *AAT*. Several key hepatic genes are direct downstream transcriptional targets of *HNF1α* including *ALB, AAT, AFP*, α- and β-fibrinogen, transthyretin, and pyruvate kinase [52-58]. Therefore, *Pdx1* induced down-regulation of *HNF1α* expression can affect these downstream target genes and may be important in the process of dedifferentiation of hepatic cells by down-regulating expression of an array of genes that determine the hepatocyte phenotype.

Our results also suggest that the mechanism for down-regulation of *HNF1α* is by affecting expression of its major activator *HNF4α*. (Note: See The Sladek Lab webpage for detailed information about *HNF4α* structure and function at http://www.sladeklab.ucr.edu/HNF4.shtml). It was demonstrated that specific *HNF4α* isoforms function differently in liver than in beta cells (**Figure 6A**, **6B**). In Huh7 cells, the activity of specific *HNF4α* isoforms appears to rely on the N-terminal domain that results from using different promoters (P1 vs. P2). *HNF4α* isoforms that are expressed using the P1 promoter contain exon 1A and are the strongest activators in Huh7 cells. The C-terminal domains that resulted from alternative splicing did not significantly alter the function of *HNF4α* in the context of our experiment in Huh7 cells. In INS1 cells the C-terminal modifications appear to play a major role in the function of *HNF4α* (**Figure 6B**). P1 driven isoforms are still stronger activators in INS1 cells when compared to their corresponding P2 driven isoforms (with similar C-terminal ends) but having the truncated C-terminal end that stops in exon 8 increases the activity independent of the first exon. The isoform driven by the P2 promoter that terminates in exon 8 (*HNF4α9*) is a stronger activator than the P1 driven isoform that include

exons 9 and 10 (*HNF4α2*). This suggests that the C-terminal modifications of *HNF4α* due to alternative exon splicing may have a beta cell-specific function.

According to the real time RT-PCR data (**Figure 6A**), *HNF4α* isoforms that are stronger transcriptional activators containing exon 1A (AF-1 activation domain) or terminating in exon 8 (*HNF4α2, HNF4α3, or HNF4α9*) are suppressed by treatment with *Pdx1* while *HNF4α* isoforms that are weaker transcriptional activators that contain exon 1D and exons 9 and 10 (*HNF4α8*). Exons 9 and 10 comprise the inhibitory F domain. It appears that *HNF4α* dependent regulation of *HNF1α* in hepatocytes requires stronger activators (P1 driven) than in beta cells which predominantly rely on P2 driven isoforms. This may be due to cell specific mechanisms for regulating this gene. For example, the transcription factor *Nkx6.1*, which is expressed in beta cells but not in hepatocytes [59], has been shown to regulate *HNF1α* by binding to its distal promoter [38]. We also produced evidence (**Figure 6D**) that promoter specific isoforms can compete with each other for activation of their target genes. It is possible that induced expression of *HNF4α* from the P2 promoter may suppress P1 expression during the reprogramming process of hepatocytes to IPCs, as the opposite has been shown during rodent liver development [60]. Briefly, ectopically expressed *Pdx1* protein in hepatocytes activates *HNF4α* P2 isoforms by binding directly to the P2 promoter resulting in increased expression of P2 isoforms that are weak activators of *HNF1α,* resulting in suppression of *HNF1α* activity via a competitive binding with *HNF4α* P1 isoforms. Ectopic *Pdx1* expression may also induce P2 CpG demethylation and increase P1 DNA methylation resulting in increased P2 transcripts in hepatocytes. By studying HNF gene regulation in future studies, we also may gain insight into the epigenetic mechanisms of *Pdx1*-mediated hepatic phenotype dedifferentiation.

Taken together, our findings indicate that transcription factors that function in multiply cell types must be regulated at specific levels appropriate for each cell type. These new findings may prove useful in the field of cell reprogramming and provide insight for the mechanism of reprogramming hepatic cells into IPCs by *Pdx1.*

Disclosure of conflict of interest

None.

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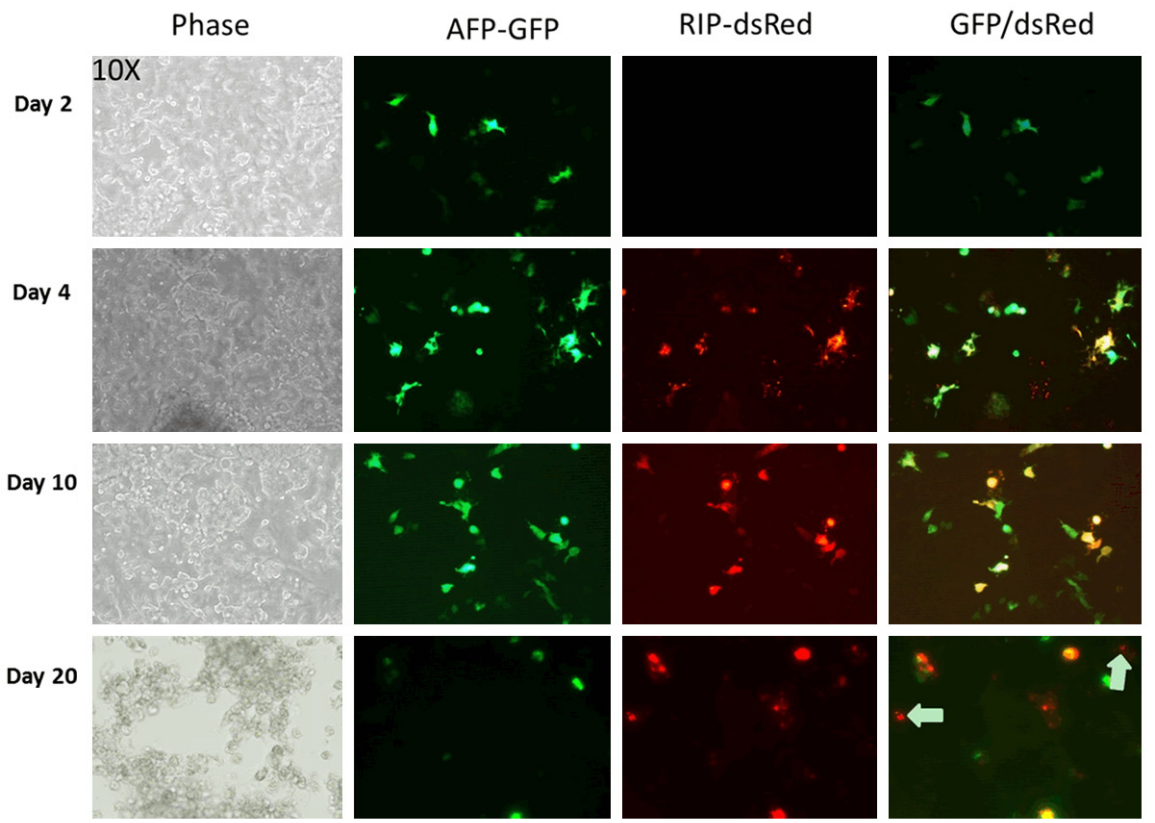

Figure S1. Identification of insulin producing cells by using a dual-color reporter system. HepG2 cells were transduced with LV-dual-color reportorm, plus LV-*Pdx1-VP16* and LV-*Ngn3*. At 20 days, rare cells (~2%) were noted to show fully transdifferentiated into insulin-producing cells (cells with only red fluorescence indicated with white arrows).

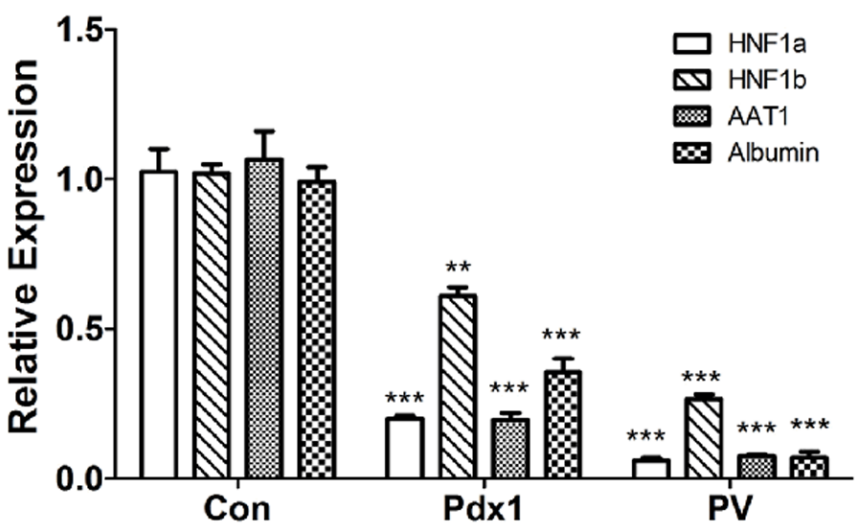

Figure S2. *Pdx1* increases *HNF4α* P2 transcripts that compete with P1 transcripts. Total RNA was collected from HepG2 cells following transduction with LV-*Pdx1* or LV-*Pdx1-VP16. HNF4α* gene expression was measured by real time RT-PCR.

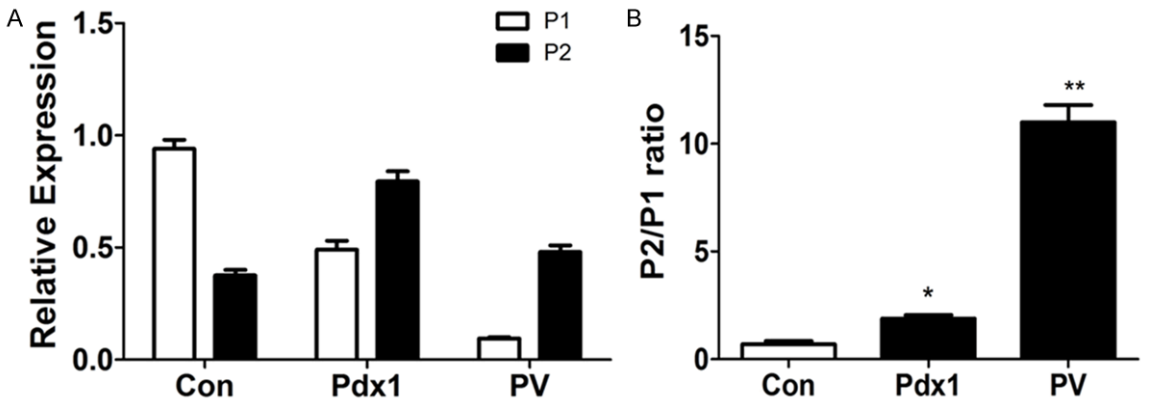

Figure S3. Down-regulation of hepatic genes (*HNF1α*, *HNF1b*, *ALB*, and *AAT*) in HepG2 cells following ectopic expression of *Pdx1*. HepG2 cells were transducted with LV-*Pdx1* or LV-*Pdx1-VP16* for 48 h. Total RNAs were extracted and subject to real-time RT-PCR analysis. The results were normalized using *GAPDH.* Experiments were done in triplicate.