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Co-expressing Pdx1 and Ngn3 induces few beta-like cells in the liver of mice

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Abstract

We have assessed whether expressing pancreatic transcription factors Pdx1 and Ngn3 in the liver could induce beta-like cells for curing type I diabetes. When co-expressed in the liver of insulin reporter mice using adenovirus, few cells in the liver were turned into beta-like cells which were marked by lacZ expression by X-gal staining. Insulin promoter assay showed that Pdx1 highly activates insulin promoter when combined with Ngn3. In ex vivo experiments to determine permissiveness of stem/progenitor and fully differentiated cells by ectopic transcription factors, it was found that more islet genes were induced in ES or bone marrow SP cells than fully differentiated NIH3T3 or MEF cells by Pdx1 or Ngn3. Our results suggest that synergistic action of Pdx1 and Ngn3 on inducing various islet genes as well as cell intrinsic factors played a crucial role in directing few liver cells into beta-like cells in vivo.

Keywords: Pdx1; Ngn3; Liver; β Cell; Gene transfer; Diabetes

The shortage of transplantable β cells is a major problem in cell therapy of type I diabetes. Many attempts were made to generate surrogate β cells from various non-pancreatic cells and liver has been reported as one of promising cell types to generate beta-like cells. Liver and pancreas arise from same endodermal region and have bipotential progenitor cells [1]. In certain pathological conditions, pancreas–liver cell switches occurs [2]. Previous studies showed that Pdx1 or NeuroD-Betacellulin expression in the liver by means of adenoviral vector could generate some insulin expressing cells in the liver and alleviated diabetic symptoms in animal models [3,4]. Active form of Pdx1 (VP16-Pdx1), combined with Ngn3 or NeuroD, induced higher insulin gene expression than using wild type Pdx1 and reversed diabetic symptoms [5]. However, it was not determined whether those insulin expressing cells are beta-like cells which are positive for other β cell markers. Insulin expressing cells in those studies were determined by immunostaining, and there have been issues of non-specific staining or sensitivity of immunostaining.

In this study we generated lineage specific transgenic mice which mark beta-like cells by lacZ expression and assessed whether expressing Pdx1 or Ngn3 in the liver via adenoviral vectors could initiate a cascade of events leading to the formation of pancreatic beta-like cells in the liver of mice. Pdx1 and ngn3 are major initiators of endocrine cell differentiation during embryonic development of pancreas. The embryonic pancreas arises within the broad foregut epithelial expression domain of Pdx1, and targeted disruption of the pdx1 gene in mice results in animals that lack a pancreas [6,7]. In adults, the expression of pdx1 is restricted to β cells and some δ cells in the pancreas, where it regulates genes involved in β-cell identity and function, such as insulin, glut2, glucokinase, polypeptide (PP) and somatostatin, and pax4. Expression of Ngn3 in the embryo is high at around e15.5 and turns off before final differentiation. Ngn3 is expressed in islet cell precursor cells and is crucial for endocrine cell differentiation [8–10]. Knockout of ngn3 prevents the differentiation of all endocrine cells in the pancreas [11].

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Our results showed that co-expressing Pdx1 and Ngn3 induced few beta-like cells in the livers of transgenic mice. Ex vivo studies showed that combined expression of Pdx1 and Ngn3 highly transactivated insulin1 and insulin2 promoters and induced more islet genes in stem/progenitor (ES and SP) cells than fully differentiated NIH3T3 and MEF cells. Our results suggest that the ability of co-expressing Pdx1 and Ngn3 to induce various islet cell-specific genes initiated series of events directing few liver cells into beta-like cells.

Materials and methods

Cloning. Retroviral constructs encoding mouse pdx1, mouse ngn3, and hamster beta2 were generated by inserting PCR amplified cDNAs into pCMMP based retroviral vector. Plasmids encoding a luciferase gene under insulin promoter (pRIP1-lucf and pRIP2-lucf) were generated using 410 base pairs of the rat insulin I gene promoter [12] and 668 base pairs of the rat insulin2 gene promoter [13], respectively.

Transgenic mice. The insulin promoter driven reporter plasmid, pRIP2-lacZ, was constructed by inserting the 668 base pairs of the rat insulin2 promoter upstream of a DNA fragment containing the human β-globulin gene intron, a nuclear localizing lacZ gene, and the SV40 late gene polyadenylation signal. Transgenic mice were generated (Dana-Farber Cancer Institute Transgenic Facility, Boston, USA) and maintained by breeding with FVB mice (Taconic, Hudson, NY, USA).

Animals and recombinant adenoviruses. Ad-Pdx1 and Ad-Ngn3 were constructed using mouse pdx1 and mouse ngn3 cDNAs. Transgenic mice 4–16 weeks old (FVB background) were treated with $4–10 \times 10^9$ plaque-forming units of recombinant adenoviruses intravenously by retroorbital injection and were sacrificed 2–7 days later. All mouse experiments were performed in accordance with the guidelines of the Harvard Medical School Animal Care and Use program.

Immunohistochemistry and X-gal staining. Liver and pancreas tissues were fixed for 3–4 h in 4% paraformaldehyde at 4 °C, and cryoprotected in 30% sucrose in PBS overnight. Guinea pig anti-Ngn3, guinea pig anti-insulin (1:200, Dako), rabbit β-galactosidase (1:100), guinea pig anti-rat C-peptide (1:500, Linco), and rabbit anti-cytokeratin (1:500, DAKO) were used to stain liver sections, followed by fluorescein- (1:100) or Cy3- (1:600) conjugated secondary antibodies (Jackson Laboratories). For cytokeratin staining, sections were pre-treated with protease K, and the avidin–biotin complex (ABC) method was used. For X-gal staining, sections were fixed with 0.5% glutaraldehyde and stained overnight at 37 °C followed by counter-staining with eosin solution (Sigma).

Retrovirus production and infection. Retroviral vector stocks were produced by transient transfection using calcium phosphate. For infection of ES, the cells were plated into gelatine coated six well plates and transduced with virus plus polybrene (8 μg/ml) for 8 h. SP cells were isolated as previously described [14], and SP cells were infected with each virus for 8–10 h after overnight activation with cytokines. SP cells were prepared from C57BL/6 mice, and cells were infected in IMDM based medium containing cytokines (50 ng/ml SCF, 10 ng/ml IL6, and 20 ng/ml Flt3) at a multiplicity of infection (MOI) of 50–100. After 8–10 h of infection, the medium was changed, and cultures were maintained for 18 h before isolation of RNA.

RT-PCR. Total RNA was isolated with the Qiagen RNA isolation kit (Qiagen, Valencia, CA, USA), followed by treatment with DNase for 40 min at 37 °C (Ambion, Austin, TX, USA). cDNA was prepared with Superscript RT (Invitrogen, Carlsbad, CA, USA). PCR primer sequences for the insulin1, insulin2, glucagon, polypeptide, pdx1, and β-actin were the same as those used by Lumelsky et al. [17]; and those for ngn3 and neuroD/beta2 were the same as those used by Huang et al. [18]. Primer sets used for the other genes: somatostatin (220 bp), CTATCCAGGAA CTGGCCAAG and CTAACAGGATGTGAATGTCTTC; pax6 (289 bP); CAACACGTACAGTGCTTTGCCAC and GAGGCCAGTACTGAG ACATGTC; pax4 (290 bp); GCTTCCCAGGACCTGACAG and GCCTCCAATCAGATGATGCAC; Nkx2.2 (204 bp); CACAGGT CAAGATCTGGTTCCAG and GTTGTACTGCATGTGCTGCAGC; Nkx6.1 (240 bp); GTTGGACAAAGATGGGAAGAG and CTCC GAGTCCTGCTTCTTC.

cDNA samples corresponding to 400 ng of RNA from NIH3T3 and MEF and 200 ng from ES cells were used for each primer set by 35 cycles of PCR. In the case of SP cells, cDNA samples corresponding to 100 ng RNA were used and 38 PCR cycles were performed. Seventy percent of SP cells were infected, as determined by FACS analysis.

Results

Synergistic transactivation of insulin promoter by Pdx1 and Ngn3

First we determined the transcriptional activity of single or combination of pdx1 and ngn3 on insulin1 and insulin2 genes. Reporter constructs which have luciferase gene under rat insulin1 or insulin2 promoters were made and examined promoter activity by measuring luciferase activity after expressing transcription factors. NIH3T3 cells transfected with reporter plasmid was used as a control for fold activation. Retrovirus encoding GFP was used as a control for viral infection.

As shown in Fig. 1, Pdx1 alone weakly transactivated insulin1 and insulin2 promoters. Ngn3 alone did not show any activity on insulin promoter, however, co-expressing Pdx1 and Ngn3 synergistically transactivated both insulin promoters (Fig. 1A and B). Beta2, which was known to transactivate insulin promoter with Pdx1 synergistically [15], was used to compare its activity with Ngn3. We found that co-expression of Pdx1 and Beta2 transactivated both insulin promoters similar to that of co-expressing Pdx1 and Ngn3.

Characterization of insulin reporter transgenic mice

To examine the effect of pancreatic transcription factors to induce pancreatic beta-like cells in the liver of mice, insulin reporter transgenic mice (RIP-lacZ) were generated to detect beta-like cells (Fig. 2A). Beta-like cells induced in the liver, if any, are marked by lacZ expression in the nuclei of cells.

X-gal staining (Fig. 2B) in the adult pancreas (4 weeks) showed strong lacZ expressing cells in the islet of pancreas. Co-staining with antibodies against insulin and beta-galactosidase verified that approximately 80% β cells expressed lacZ gene and all lacZ expressing cells in the pancreas are insulin expressing β cells (Fig. 2B). There was no lacZ expressing cells in the liver of RIP-lacZ transgenic mice.

Co-expressing Pdx1 and Ngn3 generates beta-like cells in the livers of mice but not by Pdx1 or Ngn3 alone

Adenoviruses containing pdx1 (Ad-Pdx1) and ngn3 (Ad-Ngn3) were injected into transgenic mice intravenously by retroorbital injection to infect livers of mice.

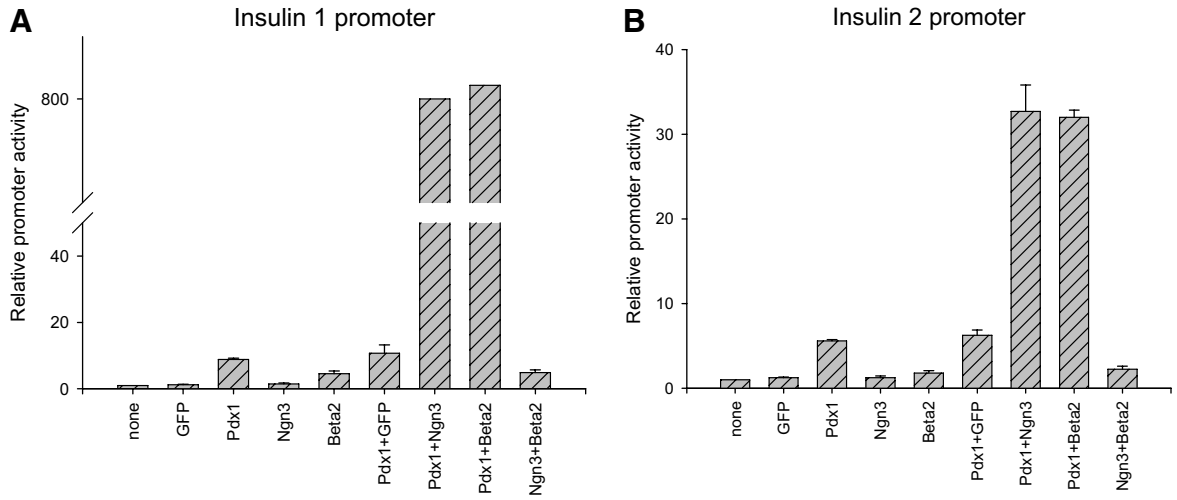

Fig. 1. NIH3T3 cells were infected with each virus as indicated, and pRIP1-lucf or pRIP2-lucf was transfected 6 h post-infection. Cells were lysed 24 h post-transfection, and luciferase activity was measured. The level of luciferase expression by reporter plasmid was used as a control for calculating fold activation. Data are means ± SD of two independent experiments.

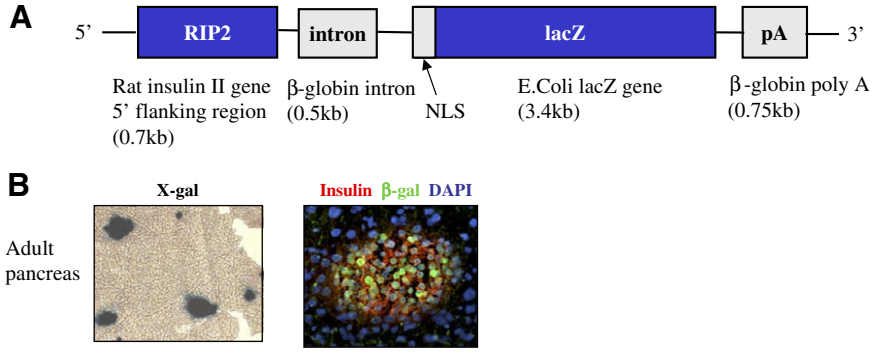

Fig. 2. Characterization of RIP-lacZ mice. (A) Diagram of the recombinant DNA plasmid composed of rat insulin2 promoter and lacZ reporter transgene. NLS, nuclear localization signal. (B) Adult pancreas stained with X-gal (left). Immunofluorescence staining of pancreas section for β-galactosidase (green), insulin (red), and DAPI (blue) is on the right side. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Ad-GFP was used as a control for adenoviral infection. The livers of mice injected with each virus were analyzed 2–7 days after viral injection. Beta-like cells were identified by staining liver sections with X-gal to detect lacZ gene expression. GFP, Pdx1, and Ngn3 expressed in approximately 70% of liver cells when analyzed at day 3 after adenoviral injection (data not shown). There was no endogenous Pdx1 or Ngn3 in the livers of non-infected transgenic animals. No lacZ expressing cells were found in all livers of mice injected with either Ad-Pdx1 ($n = 19$) or Ad-Ngn3 ($n = 3$) alone.

Interestingly few lacZ positive cells were found in the livers of transgenic mice co-injected with Ad-Pdx1 and Ad-Ngn3 (Fig. 3A). Five out of seven mice injected with both Ad-Pdx1 and Ad-Ngn3 viruses showed lacZ positive cells (Table 1). No lacZ positive cells were found in the non-transgenic livers infected with the same virus, confirming that these lacZ expressing cells result from expression of the lacZ gene under the insulin promoter and not from endogenous β-galactosidase activity. The lacZ expressing cells were found in single or several cell clusters in each section throughout the liver. There were approximately 26 lacZ positive cells (~0.1% of the hepatocytes) per section, in which 20–30% of the liver cells co-expressed both Pdx1 and Ngn3 when analyzed at 5 days post-injection (Fig. 3B and Table 1, mouse G). C-peptide positive cells were rarely detected by immunostaining (Fig. 3C). The number of lacZ expressing cells in the liver of each mouse injected with Ad-Pdx1 and Ad-Ngn3 varied (Table 1). To determine whether the lacZ positive cells were duct cells, we co-stained liver sections stained for X-gal with antibody to cytokeratin, which stains ductal cells in the liver (Fig. 3D). Some lacZ positive cells were located near hepatic duct (Fig. 3D, right) but were not co-stained, indicating that they were not duct cells. LacZ positive cells in the liver have morphology of hepatocytes.

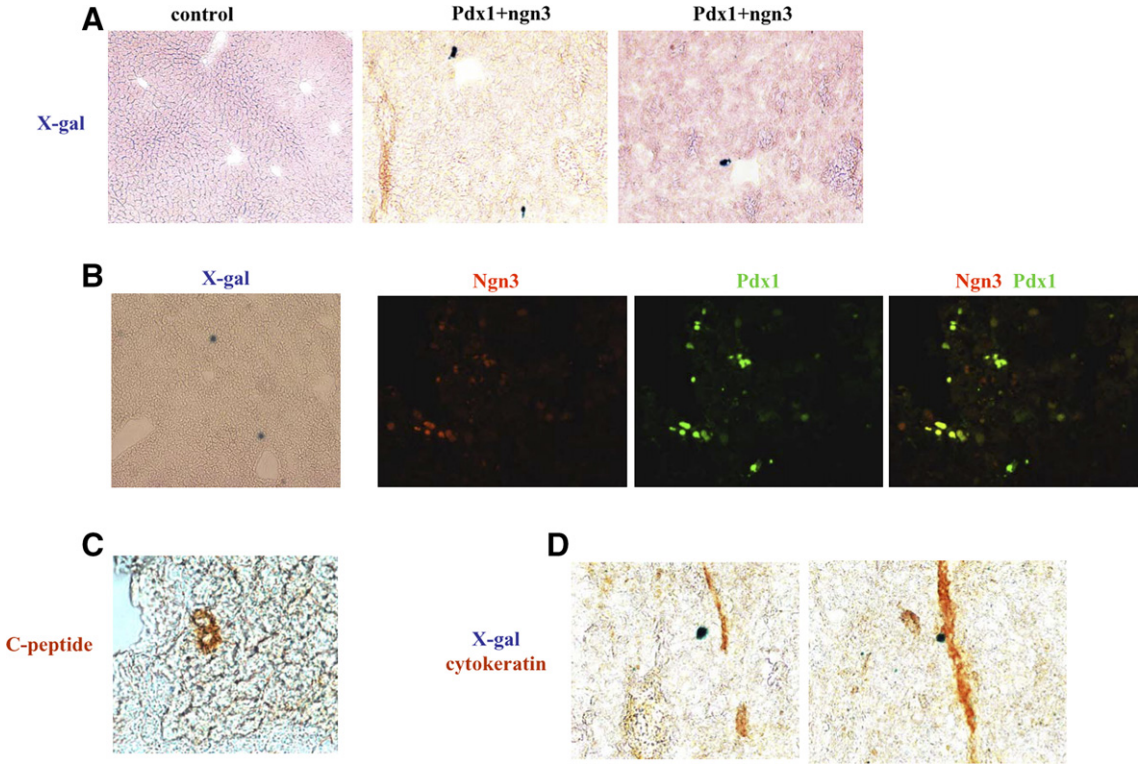

Fig. 3. Beta-like cells induced in the liver of mice co-injected with Ad-Pdx1 and Ad-Ngn3. (A) X-gal staining of liver of transgenic mice injected with no virus (control) and with adenoviruses encoding Pdx1 and Ngn3 (mouse E). (B) Liver sections (mouse G) stained with X-gal (left) and co-stained with rabbit polyclonal Pdx1 antibody (green) and guinea pig polyclonal Ngn3 antibody (red). (C) Insulin protein was detected by immunostaining with guinea pig anti-rat C-peptide. (D) Liver sections co-stained with X-gal and cytokeratin antibody. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Table 1
Beta-like cells induced in the liver of mice co-expressing Pdx1 and Ngn3 by means of adenovirus

Virus	Mouse	Age of mouse (weeks)	Viral load (PFU)	Analysis (days)	lacZ-positive cells/section
Ad-Pdx1 + Ad-Ngn3	A	6	8×10^9	2	0
	B	8	8×10^9	2	0
	C	4	7×10^9	3	18
	D	10	10×10^9	3	8
	E	10	10×10^9	3	11
	F	16	8×10^9	3	1
	G	8–10	8×10^9	5	26

Transgenic mice were co-injected with Ad-Pdx1 and Ad-Ngn3 intravenously and lacZ expressing cells were analyzed 2–7 days post-injection by X-gal staining. Analysis: time livers were harvested for analysis after viral injection. The lacZ positive cells per section are an average of 10–30 liver sections (thickness; 12 μm).

Effect of Pdx1 and Ngn3 on islet gene expression in various cell types

As our in vivo work in the liver of mice using Pdx1 and Ngn3 induced only a few lacZ positive cells compared to large number of pdx1–ngn3 co-expressing cells, we questioned whether this occurred in undifferentiated stem/progenitor cells residing in the liver of mice. In this regard, we assessed the ability of Pdx1 or Ngn3 to induce islet gene expression in a variety of cell types and compared whether islet genes are induced higher in stem or progenitor cells than in fully differentiated cells. ES or SP cells were transduced by retrovirus expressing Pdx1 or Ngn3 and subsequently RNA was prepared from the transduced cells to analyze for the expression of islet genes by RT-PCR. Expression of Pdx1 and Ngn3 in retroviral construct was confirmed by Western blot analysis (data not shown). Efficiency of gene transfer in each cell type was traced by GFP which co-express with either Pdx1 (retro-Pdx1) or Ngn3 (retro-Ngn3). Retrovirus encoding GFP was used as a control for retroviral infection. The effect of pdx1 and ngn3 on inducing islet genes in stem/progenitor cells were compared to fully differentiated cells such as NIH3T3 cells and murine embryonic fibroblasts (Table 2).

Various spectrum of islet genes were induced in each cell type. Generally more islet genes were inducible in stem or progenitor cells such as ES and SP cells than fully differentiated NIH3T3 or MEF cells by ectopic expression of Pdx1 or Ngn3. In contrast to other cell types, ES cells already expressed various transcription factors, such as pax6, neuroD1/beta2 and nkx6.1, insulin2, and polypeptide, and their level of expression was further increased by expressing ectopic transcription factors (data not shown). Overexpression of Pdx1 in ES cells increased the expression of polypeptide, which is one of hormones expressing in the islet

of pancreas. Ngn3 expression in ES cells induced pax4, and nkx2.2 genes which is important transcription factors for endocrine cells differentiation and somatostatin which expresses in the delta cells in the islet of pancreas. Co-expression of Pdx1 and Ngn3 induced proinsulin1 and somatostatin genes and appeared to increase the expression of proinsulin2, polypeptide, neuroD1/beta2, and pax6 genes. BM SP cells do not express islet genes, but Pdx1 expression induced proglucagon, pax6, and polypeptide genes, and Ngn3 expression induced neuroD1/beta2, pax4, pax6, and nkx2.2. Co-expression of Pdx1 and Ngn3 induced proglucagon, polypeptide, neuroD1/beta2, pax4, pax6, and nkx2.2 genes in SP cells.

Discussion

In this study we have tried to direct liver cells of mice into islet phenotype in vivo by expressing pancreatic transcription factors Pdx1 or Ngn3. We found that few beta-like cells were generated by co-expressing Pdx1 and Ngn3 in the liver of transgenic mice. We speculate that beta-like cells marked by lacZ expression result from induction of downstream genes of pdx1 and ngn3 in the liver as well as synergistic transactivation of insulin promoter by co-expressing Pdx1 and Ngn3. Insulin promoter assay showed that Pdx1, when combined with Ngn3, activates both insulin1 and 2 promoters much higher than pdx1 alone (10- and 6-fold, respectively). The number of lacZ expressing cells was much smaller than the number of infected cells. This indicates that simple transactivation of insulin gene by co-expressing pdx1 and ngn3 is not sufficient to turn on lacZ gene in the liver. We speculate that lacZ induction occurred in stem/progenitor cells in the liver rather than fully differentiated cells. Stem/progenitor cells have open chromatin structure due to enriched chromatin regulators and open chromatin structure enables transcription factors to access their target genes. This is supported by our ex vivo result that more islet specific genes are induced in stem/progenitor cells such as ES and BM SP cells by co-expressing Pdx1 and Ngn3 than in fully differentiated MEF and NIH3T3 cells.

Pdx1 alone did not induce beta-like cells in the liver. Our results do not completely contrast to previous report [3] that Pdx1 generates insulin expressing cells in the liver as we also found that Pdx1 could transactivate insulin promoter and induce various islet genes. However, our result does not support that pdx1 alone is sufficient to generate beta-like cells in the liver. We speculate that cascade events induced by pdx1 are not sufficient to induce various genes required for generating beta-like phenotype marked by lacZ expression in the liver of transgenic mice. As more than 80% of pancreatic β cells in transgenic mice are marked by lacZ gene expression, and this lacZ marker is more sensitive to detect insulin expression than immunostaining, it is unlikely that we missed all the beta-like cells induced by pdx1. We cannot exclude the possibility that mouse strain difference (FVB versus BL/6) or use of diabetic mice resulted in contradictory result using Ad-Pdx1.

C-peptide positive cells were rarely detected in the livers of mice co-expressing Pdx1 and Ngn3. This indicates that small portion of liver cells was able to express and retain insulin protein detectable by immunostaining using antibody to C-peptide. This indicates that X-gal staining is more sensitive to detect beta-like cells than insulin immunostaining, suggesting that most lacZ expressing cells have much lower level of insulin protein than pancreatic β cells. Diabetic or hepatic regeneration condition of mice when combined with Pdx1 or other transcription factors involved in pluripotency of stem cells may lead to generate more robust and true pancreatic beta-like cells in the liver.

Can these lacZ expressing cells induced in the liver by co-expressing Pdx1 and Ngn3 alleviate diabetic symptom? As liver is a large organ, it is possible that few lacZ expressing cells per section can contribute to reduce the level of blood glucose. In fact, there is a report that co-expressing Pdx1 and Ngn3 induced insulin gene detectable by Northern blot, alleviated diabetic symptom and showed glucose tolerance [5]. The use of active form of Pdx1 (VP16-

Table 2
Islet gene induction by Pdx1 or Ngn3 in various cell types

Gene expression	Cell type															
	NIH3T3				MEF				ES				BM SP			
	G	P	N	P + N	G	P	N	P + N	G	P	N	P + N	G	P	N	P + N
Proinsulin1	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−
Proinsulin2	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
Proglucagon	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
Somatostatin	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Polypeptide	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	+
Glut2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NeuroD1/beta2	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+
Pax4	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+
Pax6	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+
Nkx2.2									−	−	+	−	−	−	+	+

NIH3T3, MEF, ES, and BM SP cells were infected with retroviruses encoding GFP (G), pdx1 (P), and ngn3 (N), and RNA was isolated to analyze islet gene expression by RT-PCR. The expression of islet genes by each transcription factor is indicated as follows. +, expression of gene; −, no expression.

Pdx1) combined with NeuroD/Ngn3 has been reported to produce higher insulin gene than wild type Pdx1 in the liver of mice [5,16] and generate 3% C-peptide positive cells in the liver. This may be due to induction of all binding partners of Pdx1 by using active form of Pdx1 (VP16-Pdx1) and synergistic transactivation of insulin gene by VP16-Pdx1 and NeuroD/Ngn3.

Taken together, in this study we demonstrated that using lineage specific transgenic mice, beta-like cells were made in the liver of RIP-lacZ transgenic mice by co-expressing Pdx1 or Ngn3. Co-expression of Pdx1 and Ngn3 highly activated insulin promoter and induced many islet genes including insulin genes in stem/progenitor cells. This implicates that few lacZ gene expressing cells in the livers of transgenic mice were induced in stem/progenitor cells, which are permissive to express many islet genes involved in β-cell identity by their existing factors and open chromatin structure.

Acknowledgments

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