

Analysis of the transcription factor cascade that induces endocrine and exocrine cell lineages from pancreatic progenitor cells using a polyoma-based episomal vector system

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ABSTRACT

Aims/Introduction: We recently established a strategy for isolating multipotential duct-like cells, called pdx-1-positive pancreatic cell-derived (PPPD) cells, from the pancreas. To analyze the molecular mechanisms of pancreatic cell differentiation, we introduced a polyoma-based episomal vector system into PPPD cells.

Materials and Methods: PPPD cells were stably transfected with a polyoma large T (PLT)-expressing plasmid vector, which included the polyoma origin of replication, to generate PLT-PPPD cells. Various cDNA for pancreas-related transcription factors were subcloned into the expression plasmid pPyCAG, which included the polyoma origin of replication. PLT-PPPD cells were stably transfected with the resulting plasmid vectors and then subjected to gene and protein expression analyses.

Results: The coexpression of *Mafa*, *Neurod1* and *Ipf1* induced *Ins1* and *Ins2* expression in PLT-PPPD cells. The forced expression of *Pax6* alone induced the expression of glucagon. The coexpression of *Neurod1* and *Isl1* induced *Ins2* and *Sst* expression. In contrast, the expression of *Ptf1a* and *Foxa2* induced the expression of exocrine markers *Cpa1* and *Amy2*. Transfections with multiple transcription factors showed that *Isl1* is required for the differentiation of both insulin-positive cells and somatostatin-positive cells. In addition, *Foxa2* induced the differentiation of glucagon-positive cells and inhibited the differentiation of insulin-positive and somatostatin-positive cells. PLT-PPPD cells allow episomal vector-based gene expression and should be useful for studying the transcription factor cascades involved in the differentiation of pancreatic cell types *in vitro*.

Conclusions: Our coexpression study showed novel critical roles for *Isl1* and *Foxa2* in the differentiation of PPPD cells into endocrine cells. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00136.x, 2012)

KEY WORDS: Gene delivery systems *in vitro*, Pancreatic differentiation, Transcription factors

INTRODUCTION

The differentiation of various cell types is elicited by cascades involving a number of transcription factors, and the functional analysis of the genes encoding these transcription factors is essential for understanding the molecular mechanisms of development and differentiation. The introduction of transcription factor genes into cells is a major strategy for analyzing the roles of transcription factors in specific cell types. Traditionally, exogenous genes have been borne on expression plasmids that are introduced into cells by liposome-mediated transfection or electroporation. However, these methods are typically plagued by low transfection efficiency and lack of sustained gene expression, and they yield stably transfected cells at only a low efficiency.

The present study was undertaken to evaluate the use of an episomal vector to obtain stable and prolonged gene expression for examining the transcription factors involved in pancreatic cell differentiation.

Gassmann *et al.*¹ developed episomally maintained vectors to promote efficient gene expression in embryonic stem (ES) cells. In this system, a plasmid vector called pMGD20neo is maintained extrachromosomally by the presence of polyoma virus DNA sequences, including the origin of replication, harboring a mutant enhancer (PyF101) and a modified version of the polyoma early region (LT20), which encodes the large T antigen. Interestingly, when introduced into cells that already contain pMGD20neo, additional plasmids containing a polyoma ori are maintained in a similar episomal state, owing to the large T antigen produced from pMGD20neo. Thus, the polyoma-based episomal vector system is a very convenient method for stably transfecting ES cells with plasmids expressing various genes. This system has been used to screen for novel genes

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involved in maintaining the undifferentiated state of ES cells^{2–4} and to induce the differentiation of cells of a specific lineage⁵. Camenisch *et al.*⁶ also reported that this polyoma-based episomal vector can replicate in cell lines other than ES cells, including the mouse embryonic carcinoma F9, mouse erythro-leukemia (MEL), mouse renal adenocarcinoma (RAG) and mouse L-fibroblast (L-929) lines. We recently developed a method for isolating and propagating pancreatic epithelial cells from adult mice⁷. These cells, called pdx-1-positive pancreatic cell-derived (PPPD) cells, grow continuously in serum-free DMEM/F12 medium when cyclic adenosine monophosphate (cAMP) signaling is stimulated. In these cells, the adenovirus-mediated expression of Neurogenin 3 (Ngn3) and Ptf1a induces the expression of insulin and somatostatin, and of carboxypeptidase A, respectively. Furthermore, albumin production is induced by dexamethasone treatment or by long-term culture in serum-containing medium. Thus, PPPD cells isolated from adult mice are multipotent, able to differentiate into endocrine cells, exocrine cells and hepatocytes. To carry out a detailed analysis of the transcription factors involved in the differentiation of specific cell lineages from these cells, effective gene transfer is essential. Therefore, here we developed a method to deliver exogenous genes into PPPD cells efficiently and stably, using an episomal vector system.

MATERIALS AND METHODS

Establishment of PPPD Cells Expressing the Polyoma Large T Antigen

The isolation and characterization of pancreatic cells from mice was previously described⁷. In the original method, PPPD cells were grown under the serum-free condition, but the growth rate was quite slow, which was not suitable for selecting stably transfected cells. We found that fetal calf serum improved their growth, but it also induced their differentiation into albumin-producing cells. Knockout serum replacement (KSR; Invitrogen, Carlsbad, CA, USA) is a serum replacement reagent developed for maintaining ES cells with minimum stimulation of differentiation. The addition of 5% KSR to the medium greatly improved the growth rate of PPPD cells without affecting their morphology or expression of pancreatic endocrine and exocrine marker genes (data not shown). Therefore, we used the serum-free medium (DMEM/F-12 medium supplemented with 100 ng/mL cholera toxin, 5 µg/mL insulin-transferin-selenium, 0.2% bovine serum albumin and 25 ng/mL keratinocyte growth factor containing 5% KSR for the culture of PPPD cells. The culture was incubated at 37°C in a humidified 5% CO₂ and 95% air atmosphere, and the medium was changed every 2 or 3 days.

The episomal vector pMGD20neo was recovered from MG1.19 ES cells¹ by Hirt's method⁸. PPPD cells were transfected with the pMGD20neo plasmid, followed by selection with 200 µg/mL G418. After 2 weeks of selection, the G418-resistant PPPD cells were designated as polyoma large T-expressing (PLT)-PPPD cells. The PLT-PPPD cells were cultured with

200 µg/mL G418 and could be maintained for several months without any change in their morphology or proliferative capacity.

Transfection of pPyCAG Vectors Expressing Transcription Factor Genes

The cDNA for transcription factors were amplified by proof-reading polymerase chain reaction (PCR), subcloned into pCR4 vectors (Invitrogen), sequenced and introduced into the pPyCAG expression vector^{9,10}. The pPyCAG vector was constructed from pCAGGS¹¹ by inserting a puromycin-resistance cassette and a polyoma ori DNA fragment (Figure 1a). A series of expression vectors was constructed by replacing the stuffer of the pPyCAG vector with the transcription factor cDNA. The pPyCAG vectors bearing transcription factor genes were transfected into PLT-PPPD cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The medium contained 1.5 µg/mL puromycin and was renewed every 2–3 days until 7–8 days post-transfection.

Reverse Transcription PCR Analysis

Total RNA was extracted from cultured cells by the acid guanidinium-phenol-chloroform method. cDNA was prepared from the total RNA using a ReverTra Ace-α kit (Toyobo, Tokyo, Japan) with oligo dT primers, according to the manufacturer's instructions. The primer sequences and PCR conditions used for reverse transcription (RT)-PCR were previously described^{12,13}. PCR was carried out with Taq DNA polymerase (Promega, Madison, WI, USA) within the log phase of the reaction (25–30 cycles).

Immunohistochemical Analysis

Cells were washed four times with PBS, fixed for 15 min in 4% paraformaldehyde and then incubated with Blocking One (Nacalai, Kyoto, Japan). Immunofluorescence staining was carried out using the primary antibodies and second antibodies (listed in Table S1). Nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). The stained cells were examined using a fluorescence microscope (Olympus, Tokyo, Japan). We have confirmed no staining for insulin, glucagon, somatostatin or amylase was observed when pPyCAG-empty vector was transfected in each experiment (data not shown).

RESULTS

Characterization of PLT-PPPD Cells

PPPD cells were transfected with the pMGD20neo plasmid and then selected with G418. The G418-resistant PPPD cells were designated PLT-PPPD cells. The PLT-PPPD cells were cultured in the presence of G418 and were maintained without any change in morphology or proliferative capacity for more than several months. The expression of the polyoma T antigen in the PLT-PPPD cells was confirmed by immunostaining (Figure 1b,c). The expression of pancreatic marker genes was examined by RT-PCR and was almost the same as in the

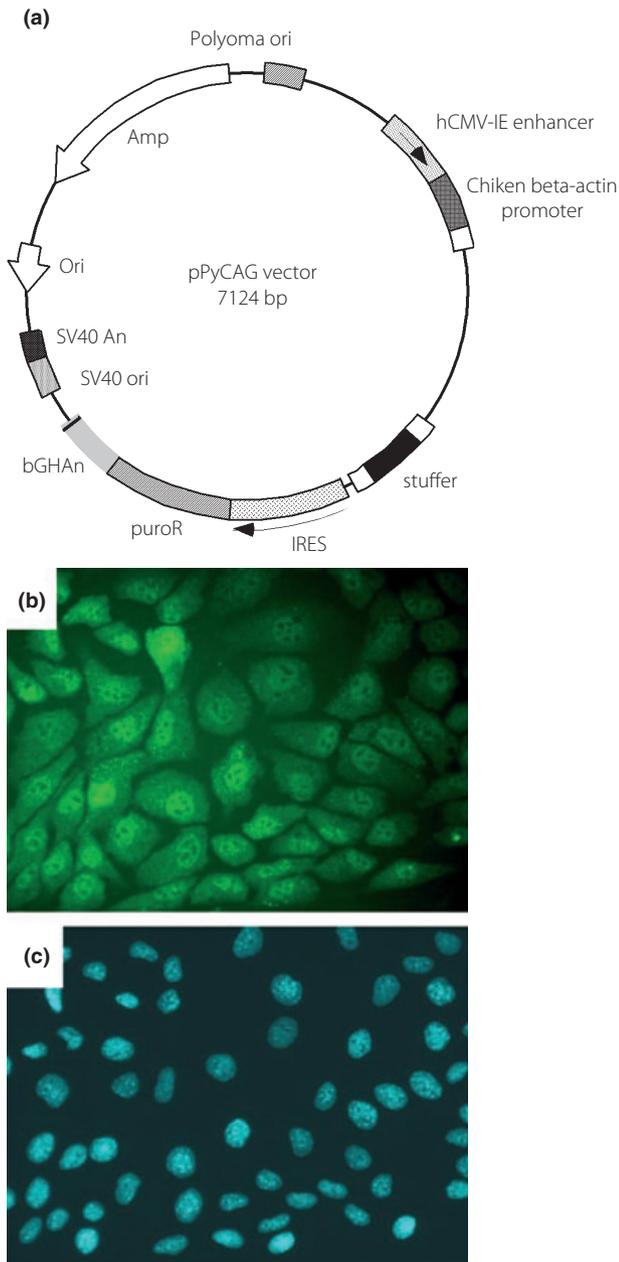


Figure 1 | Polyoma T antigen expression in polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPP) cells and construction of the pPyCAG vector. (a) Episomal plasmid vector pPyCAGIP containing a puromycin-resistance cassette and polyoma ori DNA fragment. (b) PLT-PPP cells were (c) stained with an anti-polyoma virus T antigen antibody.

parental PPPD cells: neither the PPPD nor the PLT-PPP cells expressed insulin1 (*Ins1*), insulin2 (*Ins2*), glucagon (*Gcg*), somatostatin (*Sst*), carboxypeptidase1 (*Cpa1*) or amylase2 (*Amy2*). Immunostaining showed that the PLT-PPP cells expressed CK19 and Ip1-f, similar to PPPD cells (data not shown).

The pPyCAG plasmid vector, the expression vector for exogenous genes of interest, included the polyoma ori, which allows episomal maintenance of the plasmid in polyoma T antigen-producing cells^{9,10}. Selection with puromycin was started 48 h after transfection of the plasmid. Transfection of a pPyCAG plasmid expressing enhanced green fluorescence protein (EGFP) showed that the efficiency of transfection was strikingly higher (40–50% of the total cells) in PLT-PPP cells than in PPPD cells (4–5% of the total cells), probably because the introduced plasmid could be readily amplified in them (Figure 2a,b). The duration of expression vector maintenance was also examined using the pPyCAG-EGFP vector, and EGFP fluorescence could be detected even 2 months after transfection (Figure 2c,d). We also examined the effect of cotransfecting multiple genes, using our episomal vector system. The cotransfection of pPyCAG-EGFP and pPyCAG-dsRed2 resulted in the coexpression of EGFP and dsRed2 proteins in 40–50% of the PLT-PPP cells after 48 h of puromycin selection (Figure 2e–h) and the single positive cell for EGFP or dsRed2 cells were rare (1–5% of the cells).

Effects of Expressing Transcription Factor Genes on the Differentiation State of PLT-PPP Cells

To analyze the effects of transcription factor gene expression on the differentiation of pancreatic cell lineages from PLT-PPP cells, pPyCAG vectors expressing various transcription factor genes were introduced into PLT-PPP cells, and their gene expression pattern was examined (Figure 3). In experiments in which a single expression vector was introduced, the expression of *Neurod1* induced the expression of the *Ins2* gene, *Pax6* induced expression of the glucagon gene (*Gcg*), and *Ptf1a* induced expression of the *Cpa1* gene, but none of the other genes examined, including *Isl1*, *Mafa*, *Ip1f* (Figure 3), *Pax4*, *Nkx2-2*, *Nkx6-1*, *Hnf6* or *Foxa2* (data not shown) affected the expression of the pancreatic genes *Ins1*, *Ins2*, *Gcg*, *Sst*, *Cpa1* or *Amy2*.

In contrast, the co-introduction of two or three genes enhanced or induced the expression of several pancreatic genes whose expression was not altered by single gene introduction. For example, *Neurod1*, *Mafa* and *Ip1f* encode transcription factors that synergistically stimulate insulin gene transcription. When all three of these genes were co-introduced into PLT-PPP cells, the expression of both the *Ins2* and the *Ins1* gene was induced, although the level of *Ins2* gene expression in these PLT-PPP cells was 1–5% of those of mouse islet cells analyzed by realtime PCR (data not shown).

Neurod1 is one of the first transcription factors to be induced by *Ngn3*, which is expressed in the pancreatic endocrine lineage. To elucidate the transcriptional cascade of the pancreatic endocrine fate, we introduced various transcription factors in combination with *Neurod1* (Figure 4). *Ins2* expression was enhanced by *Neurod1*'s coexpression with *Isl1* and *Ip1f*. The expression of *Mafa* with *Ip1f* and *Neurod1* enhanced the expression of *Ins2*. In contrast, the co-introduction of *Foxa2* with *Isl1* and *Neurod1*

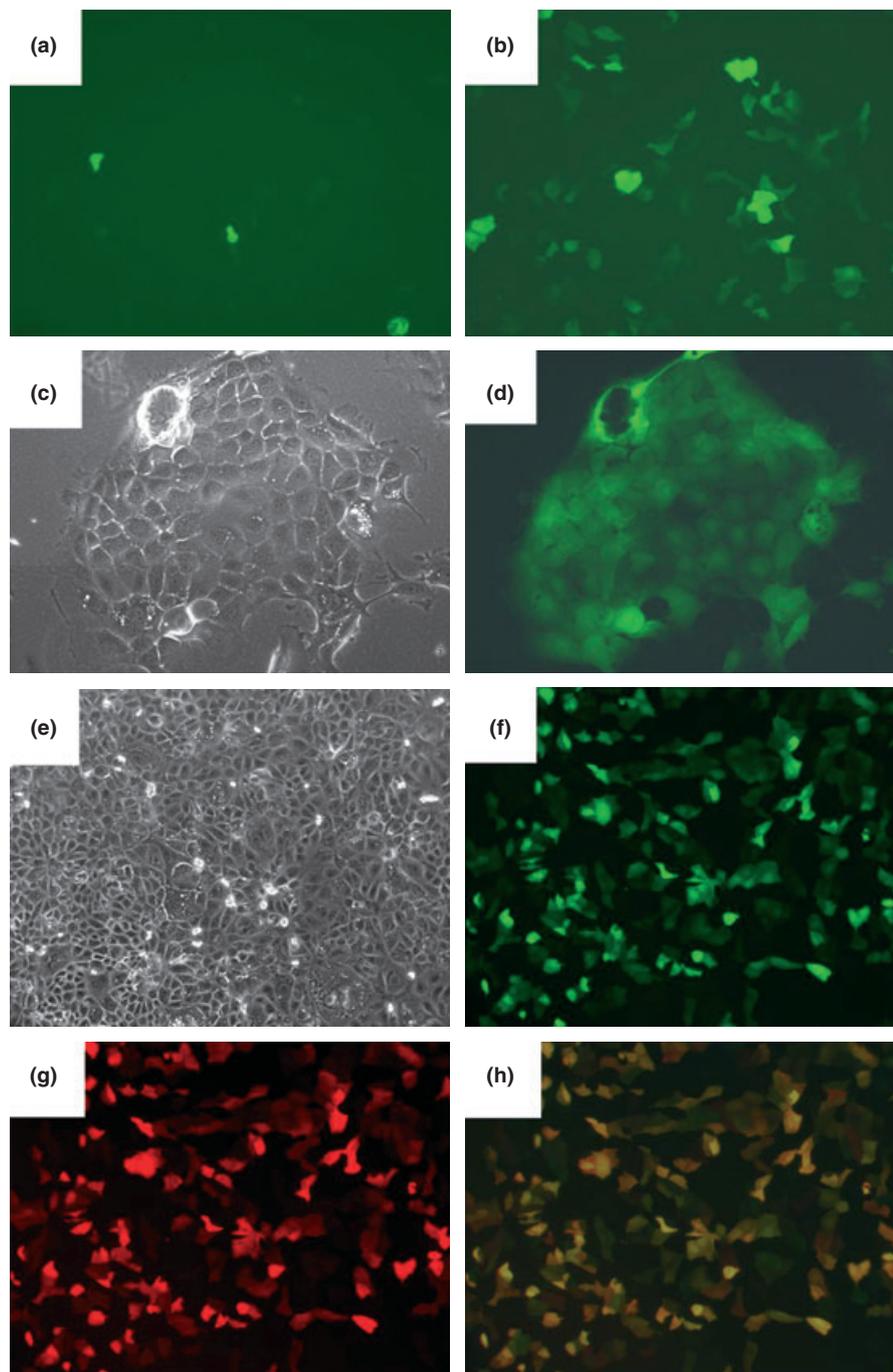


Figure 2 | High efficiency of transfection in polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPPD) cells using episomal vectors harboring polyoma ori. Enhanced green fluorescence protein (EGFP)-expressing cells 24 h after pPy-CAG-EGFP vector transfection into (a) PPPD and (b) PLT-PPPD cells. The expression of EGFP was more prominent in the PLT-PPPD than in the PPPD cells. (c,d) The expression of EGFP was maintained in PLT-PPPD cells for more than 1 month. (e–h) Successful simultaneous transfection of multiple plasmids was observed. Two expression vectors, pPy-CAG-EGFP and pPy-CAG-dsRed2, were transfected into PLT-PPPD cells. (f)EGFP expression, (g) dsRed2 expression and (h) their merged image showed that most of the transfected cells expressed both EGFP and dsRed proteins.

suppressed *Ins2* expression and induced the expression of *Gcg*. *Gcg* expression was also induced by *Foxa2* and *Mafa*, and by *Isl1* and *Mafa* coexpressed with *Neurod1*. Interestingly, the

Isl1-induced *Gcg* expression was suppressed by the addition of *Ipf1*, whereas somatostatin (*Sst*) gene expression was induced by *Isl1*, in the *Neurod1*-coexpression condition. This induction was

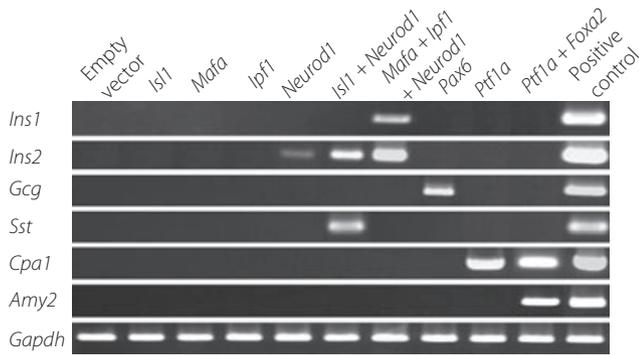


Figure 3 | Reverse transcription polymerase chain reaction analysis of differentiated polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPPD) cells transfected with various transcription factor genes. PLT-PPPD cells were transfected with pPyCAG expression vectors. Total RNA was extracted from the PLT-PPPD cells 72 h after transfection. Empty vector, RNA from PLT-PPPD cells transfected with pPyCAG empty vector was used as a negative control; positive control, RNA from whole pancreas or islet cells was used as a template.



Figure 4 | Reverse transcription polymerase chain reaction analysis of differentiated polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPPD) cells cotransfected with the *Neurod1* gene and various other transcription factor genes. PLT-PPPD cells were cotransfected with various pPyCAG expression vectors and the *Neurod1*-expressing vector. Total RNA was extracted from the PLT-PPPD cells 72 h after transfection. Empty vector, RNA from PLT-PPPD cells transfected with pPyCAG empty vector was used as a negative control; positive control, RNA from whole pancreas or islet cells was used as a template.

suppressed by *Mafa*, but unaffected by the addition of *Ipfl*. *Ppy* (*Ppy*) expression was induced by the addition of *Isl1* and *Ipfl* with *Neurod1*.

Immunofluorescence analysis showed that PLT-PPPD cells transfected with *Neurod1*, *Mafa* and *Ipfl* stained positively for C-peptide (4–5% of the cells were positive for C-peptide), showing that these cells produced insulin de novo (Figure 5a). In addition, measurement of the insulin content by ELISA showed that the PLT-PPPD cells transfected with *Neurod1*, *Mafa* and *Ipfl* contained significantly enhanced levels of insulin compared with cells transfected with the control vector (99.2 ± 37.6 vs 5.9 ± 3.7 ng/ 10^5 cells), although they did not secrete the detectable insulin by ELISA and not express *Glut2* or the glucokinase

gene (data not shown). Interestingly, when PLT-PPPD cells transfected with *Neurod1*, *Mafa* and *Ipfl* were stained with an antibody against pancytokeratin, a marker for duct cells, the insulin-producing cells were only rarely costained (Figure 6a,b).

Introduction of the *Pax6* gene alone into PLT-PPPD cells induced the expression of *Gcg* (Figure 3). The expression of glucagon was verified by immunofluorescence analysis (70–80% of the cells were positive for glucagon; Figure 5b).

Introduction of the *Isl1* gene together with the *Neurod1* gene into PLT-PPPD cells induced the expression of the *Sst* and *Ins2* genes (Figure 3). The expression of somatostatin was verified by immunofluorescence analysis (10–20% of the cells were positive for somatostatin; Figure 5c). Immunofluorescence analysis also showed a number of cells that produced somatostatin or insulin (Figure 6c,d). However, each hormone-producing cell was positive for either somatostatin or insulin, and there were no multi-hormone-producing cells. At present, we do not know why the same set of transcription factors induced different cell populations, but it is possible that the dose of each transcription factor was different among the transfected cells, and their levels affected the differentiation status of the PLT-PPPD cells.

Ptf1a alone induced *Cpa1*, but not expression of the amylase gene. Interestingly, transfection of the *Foxa2* gene in addition to the *Ptf1a* gene did induce amylase gene expression (Figure 3). Immunostaining showed that amylase protein was also present in the transfected cells (10–20% of the cells were positive for amylase; Figure 5d).

When cells differentiate, they usually lose their capacity to proliferate and they looked flattened cells. To examine the proliferative capacity of the cells that had been transfected with transcription factor genes, we carried out Ki67 staining. In the case of cells cotransfected with *Neurod1*, *Mafa* and *Ipfl* (Figure 7a–d) or with *Ptf1a* and *Foxa2*, most of the undifferentiated cells were Ki67-positive, but only a few differentiated cells were Ki67-positive (Figure 7e,f). Thus, the differentiated cells appeared to lose their proliferative capacity.

DISCUSSION

In the present study, we established an experimental system suitable for the high-throughput analysis of transcription factor genes involved in the differentiation of pancreatic precursor cells, PPPD cells, using an episomal vector.

In PLT-PPPD cells, the efficiency of gene transfection was markedly improved by using the episomal vector (Figure 2a,b). This was probably because the T antigen present in the PLT-PPPD cells readily promoted the replication of plasmids containing polyoma ori when they entered the nuclei. After puromycin selection, the transfectants stably expressed the transgenes for more than 2 months. Furthermore, multiple transgenes could be efficiently transfected into one cell (Figure 2e–h). Thus, combining the episomal vector system with PLT-PPPD cells provides a useful tool for analyzing the effects of transcription factor gene expression on the differentiation of pancreatic cells.

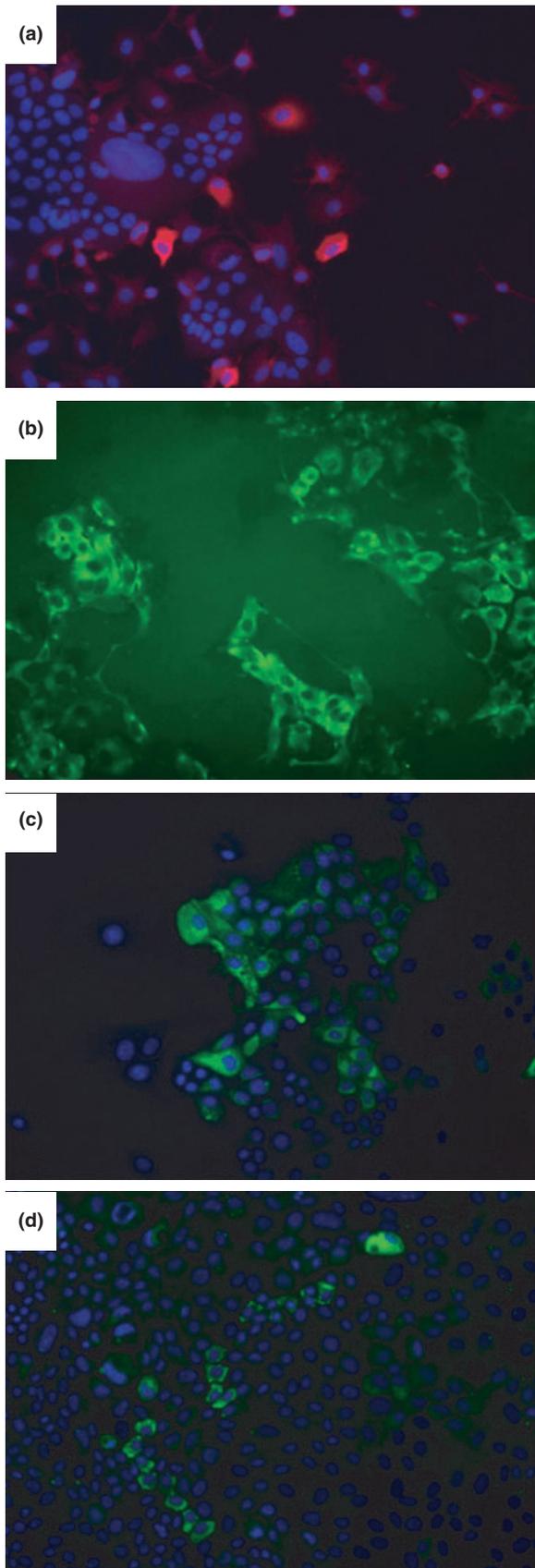


Figure 5 | Immunostaining of polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPPD) cells transfected with various transcription factor genes. (a) pPyCAG vectors expressing *Ipfl*, *Neurod1* and *Mafa* were cotransfected into PLT-PPPD cells. The cells were stained with an anti-C-peptide antibody (red) and the nuclei were stained with DAPI (blue). (b) A pPyCAG vector expressing *Pax6* was transfected into PLT-PPPD cells. The cells were stained with an anti-glucagon antibody (green). (c) pPyCAG vectors expressing *Isl1* and *Neurod1* were cotransfected into PLT-PPPD cells. The cells were stained with an anti-somatostatin antibody (green) and the nuclei were stained with DAPI (blue). (d) pPyCAG vectors expressing *Ptf1a* and *Foxa2* were cotransfected into PLT-PPPD cells. The cells were stained with an anti-amylase antibody (green) and the nuclei were stained with DAPI (blue).

The combined transfer of the *Ipfl*, *Neurod1* and *Mafa* genes induced the expression of not only the *Ins1* gene, but also the *Ins2* gene, at high levels, without inducing other pancreatic genes, such as *Gcg*, *Sst*, *Cpa1* or *Amy2*. Importantly, we detected C-peptide in these cells, which showed the de novo synthesis of insulin in them. Although PLT-PPPD cells expressed the *Ipfl* gene (data not shown), as the original PPPD cells did⁷, the transfer of just the *Neurod1* and *Mafa* genes did not induce the expression of insulin genes. It is possible that the endogenous expression of *Ipfl* was too low to induce expression of the insulin gene, so transfer of the additional *Ipfl* gene was required to induce it.

The *Ipfl*, *Neurod1* and *Mafa* transcription factor genes were previously shown to synergistically induce insulin gene expression^{14–16}. Furthermore, the simultaneous introduction of these three genes into the liver leads to the neogenesis of insulin-producing cells¹⁷. It was recently reported that this combination of transcription factor genes also induces the production of insulin in pancreatic acinar AR42J cells, and in IEC-6 cells, an intestinal endodermal cell line¹⁸.

Our experiments in which transcription factors were transfected in combination with *Neurod1* showed that either *Isl1* or *Ipfl* increased the *Ins2* gene expression, whereas *Mafa* in combination with *Neurod1* did not. However, *Mafa* was required for *Ins1* gene expression when *Ipfl* and *Neurod1* were cotransfected. Thus, *Mafa*'s promotion of insulin gene transcription appeared to occur in the context of coexpressed *Neurod1* and *Ipfl*. Indeed, a *Mafa* knockout mouse has pancreatic islets that appear normal, but their insulin secretory response to glucose gradually deteriorates¹⁹. Thus, *Mafa* might be a determinant factor for the final differentiation of pancreatic β -cells.

In contrast, *Foxa2* suppressed the *Ins2* gene expression induced by *Neurod1* and *Isl1*. Consistent with our results, the expression of insulin and islet amyloid polypeptide is markedly suppressed after the induction of *Foxa2* in INS1 cells, an insulinoma cell line, whereas *Gcg* mRNA levels are significantly increased, and the forced expression of *Foxa2* blunts the insulin release from INS1 cells *in vitro*²⁰. Concordantly, the deletion of *Foxa2* stimulates insulin release from islets *in vivo*²¹. Thus,

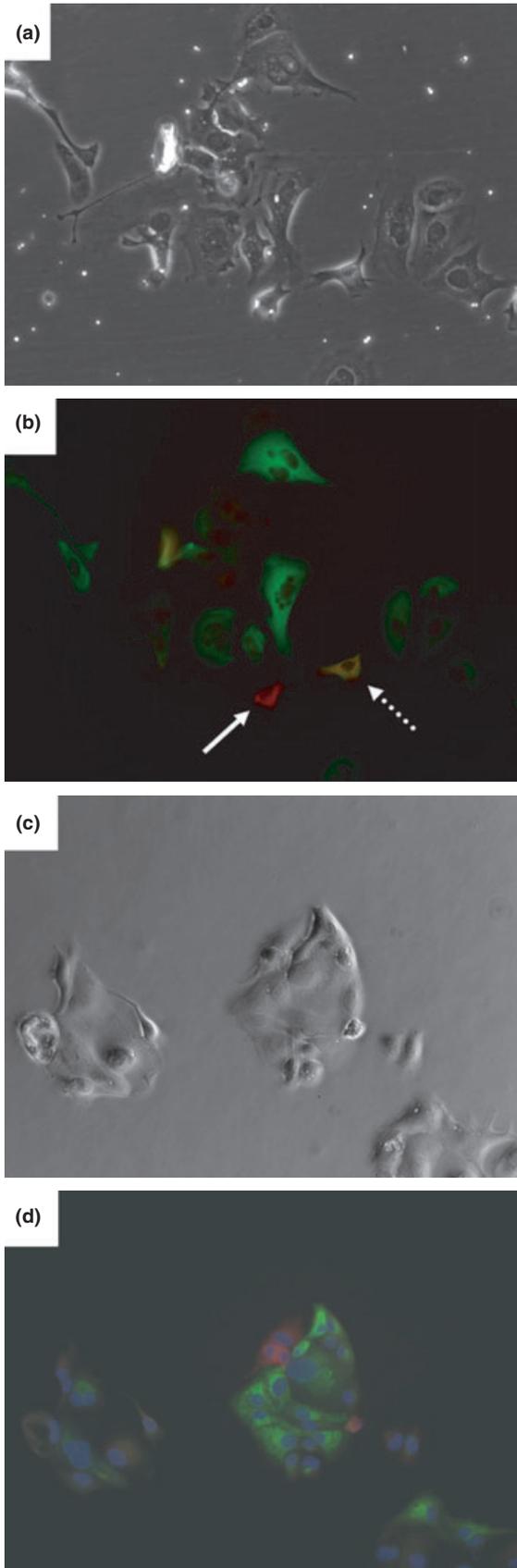


Figure 6 | Characterization of the differentiated polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPPD) cells. (a,b) pPyCAG vectors expressing *Ipfl*, *Neurod1*, and *Mafa* were cotransfected into PLT-PPPD cells. (a) Phase contrast view of the differentiated cells. (b) Cells were stained with anti-insulin (red) and anti-pancytokeratin (green) antibodies. Solid arrow shows cells stained with anti-insulin, but not with anti-pancytokeratin. Dotted arrow shows cells stained with both anti-insulin and anti-pancytokeratin. (c,d) pPyCAG vectors expressing *Isl1* and *Neurod1* were cotransfected into PLT-PPPD cells. (c) Phase contrast view of the differentiated cells. (d) Cells were stained with anti-insulin (red) and anti-somatostatin (green) antibodies. Nuclei were stained with DAPI (blue). There were no cells co-stained with the anti-insulin and anti-somatostatin antibodies.

Foxa2 not only inhibits the insulin secretory mechanism in pancreatic β -cells, as shown in previous reports, but also plays an inhibitory role in β -cell differentiation, as shown in our present study.

Pax6 was the only transcription factor that could induce the expression of a pancreatic gene and its protein without the co-introduction of another transcription factor gene. *Pax6* stimulates *Gcg* expression^{22–24} and is essential for the development of glucagon-producing pancreatic α -cells^{25,26} and enteric L cells²⁷. We showed that introduction of the *Pax6* gene alone was sufficient to induce the differentiation of PPPD cells into glucagon-producing cells. Consistent with our results, the introduction of the *Pax6* gene by adenoviral vector induces the expression of the proglucagon gene in both immortalized enteroendocrine cells and in non-transformed intestinal epithelium²⁸.

Foxa2 enhanced *Gcg* gene expression, but suppressed the *Ins2* expression that was induced by the cotransfection of *Isl1* and *Neurod1*. Consistent with our results, a *Foxa2* conditional knockout mouse shows reduced numbers of glucagon-producing α -cells²⁹. Thus, *Foxa2* might be another determinant factor for islet cell lineage, by inducing pancreatic α -cell differentiation and repressing pancreatic β -cell differentiation.

Although *Mafa* is a crucial transcription factor for the maintenance of pancreatic β -cell function, *Gcg* gene expression was also induced by the cotransfection of the *Neurod1* and *Mafa* genes in our study. Indeed, the *Mafa* protein is reported to bind to the G1 element of the glucagon promoter, although its binding is weaker than that of *Mafb*³⁰. Thus, in our system, *Mafa* overexpression appeared to induce *Gcg* gene expression, and this induction was inhibited by the additional transfection of the *Ipfl* gene. Consistent with this observation, the overexpression of *Ipfl* suppresses the expression of *Gcg* in INSR α -cells, a subclone of INS-1 cells in which both the insulin and glucagon genes are expressed³¹, and in InRIG9, a glucagonoma cell line³². Thus, our results suggest that *Ipfl* gene expression induces pancreatic β -cell differentiation and suppresses that of pancreatic α -cells.

Another interesting finding was the differentiation of somatostatin-producing cells after the introduction of the *Isl1* and

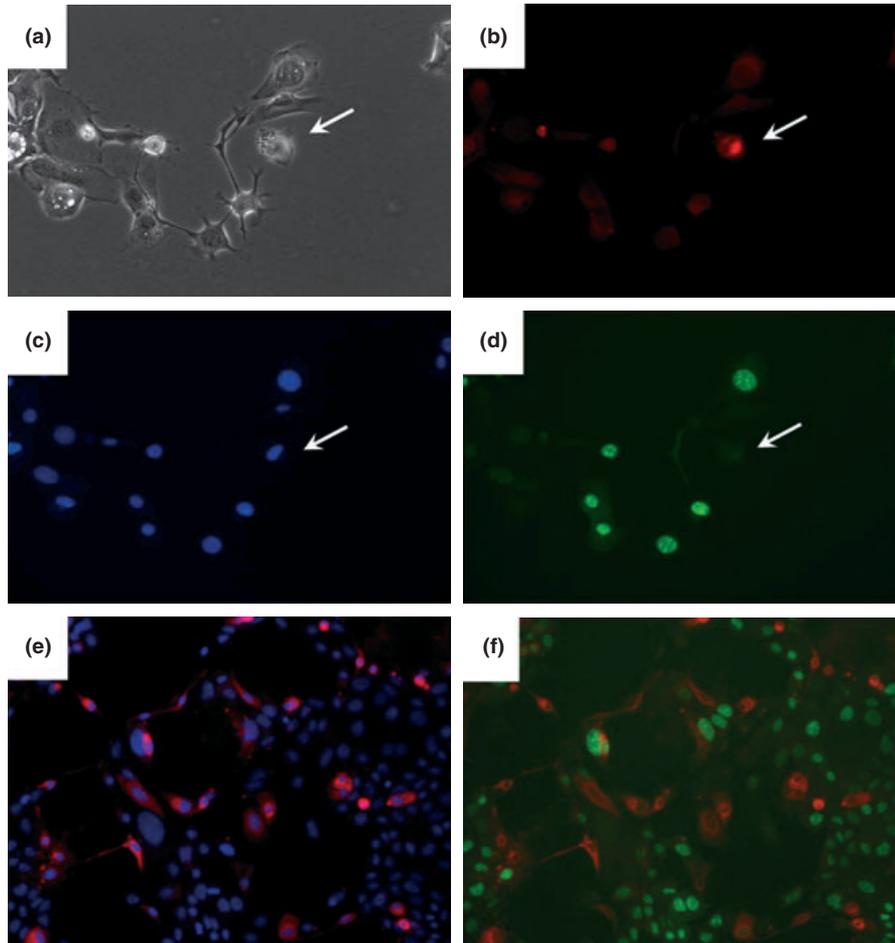


Figure 7 | Proliferative capacity of the differentiated polyoma large T pdx-1-positive pancreatic cell-derived (PLT-PPPD) cells. Proliferative capacity of the (a–d) insulin-positive and (e,f) amylase-positive cells. (a–d) pPyCAG vectors expressing *Ipfl1*, *Neurod1* and *Mafa* were cotransfected into PLT-PPPD cells. (a) Phase contrast view of the differentiated cells. (b) Cells stained with an anti-insulin antibody (red). (c) Nuclei were stained with DAPI (blue) or (d) with an anti-Ki67 antibody (green). Solid arrow shows cells stained with insulin, but not Ki67. (e,f) pPyCAG vectors expressing *Ptf1a* and *Foxa2* were cotransfected into PLT-PPPD cells. (e) Cells stained with an anti-amylase antibody (red) and nuclei stained with DAPI (blue). (f) Cells stained with anti-amylase (red) and anti-Ki67 (green) antibodies. Most of the amylase-positive cells were not stained with the anti-Ki67 antibody.

Neurod1 genes. This combination of introduced genes induced the expression of both *Sst* and *Ins2*. Our immunohistological analysis showed insulin-only-producing cells and somatostatin-only-producing cells. *Isl1* is an important transcription factor for the development of pancreatic islets; it is expressed in every hormone-producing cell in them³³ and in the progenitor cells of pancreatic endocrine cells³⁴. Thus, *Isl1* is an important factor for the cell lineage determination of pancreatic endocrine cells. In our experiments, the single introduction of the *Isl1* gene into PPPD cells did not induce the expression of any islet hormone genes, but its coexpression with *Neurod1* induced the expression of the *Sst* gene. Indeed, *Isl1* is known to promote *Sst* gene expression^{35,36}. Our report is the first to show that exogenous *Isl1* gene expression induced the differentiation of somatostatin-producing cells from pancreatic cells, suggesting that *Isl1* plays a role in the development of pancreatic δ -cells.

Interestingly, the additional transfection of the *Mafa* gene, along with the combination of *Isl1* and *Neurod1*, suppressed the *Sst* gene expression, whereas the additional transfection of *Ipfl1* did not. This result is reasonable, considering that *Ipfl1*, but not *Mafa*, is endogenously expressed in the pancreatic δ -cells. The coexpression of *Foxa2* also inhibited the somatostatin gene expression. Thus, both *Mafa*, an inducer for pancreatic β -cells, and *Foxa2*, an inducer for pancreatic α -cells, inhibited the differentiation into pancreatic δ -cells.

PP is another hormone that is secreted from the pancreatic islets. There are only few reports on the development of pancreatic PP cells. In the early developing pancreas, Pdx-1 protein is expressed in both somatostatin-producing and PP-producing cells³⁷. Furthermore, a recent study using *Nkx2.2*- and *Neurod1*-knockout mice showed that the *Neurod1* gene is important for the development of pancreatic α - and PP cells³⁸. In our

experiments, the PP gene was induced by the coexpression of *Neurod1* and *Ipf1*, whereas it was suppressed by *Foxa2* or *Mafa*. Because *Neurod1* and *Ipf1* induced not only the PP gene, but also the *Ins2* gene, this combination is not a determinant for the differentiation of pancreatic PP cells. The repressive effect of the *Foxa2* and *Mafa* genes on PP cell differentiation is noteworthy, as it resembles the repressive effect on the δ -cell differentiation, described above.

In our experiments, *Ptf1a* could induce *Cpa1*, but not amylase gene expression in PPPD cells, although *Ptf1a* is known to be a determinant factor for pancreatic exocrine cell differentiation^{39,40}. A recent report showed that a population in the embryonic pancreas that is *Pdx1(+)* *Ptf1a(+)* *cMyc(High)* *Cpa1(+)* and negative for differentiation markers represents potent multipotent progenitors in the pancreas⁴¹. Thus, *Cpa1* is a possible marker for endodermal progenitor cells. It is therefore not surprising that there were *Cpa1*-positive and *Amy2*-negative cells, which might represent or resemble these progenitors, among the differentiated PPPD cell populations.

In PPPD cells, *Foxa2* gene expression in combination with the *Ptf1a* gene was required for amylase gene expression. The *Foxa2* gene is expressed in pancreatic exocrine cells⁴², in which *Foxa2* and *Ptf1a* gene coexpression induces the α -amylase gene⁴³. Therefore, the differentiation into pancreatic exocrine cells would be achieved by the combination of the *Foxa2* and *Ptf1a* genes.

The present study showed that the expression of transcription factors could induce the differentiation of PPPD cells into the exocrine cell lineage and each of the endocrine cell lineages, including insulin-producing cells, glucagon-producing cells and somatostatin-producing cells. Figure 8 shows the differentiation pathways from PPPD cells into the exocrine and endocrine cell lineages revealed by the expression of transcription factors, which are mostly consistent with the proposed pathways of cell-lineage specification in the developmental pancreas determined by gene ablation studies^{44–47}. The importance of *Ipf1*, *Neurod1*

and *Mafa* in β -cell specification and of *Pax6* in α -cell specification was also confirmed by the present study, as described earlier. Furthermore, our analysis suggests the presence of some kinds of intermediate cells during the differentiation of progenitor cells into specific endocrine cell lineages.

Finally, to obtain evidence that the transfected PLT-PPPD cells were indeed differentiating, and not merely expressing marker genes while maintaining their stem-cell identity, we examined their proliferation. As expected for terminally differentiated cells, we found little proliferation among the cells that expressed endocrine or exocrine markers because of the exogenously introduced transcription factor genes, consistent with the idea that the marker-expressing cells were differentiated.

In conclusion, we established a novel experimental system for investigating the effects of transcription factor gene expression on pancreatic stem/precursor cells. This system should provide a convenient tool for investigating the transcription factor cascades in pancreatic cell lineage determination.

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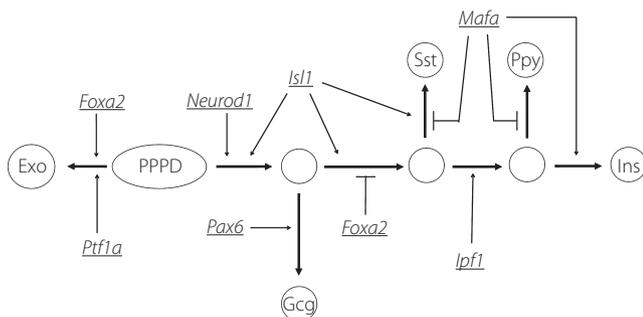


Figure 8 | Schematic view of the pdx-1-positive pancreatic cell-derived (PPPD) cell differentiation induced by transcription factors. Transcription factors control the differentiation of PPPD cells into pancreatic exocrine and endocrine cell lineages. The differentiation pathways shown by the expression of transcription factors in PPPD cells are schematically presented.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Antibodies used in this study.

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