Regulated Expression of pdx-1 Promotes In Vitro Differentiation of Insulin-Producing Cells From Embryonic Stem Cells

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Embryonic stem (ES) cells can differentiate into many cell types. Recent reports have shown that ES cells can differentiate into insulin-producing cells. However, the differentiation is not efficient enough to produce insulin-secreting cells for future therapeutic use. Pdx-1, a homeodomain-containing transcription factor, is a crucial regulator for pancreatic development. We established an ES cell line in which exogenous pdx-1 expression was precisely regulated by the Tet-off system integrated into the ROSA26 locus. Using this cell line, we examined the effect of pdx-1 expression during in vitro differentiation via embryoid body formation. The results showed that pdx-1 expression clearly enhanced the expression of the insulin 2, somatostatin, Kir6.2, glucokinase, neurogenin3, p48, Pax6, PC2, and HNF6 genes in the resulting differentiated cells. Immunohistochemical examination also revealed that insulin was highly produced in most of the differentiated ES cells. Thus, exogenous expression of pdx-1 should provide a promising approach for efficiently producing insulin-secreting cells from human ES cells for future therapeutic use in diabetic patients. *Diabetes* 53: 1030–1037, 2004

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst-stage embryos and contribute to all adult tissues, including germ cells, when reintroduced into host blastocysts (1). To date, ES cells have been shown to differentiate in vitro into various cell lineages, including those of hematopoietic precursors, heart muscle, skeletal muscles, endothelium, and neural cells (2,3). In most cases, these cells were differentiated via embryoid bodies (EBs) produced when the ES cells are grown as aggregates in suspension (2,4).

Recently, we and other groups have reported that ES cells can differentiate into insulin-producing cells. Soria and colleagues (5,6) reported that mouse ES cells transfected with the β-geo gene driven by the human insulin promoter differentiate into insulin-producing cells. Lumelsky et al. (7) developed a five-stage method, including a selection step for nestin-positive cells, for the differentiation of mouse ES cells into insulin-secreting cells, although there is an argument that insulin detected in the differentiated cells was concentrated from the culture medium (8). Recently, we reported the differentiation of insulin-producing cells from a feeder-free ES cell line harboring the β-geo gene under the control of the mouse insulin 2 promoter by a method similar to the five-stage method. The differentiated cells that were derived from this ES cell line expressed not only the insulin 2, glucagon, somatostatin, and PP genes, but also the genes encoding p48, amylase, and carboxypeptidase A, which are exocrine cell-specific. These results suggested that ES cells can differentiate not only into endocrine cells but also into exocrine cells of the pancreas (9). Recent reports showed that growth inhibitors or the expression of Pax4 promoted the differentiation of insulin-producing tissue from embryonic stem cells (10,11). Using human ES cells in both adherent and suspension culture conditions, Assady et al. (12) observed spontaneous in vitro differentiation that included the generation of cells with characteristics of insulin-producing β-cells.

Organogenesis involves a sequential cascade of inductive events in association with the activation of specific transcription factors (13–15). It is also becoming clear that some transcription factors possess early functions related to the differentiation of various cell types and late functions related to their maintenance. The pancreas is an organ with two distinct functions: exocrine secretion of digestive enzymes into the gut and endocrine secretion of hormones into the bloodstream (16). A number of transcription factors are specifically involved in the development and commitment of progenitor cells to either the exocrine or endocrine pancreas (17). Pdx-1 is homologous to a *Xenopus* endoderm-specific homeodomain protein, XIHbox8. In fact, pdx-1 transactivates insulin gene expression through conserved enhancer elements, and is also an essential regulator of pancreatic development. In mice lacking pdx-1, the development of the pancreas is blocked at a very early stage, and they have no or diminished numbers of endocrine cells in the rostral duodenum and stomach (18,19). Therefore, pdx-1 expression seems likely to be very important in the in vitro differentiation of ES cells along pancreatic cell lineages.

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bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; Dox, doxytetracycline; EB, embryoid body; EGFB, enhanced green fluorescent protein; ES, embryonic stem; IRES, internal ribosome entry site; LIF, leukemia inhibitory factor; tTA, tetracycline-regulated transcriptional activator; TUNEL, transferase-mediated dUTP nick-end labeling.

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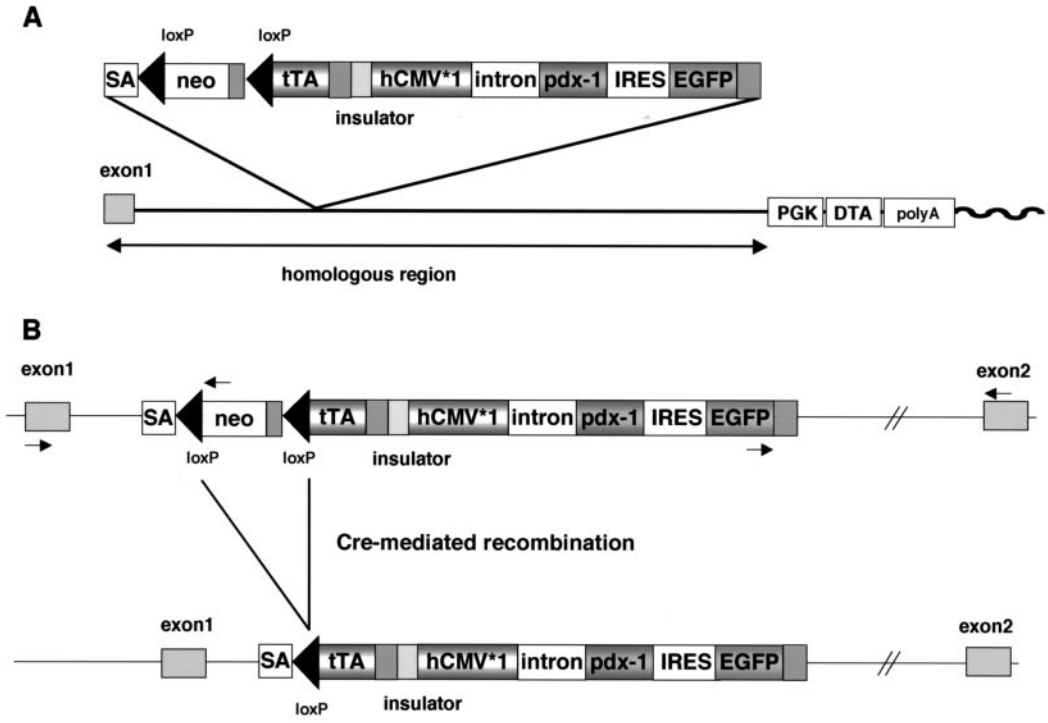

FIG. 1. Strategy for generating ES cells (RTF-pdx-1) that show regulable expression of pdx-1 by tetracycline. We integrated the Tet-off regulation unit for pdx-1 expression into the ROSA26 locus. See RESEARCH DESIGN AND METHODS for a full description of this unit. The resulting knock-in vector was linearized by *Swa*I digestion and used to transfect ES cells by electroporation. ES cells were selected with G418. Correctly targeted clones were identified by long-PCR.

We noted that the levels of pdx-1 expression were very low throughout the differentiation stages (9). Thus, we expected the strategy of leading the ES cells to express the transcription factor pdx-1 during in vitro differentiation would improve the efficiency of producing cells of the pancreatic lineage, and especially β-cells. Based on these considerations, we attempted to inducibly express exogenous pdx-1 during the in vitro differentiation of ES cells.

First, we produced an ES cell line in which exogenous pdx-1 expression was precisely regulated by the Tet-off system integrated into the ROSA26 locus. Transgenes integrated into this locus are expressed without any tissue- or developmental stage-specificity (20). The resulting ES clones constitutively and homogeneously expressed tetracycline-regulated transcriptional activator (tTA) under the ROSA26 promoter. The tTA protein activated the downstream CMV*1 promoter to produce pdx-1 as well as enhanced green fluorescent protein (EGFP). Tetracycline or doxycycline (Dox) can bind to tTA and inhibit this activation. Using this cell line, we examined the effect of expressing pdx-1 on the in vitro differentiation of ES cells via EB formation. We found that pdx-1 expression clearly enhanced the differentiation of insulin-producing cells, which was accompanied by the increased expression of various pancreatic markers.

RESEARCH DESIGN AND METHODS

DNA construct. A backbone knock-in vector for the ROSA26 locus was kindly provided by Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, Washington) (20,21). We integrated the Tet-off regulation unit for pdx-1 expression into this vector (Fig. 1*A*). This unit included a splice-acceptor sequence; a loxP-flanked neomycin phosphotransferase gene including a polyA signal; the tTA gene from pUHD15–1; a polyA signal; an insulator sequence; the CMV*1 promoter, which is responsive to tTA; the rabbit β-globin second intron; mouse pdx-1 cDNA; an internal ribosome entry site (IRES); EGFP cDNA; and a polyA signal. The pdx-1 cDNA was obtained from an RNA sample of MIN6 pancreatic β-cells (22) by RT-PCR. The insulator sequence, which was derived from the chicken β-globin gene (23), was inserted between the tTA expression unit and the tTA-responsive pdx-1 expression unit to suppress the possible interaction of these two units. The resulting knock-in vector was amplified in *E. coli* SURE cells (Stratagene, La Jolla, CA), purified using a CONCERT plasmid purification kit (Life Technologies, Rockville, MD), linearized by *Swa*I digestion, and used to transfect ES cells.

ES cell culture and transfection. The murine ES cell line, EB3, which was derived from E14tg2a ES cells, was maintained without feeders in Glasgow minimum essential medium supplemented with 10% FCS (Equitech-Bio, Kerrville, TX), 2-mercaptoethanol, and leukemia inhibitory factor (LIF) (9). RTFN-pdx-1 ES cells were established by introducing the above knock-in vector into the ROSA26 locus of EB3 cells. Briefly, EB3 ES cells were electroporated with the linearized knock-in vector and selected with G418 (150–200 μg/ml). G418-resistant colonies were picked, and ES clones carrying a targeted integration of the vector in the ROSA26 locus were identified by long-PCR analysis (see Fig. 1) using the following primers: forward (ROSA26 locus 1st exon), 5′-CCTCGGCTAGGTAGGGGATCGGGACTCT-3′; reverse (neomycin phosphotransferase gene), 5′-CGGAGAACCTGCGTGCAATCCATCTT GTTC-3′; forward (EGFP), 5′-GGATCACTCTCGGCATGGACGAGCTGTAC-3′; and reverse (ROSA26 locus 2nd exon), 5′-AGCCTTAAACAAGCACTGTCCTGTC CTCAAG-3′. The PCR cycles consisted of one cycle at 94°C for 1 min, 32 cycles at 98°C for 20 s, 66°C for 30 s, 68°C for 4 min, and one cycle at 72°C for 10 min. We obtained two knock-in clones out of 43 G418-resistant colonies. Cre recombinase was expressed in these clones in the presence of Dox (1 μg/ml) by transient transfection of the pCAG-cre-IRES-puro plasmid to remove the loxP-flanked neomycin phosphotransferase gene (24) (Fig. 1*B*). The resulting ES clones were designated RTF-pdx-1 cells.

In vitro differentiation procedure. RTF-pdx-1 ES cells were subjected to an in vitro differentiation procedure basically as described previously (9). Before the induction of differentiation (stage 0), the ES cells were cultured in the presence of Dox (Fig. 3). The cells were dissociated with 0.25% trypsin/0.04% EDTA in PBS and plated onto bacterial culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS (stage 1) in the presence or absence of Dox, as indicated. Embryoid bodies (EBs) then formed within 4–5 days. The EBs were replated onto gelatinized plates in DMEM containing 10% FCS. By the next day, most of the EBs had attached to the plates (stage 2). The medium was replaced with serum-free ITSFn medium

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(25), and the cells were cultured for 3–6 days (stage 3). The surviving cells were dissociated and replated onto gelatinized plates in media hormone mix medium; that is, DMEM/F12 (1:1) medium containing 25 μg/ml insulin, 100 μg/ml transferrin, 20 nmol/l progesterone, 60 μmol/l putrescine, 30 nmol/l sodium selenite, 10 ng/ml human keratinocyte growth factor (KGF) (Peprotech EC, London, U.K.), 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich), 25 ng/ml basic fibroblast growth factor (bFGF) (Strathmann Biotech, Hamburg, Germany), B27 supplement (Invitrogen), and 10 ng/ml nicotinamide (9), and cultured for 6–8 days (stage 4). In stage 5, the expanded cells were dissociated and replated onto gelatinized plates in media hormone mix medium without bFGF, EGF, or KGF.

RT-PCR analysis. Total RNA was prepared from the ES-derived cells at culture stages 4 and 5 by the acid guanidinium thiocyanate-phenol-chloroform method. Two micrograms of total RNA was reverse transcribed using reverse transcriptase (ReverTraAce-α; Toyobo, Osaka, Japan) with oligo dT primers. The primer sequences and PCR conditions used for the RT-PCR were previously described (9).

Immunohistochemical analysis. Cells were fixed with 1% paraformaldehyde in PBS for 10 min, washed twice with PBS, and treated with PBS containing 0.1% Triton X-100 for 10 min at room temperature for insulin immunostaining or with ethanol/acetic acid (2:1) for 5 min at −20°C for transferase-mediated dUTP nick-end labeling (TUNEL) and C-peptide immunostaining. After being blocked with 1% normal goat serum for 20 min at room temperature, the cells were incubated for 1 h at room temperature with the following primary antibodies: guinea pig anti-insulin (Dako, Kyoto, Japan), guinea pig anti-rat C-peptide (Linco Research, St. Louis, MO), and rabbit anti–pdx-1 (a gift from Dr. Y. Kajimoto, Osaka University Medical School, Japan). The cells were then washed four times with PBS and incubated with the appropriate secondary antibodies labeled with Alexa 594 (Molecular Probes, Eugene, OR) for 1 h at room temperature. After being washed twice with PBS, the cells were observed by confocal immunofluorescence microscopy using a Leica TCS SP2 confocal microscope system (Leica Microsystems, Wetzlar, Germany).

TUNEL and insulin double staining. Cells were fixed with 1% paraformaldehyde in PBS for 10 min, washed twice with PBS, and treated with ethanol/acetic acid (2:1) for 5 min at −20°C. TUNEL staining was performed using an ApopTag Fluorescein In Situ Apoptosis Detection Kit (Talron, Israel). The cells were incubated for 1 h at room temperature with guinea pig anti-insulin and then with goat anti–guinea pig IgG Alexa Fluor 594 for 1 h at room temperature. Confocal immunofluorescence microscopy was performed using the Leica confocal microscope system as above.

Assays for insulin secretion. For static incubation, stage 5 cell clusters were cultured in 12-well plates and allowed to grow for 4–6 days. The cells were washed four times and incubated in 1 ml DMEM containing 10 mmol/l HEPES buffer at pH 7.5 and 3.3 mmol/l glucose for 30 min. The cells were washed five times and incubated in 1 ml DMEM containing 10 mmol/l HEPES buffer at pH 7.5 and 3.3 mmol/l glucose for 30 min, then washed five more times and incubated in Krebs-Ringer buffer (+0.2% BSA) containing 3.3 or 33 mmol/l glucose at 37°C. The culture supernatants were collected after 1 h of incubation. The insulin concentration of the culture supernatants was determined using an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). To normalize the amount of insulin secretion, the total protein of the cells in each well was measured by the Bradford method.

Insulin content. Stage 5 cell clusters were collected from a 60-mm dish and washed twice, and half of them were homogenized with acidified EtOH. The rest were homogenized with radioimmunoprecipitation buffer. The supernatant of the first sample was neutralized with saturated $NaHCO_3$ and assayed for insulin using an enzyme-linked immunosorbent assay kit, as described above. The second sample was used to measure the protein concentration by the Bradford method in order to normalize the amount of insulin content. Each experiment was performed in duplicate.

RESULTS

Establishment of an ES cell line with tetracyclin-regulable pdx-1 expression. We could not obtain stable transfectant ES cell clones expressing pdx-1 under the β-actin promoter using the conventional transfection approach, probably because the high-level expression of pdx-1 was toxic to ES cells. We then tried to establish an ES cell line with tetracyclin-regulable pdx-1 expression by integrating the Tet-off system into the ROSA26 locus. We designed a knock-in vector that included a tTA expression unit and a tTA-responsive pdx-1 expression element (Fig. 1*A*). When this vector was integrated into the ROSA26 locus, a loxP-flanked neomycin phosphotransferase gene (neo^r) was first expressed under the ROSA26 promoter, conferring G418 resistance. ES cell clones in which this vector had been correctly knocked-in were subjected to transient expression of Cre recombinase to remove the neo^r gene (Fig. 1*B*). The resulting ES clones constitutively expressed tTA under the ROSA26 promoter and were designated the RTF-pdx-1 cell lines.

The tTA protein activates the downstream CMV*1 promoter to produce pdx-1 as well as EGFP. Tetracycline or Dox binds to tTA and inhibits this activation. The insulator sequence placed between the tTA gene and the tTA-responsive element was derived from the chicken β-globin locus (23) and included to prevent any possible interaction between these two expression units. However, we do not know the precise effect of this insulator sequence in this specific construct, since we did not test the construct lacking it. The expression of transgenes is often down-regulated and/or heterogeneous in ES cells, probably because the activity of the promoter tends to be silenced, especially after differentiation (26). However, transgene expression in this RTF-pdx-1 cell line was strictly regulated, that is, almost completely repressed in the presence of Dox, but induced in almost all cells upon withdrawal of Dox, as shown by the EGFP expression (Fig. 2*C* and *D*), Western blot analysis of pdx-1 (Fig. 2*A*), and immunostaining for pdx-1 (Fig. 2*B*).

We obtained two independent RTF-pdx-1 clones. Both clones showed inducible expression of pdx-1 and EGFP after withdrawal of Dox and gave similar results in the in vitro differentiation experiments (data not shown). Thus, in this study we show the results from one RTF-pdx-1 clone.

Effects of pdx-1 expression on in vitro differentiation. We previously applied the four-stage differentiation method, including EB formation and nestin-positive cell selection, to feeder-free ES cells and showed that ES-derived cells produced insulin in the final stage of in vitro differentiation (9). In the present study, we used basically the same protocol for in vitro differentiation (Fig. 3). We allowed RTF-pdx-1 cells to form EBs in the absence of LIF and induced the expression of pdx-1 from this initial stage of differentiation by removing Dox [Dox(−)]. In control experiments, Dox was present throughout the in vitro differentiation stages [Dox(+)], or in stage 2, but was absent in the subsequent stages [Dox(+)(−)]. The presence or absence of Dox did not affect EB formation. In the final stage of in vitro differentiation, Dox(+) cells did not proliferate well. On the other hand, Dox(−) cells could be cultured for >2 months. Thus, the exogenous pdx-1 expression appeared to affect the cell growth of the differentiated cells.

To investigate the effect of pdx-1 expression on the differentiation of ES cells, the expression of a set of marker genes for the pancreatic lineage was examined by RT-PCR at stages 4 and 5 of in vitro differentiation (Fig. 4). Two nonallelic insulin genes, insulin 1 and insulin 2, are expressed in rodents. The insulin 2 gene was detected in ES cells that differentiated without exogenous pdx-1 expression, consistent with our previous report (9), but pdx-1 expression throughout the differentiation stages clearly enhanced the insulin 2 expression.

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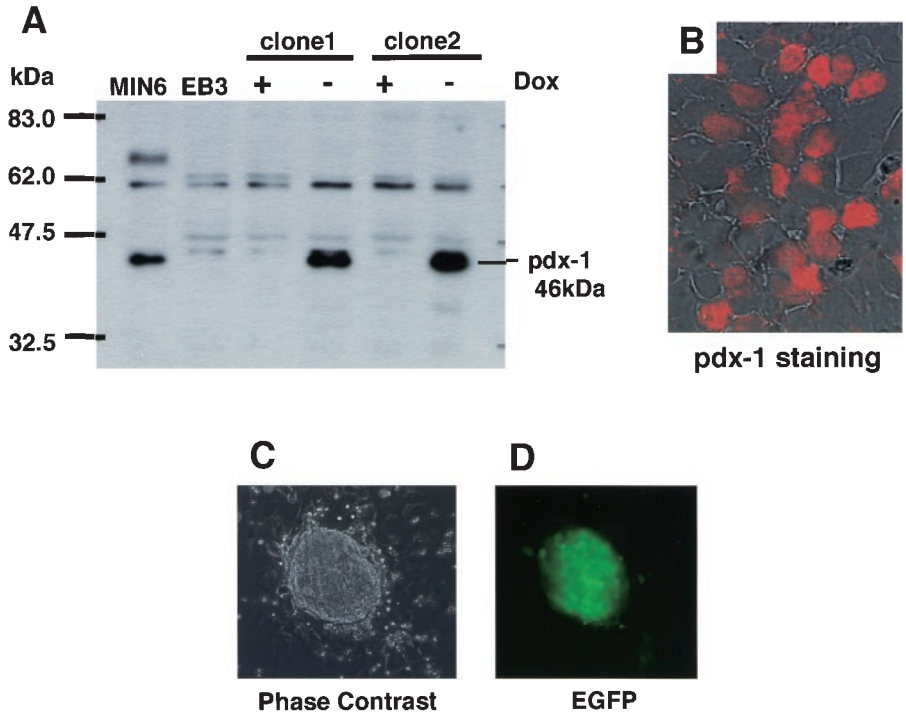

FIG. 2. Tet-regulable pdx-1 expression in RTF-pdx-1 ES cells. *A*: Western blot analysis of pdx-1 expression in RTF-pdx-1 cells. RTF-pdx-1 cells were cultured for 5 days in the absence (−) or presence (+) of Dox, then the cells were harvested and used for Western blot analysis for pdx-1 expression. *B*: Pdx-1 immunostaining of RTF-pdx-1 cells. *C* and *D*: When pdx-1 gene expression was induced, the downstream EGFP gene was also induced.

Moreover, a number of pancreas-specific genes were strongly induced by the exogenous expression of pdx-1 during in vitro differentiation. In view of the developmental lineage of pancreatic islets, it is noteworthy that not only the pancreatic β-cell–specific insulin 2 gene but also other genes specific for the pancreas were induced by pdx-1. These included the genes for somatostatin, prohormone convertase 2 (PC2), Kir6.2, glucokinase, p48, nkx2.2, HNF6, Pax4, Pax6, and neurogenin3. Thus, pdx-1 induces the expression of genes that are specific to the pancreatic cells during in vitro differentiation and drives the differentiation to pancreatic endocrine cells. In contrast, as shown in the Dox(+)(−) sample in Fig. 4, the exogenous expression of pdx-1 after EB formation did not enhance the expression of the insulin 2 gene or other genes associated with the development of the endocrine pancreas except for somatostatin and Kir6.2. Thus, the expression of pdx-1 during EB formation is important for the induction of the differentiation to the pancreatic lineage.

On the other hand, we did not observe the expression of the insulin 1, glucagon, PP gene, or Glut2 gene, which are all specific to the endocrine pancreas in vivo, even under continuous pdx-1 expression (Fig. 4). Although we do not know the reason for this, we speculate that the continuous expression of exogenous pdx-1 expression can prime pancreatic progenitors during EB formation, but may instead suppress the expression of some endocrine-specific genes in the final stage.

The fact that the pdx-1-null mutant mouse had defects in both the endocrine and exocrine cell lineages of the pancreas is compatible with the idea that the endocrine and exocrine cell types both arise from a common pool of pdx-1–positive stem cells. Exocrine pancreas-specific gene expression is under the control of the cell type–specific

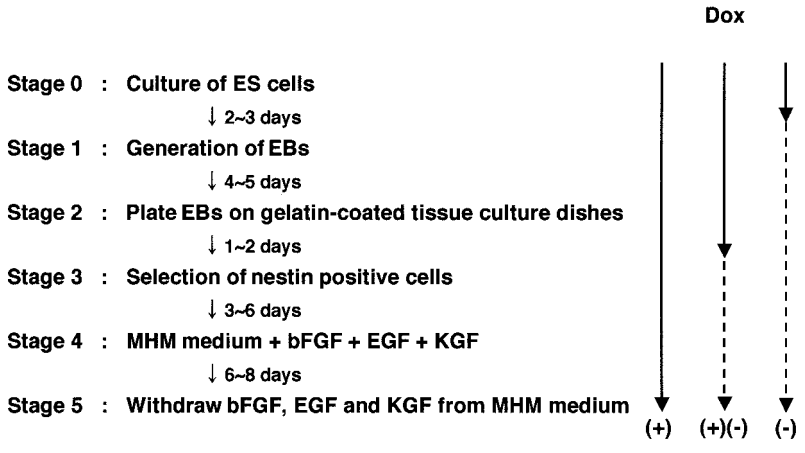

FIG. 3. Protocol for in vitro differentiation of RTF-pdx-1 cells. Stage 0 cells are undifferentiated ES cells. Stage 1 cell clusters are EBs cultured in the absence of LIF. Stage 3 cell clusters are the surviving cells grown under serum-free conditions. Stage 4 cell clusters are cultured in the presence of growth factors. Stage 5 cell clusters are cultured in the same medium without growth factors.

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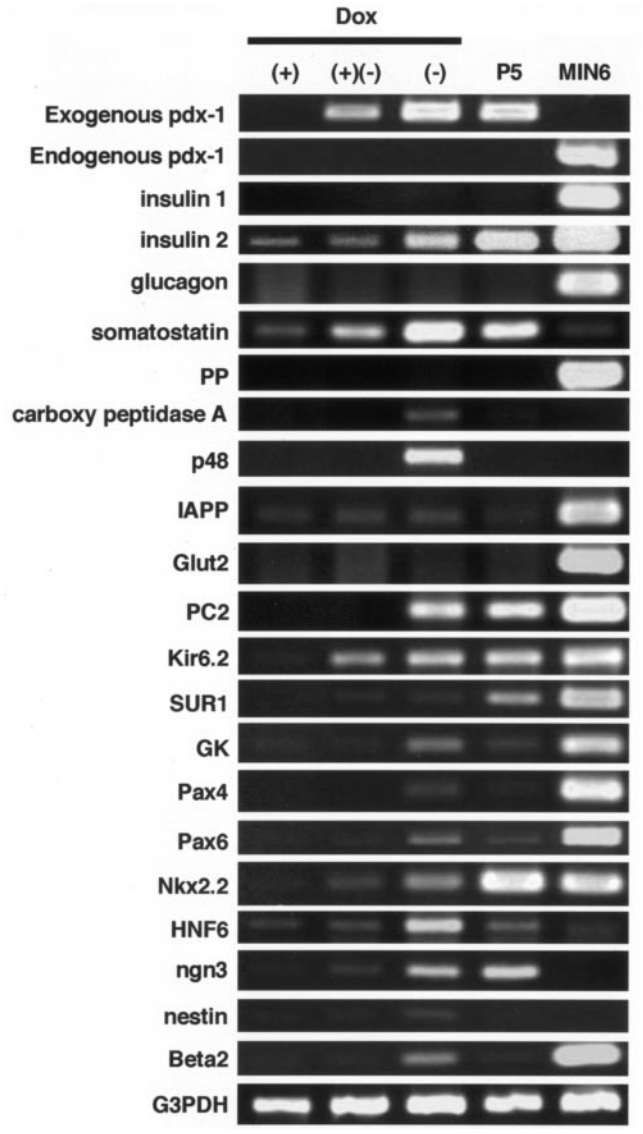

FIG. 4. Gene expression in the RTF-pdx-1 cells in stage 4 of in vitro differentiation. RT-PCR analysis was performed with RNAs from the differentiated cells. Dox(+): cultured with Dox in stages 1–4. Dox(+)(−): cultured with Dox in stages 1–2, but without Dox in stages 3–4. Dox(−): differentiated without Dox throughout the stages. P5: gene expression in the RTF-pdx-1 cells in Dox(−) stage 5 of in vitro differentiation. MIN6: RNA from MIN6 insulinoma cells served as a positive control.

transcription factor PTF (27). The p48 subunit of PTF-1 is considered to be the only cell type–specific constituent of the PTF-1 complex. In our study, p48 gene expression was dependent on the exogenous pdx-1 expression (Fig. 4). In addition to p48, the expression of carboxypeptidase A (CpA), which is one of the digestive enzymes secreted from the exocrine pancreas, was induced by pdx-1 expression, although the expression level appeared to be very low. Dox(−) stage 5 cell clusters could be maintained for >2 months by weekly serial passages. RT-PCR analysis of these cells at passage 5 is shown in Fig. 4. Interestingly, the CpA and p48 gene expression was suppressed in these cells, which continuously expressed pdx-1, even though these genes were induced only when the exogenous pdx-1 gene had been turned on during EB formation. Prolonged culture of the Dox(−) cells might inhibit the differentiation of pancreatic progenitor cells to the exocrine pancreatic lineage. Alternatively, the culture conditions used in this study might select away the cells that expressed p48 and CpA.

Immunohistochemical analysis of stages 4 and 5 cell clusters. We next performed immunostaining with an anti-insulin antibody of stage 4 or 5 cells forming aggregates or "clumps" on a gelatinized dish. The Dox(+) cell clumps showed only weak insulin immunoreactivity (Fig. 5*A*). In contrast, most cells in the Dox(−) cell clumps showed strong and homogeneous insulin immunoreactivity (Fig. 5*B*). Since there is an argument that the insulin detected in the differentiated cells was concentrated from the culture medium and that these insulin-positive cells were apoptotic (8), we performed insulin and TUNEL double staining and C-peptide staining. Most of the cells in the Dox(−) cell clumps and spheres were stained with the anti-insulin antibody, and a marginal subset of these cells was TUNEL positive, indicating apoptosis. However, most of the cells in the center of the colony and spheres were insulin-positive and -negative for TUNEL staining (Fig. 5*C*). The results of the C-peptide immunostaining confirmed that most of the cells in the Dox(−) cell clumps were producing insulin, although at lower levels than MIN6 cells (Fig. 5*D*).

Glucose-induced insulin secretion from stage 5 cell clusters. Stage 5 cell clusters were examined for glucose-induced insulin secretion (Fig. 6). They secreted insulin at 8.3 and 10.9 ng · mg protein^{-1} · h^{-1} when cultured with 3.3 and 33 mmol/l glucose, respectively. Thus, the differentiated pdx-1–expressing ES cells did not show a significant response in insulin secretion to glucose. The insulin content of the stage 5 cell clusters was estimated to be 54 ng/mg protein.

DISCUSSION

The capacity of ES cells for multilineage differentiation is partially reproduced in culture, in that ES cells can differentiate into a wide range of well-defined cell types. ES cells might represent a limitless source of specific cell types for transplantation. To date, differentiation into specific cell types has been promoted by modifying the culture conditions or by adding various growth factors or cytokines. In the present study, we adopted another approach to direct ES cells into the pancreatic cell lineage. Using the ROSA26 locus, we first established an ES cell line in which pdx-1 expression was precisely regulated by tetracycline. The use of the ROSA26 locus for the Tet-on system has already been reported by Kyba et al. (28). However, in their system, the rtTA gene was integrated into the ROSA26 locus, but the rtTA-regulated target gene was integrated into the HPRT gene. We integrated not only the tTA expression unit, but also the tTA-responsive pdx-1 expression element, into the ROSA26 locus through a single knock-in vector. The resulting ES cells, RTF-pdx-1, constitutively expressed tTA under the ROSA26 promoter. tTA protein activated the downstream CMV*1 promoter to produce pdx-1 as well as EGFP. Transgene expression in this RTF-pdx-1 ES cell line was strictly regulated, that is, almost completely repressed in the presence of Dox, but induced in almost all cells upon withdrawal of Dox, as shown by the EGFP expression (Fig. 2*C* and *D*) and

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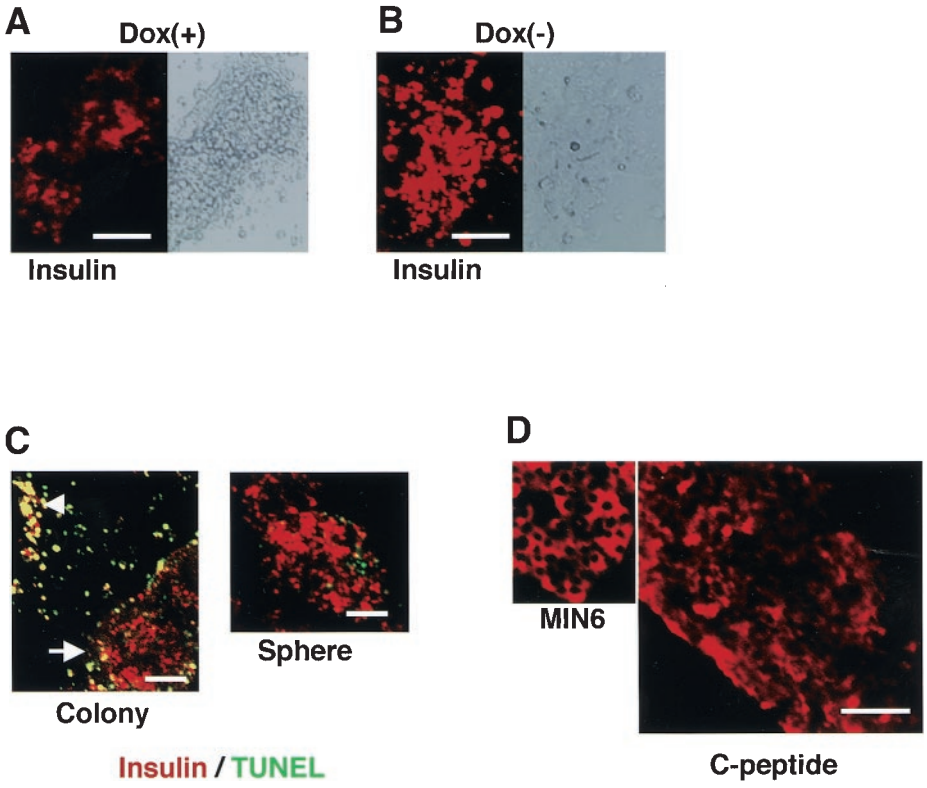

FIG. 5. Immunofluorescence analysis of differentiated RTF-pdx-1 cells. *A* and *B*: Phase-contrast and anti-insulin antibody staining of the RTF-pdx-1 cells at stage 4 of in vitro differentiation. *A*: Dox(+) stage 4 cell clusters. *B*: Dox(−) stage 4 cell clusters. *C*: Dual immunofluorescence staining using anti-insulin (red) and TUNEL (green). An arrow indicates the insulin-positive cells in the center of the colony, and an arrowhead indicates insulin and TUNEL double-positive cells. *D*: Immunofluorescence staining for C-peptide. MIN6 cells were used as the control. C-peptide was expressed in the cytoplasm of the stage 5 cell spheres, although at lower levels than in the MIN6 control. Scale bars, 50 μm.

Western blot analysis of pdx-1 (Fig. 2*A*). Furthermore, the pdx-1 protein was localized to the nucleus of ES cells (Fig. 2*B*).

It has been reported that pdx-1 regulates the expression of a number of pancreatic genes, including insulin, somatostatin, IAPP, glucokinase, Glut2, and pdx-1 itself (31). Gene expression analysis by RT-PCR showed that the expression of the insulin 2, somatostatin, and glucokinase genes were clearly enhanced by the pdx-1 expression, but insulin 1, Glut2, or endogenous pdx-1 gene expression could not be detected at any stage (Fig. 4). Two nonallelic

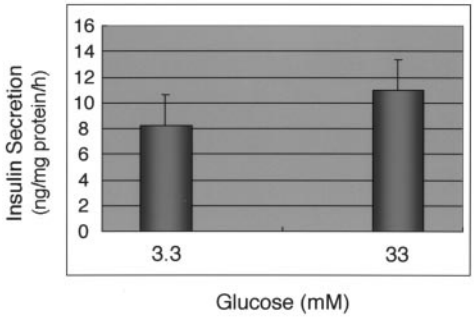

FIG. 6. Dox(−) stage 5 cell clusters cultured in 12-well plates were incubated in Krebs-Ringer buffer containing 3.3 or 33 mmol/l glucose. The culture supernatants were collected after 1 h of incubation. The insulin concentration of the culture supernatants was determined. To normalize the amount of insulin secretion, the total protein of the cells in each well was measured by the Bradford method. The experiment was performed in triplicate.

insulin genes are expressed in rodents. The insulin 2 gene is expressed in the developing brain and yolk sac as well as in the pancreatic β-cells, whereas insulin 1 gene expression is restricted to the pancreatic β-cells (29,30). Thus, we speculate that our ES cell–derived insulin-positive cells might not be fully mature, as compared with adult pancreatic β-cells, and might lack some of the transcription factors required for the activation of the insulin 1, Glut2, and pdx-1 genes, which might be the reason why the differentiated RTF-pdx-1 cells showed only a marginal insulin-secretion response to glucose, although they expressed the genes for glucokinase and Kir6.2 that are essential for glucose-induced insulin secretion.

Although the pdx-1 expression increased the levels of insulin 2 gene expression in RTF-pdx-1 cells, the amount of insulin secreted into the medium was still much lower than that from pancreatic β-cells. We transplanted differentiated RTF-pdx-1 cell clusters into streptozotocin-induced diabetic mice, but their blood glucose levels were not lowered (data not shown). On the other hand, other groups showed that implantation of ES-derived insulin-secreting cells into streptozotocin-induced diabetic mice led to correction of hyperglycemia (10,11). The reason for this difference is not clear at present, but reversing the diabetes in this mouse model will be essential to further improve the methods of differentiation and purification of insulin-producing cells.

Interestingly, the differentiated RTF-pdx-1 cells continued to grow in the absence of Dox, FBS, and bFGF.

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RT-PCR analysis showed that some pancreatic markers including insulin 2 were further enhanced at passage 5, while exocrine-specific markers were lost (Fig. 4). After these passages, cells tended to form sphere structures, which could be passaged in suspension culture. The properties of these sphere clusters are under investigation.

Recently, Blyszczuk et al. (11) showed that constitutive expression of Pax4 affects the differentiation of ES cells and significantly promotes the development of insulin-producing cells. In Pax4-overexpressing R1 ES cells, isl-1, ngn3, insulin, IAPP, and Glut2, mRNA levels increased significantly. However, there was only a marginal effect of pdx-1 expression in their system. The difference in the effect of pdx-1 expression on pancreatic endocrine cell differentiation between our and their experiments may be due to the difference in the expression levels of pdx-1. In this regard, we noted that high-level expression of pdx-1 in undifferentiated ES cells appeared toxic to the cells (data not shown). Hori et al. (10) reported that treatment of mouse embryonic stem cells with inhibitors of phosphoinositide 3-kinase, an essential intracellular signaling regulator, produced cells that resembled pancreatic β-cells in several ways. The differentiated ES cells in their system produced insulin at levels far greater than previously reported and displayed glucose-dependent insulin release in vitro. However, it is necessary to show that their differentiated cells produced insulin de novo and were not apoptotic.

The present study showed that the exogenous expression of the transcription factor pdx-1 directed ES cells into the pancreatic cell lineage during in vitro differentiation via EB formation. In normal development, pdx-1 is temporally expressed in the pancreatic buds, and later its expression becomes restricted to the pancreatic β-cells. Our study suggested that pdx-1–expressing cells predominantly differentiate into both the endocrine and exocrine pancreatic lineages, but not to the other endodermal cell lineages, because the differentiated cells did not express the gastrin or albumin gene (data not shown). It is possible that pdx-1–expressing cells also differentiated into the neuronal cell lineages, considering that our differentiation method included a selection step for nestin-positive cells. However, it seemed difficult to examine this possibility, because a number of "neuron-specific" genes have been shown to be expressed also in insulinoma cell lines and β-cells (32).

Our strategy of expressing pdx-1 might have induced the ES cells to differentiate into pancreatic progenitor cells. The induction of insulin 2 gene–expressing cells indicates that this is a feasible strategy for producing insulin-producing cells from human ES cells. Our system, based on the regulated expression of a transcription factor, provides a novel strategy for inducing differentiated cells useful for research or therapy from ES cells in vitro.

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