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...
involved in maintaining the undifferentiated state of ES cells and to induce the differentiation of cells of a specific lineage. Camenisch et al. also reported that this polyoma-based episomal vector can replicate in cell lines other than ES cells, including the mouse embryonic carcinoma F9, mouse erythroleukemia (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 mice. 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 adeno-virus-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 described. 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 proliferative capacity. 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.

Characterization of PLT-PPPD Cells

The episomal vector pMGD20neo was recovered from MG1.19 ES cells by Hirt’s method. 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. The pPyCAG vector was constructed from pCAGGS 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 guanidium-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. 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
parental PPPD cells: neither the PPPD nor the PLT-PPPD cells expressed insulin1 (Ins1), insulin2 (Ins2), glucagon (Gcg), somatostatin (Sst), carboxypeptidase1 (Cpa1) or amylase2 (Amy2). Immunostaining showed that the PLT-PPPD cells expressed CK19 and Ipf-1, 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\textsuperscript{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-PPPD 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 cotransfected 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-PPPD 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-PPPD Cells

To analyze the effects of transcription factor gene expression on the differentiation of pancreatic cell lineages from PLT-PPPD cells, pPyCAG vectors expressing various transcription factor genes were introduced into PLT-PPPD 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, Ipf1 (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 Ipf1 encode transcription factors that synergistically stimulate insulin gene transcription. When all three of these genes were co-introduced into PLT-PPPD cells, the expression of both the Ins2 and the Ins1 gene was induced, although the level of Ins2 gene expression in these PLT-PPPD 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 Ipf1. The expression of Mafa with Ipf1 and Neurod1 enhanced the expression of Ins2. In contrast, the co-introduction of Foxa2 with Isl1 and Neurod1
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
Gene transfer in pancreatic progenitors

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 Ipf1. PP (Ppy) expression was induced by the addition of Isl1 and Ipf1 with Neurod1.

Immunofluorescence analysis showed that PLT-PPPd cells transfected with Neurod1, Mafa and Ipf1 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 Ipf1 contained significantly enhanced levels of insulin compared with cells transfected with the control vector (99.2 ± 37.6 vs 5.9 ± 3.7 ng/10⁵ 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 Ipf1 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 Foxa 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 3b).

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 3c). Immunofluorescence analysis also showed a number of cells that produced somatostatin or insulin (Figure 3d,e). 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 3d).

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 Ipf1 (Figure 3a–d) or with Ptf1a and Foxa2, most of the undifferentiated cells were Ki67-positive, but only a few differentiated cells were Ki67-positive (Figure 3e,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 2a–e). 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.
The combined transfer of the Ipf1, 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 Ipf1 gene (data not shown), as the original PPPD cells did\textsuperscript{7}, 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 Ipf1 was too low to induce expression of the insulin gene, so transfer of the additional Ipf1 gene was required to induce it.

The Ipf1, Neurod1 and Mafa transcription factor genes were previously shown to synergistically induce insulin gene expression\textsuperscript{14–16}. Furthermore, the simultaneous introduction of these three genes into the liver leads to the neogenesis of insulin-producing cells\textsuperscript{17}. 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 line\textsuperscript{18}.

Our experiments in which transcription factors were transfected in combination with Neurod1 showed that either Isl1 or Ipf1 increased the Ins2 gene expression, whereas Mafa in combination with Neurod1 did not. However, Mafa was required for Ins1 gene expression when Ipf1 and Neurod1 were cotransfected. Thus, Mafa’s promotion of insulin gene transcription appeared to occur in the context of coexpressed Neurod1 and Ipf1. Indeed, a Mafa knockout mouse has pancreatic islets that appear normal, but their insulin secretory response to glucose gradually deteriorates\textsuperscript{19}. 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 \textit{in vitro}\textsuperscript{20}. Concordantly, the deletion of Foxa2 stimulates insulin release from islets \textit{in vivo}\textsuperscript{21}. Thus,
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\(^\text{22-24}\) and is essential for the development of glucagon-producing pancreatic α-cells\(^\text{25,26}\) and enteric L cells\(^\text{27}\). 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 epithelium\(^\text{28}\).

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-cells\(^\text{29}\). 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\(^\text{30}\). Thus, in our system, Mafa overexpression appeared to induce Gcg gene expression, and this induction was inhibited by the additional transfection of the Ipf1 gene. Consistent with this observation, the overexpression of Ipf1 suppresses the expression of Gcg in INSr α-cells, a subclone of INS-1 cells in which both the insulin and glucagon genes are expressed\(^\text{31}\), and in InR1G9, a glucagonoma cell line\(^\text{32}\). Thus, our results suggest that Ipf1 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
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 expression35,36. 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 Ipf1 did not. This result is reasonable, considering that Ipf1, 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
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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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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