Analysis of Insulin-Producing Cells During In Vitro Differentiation From Feeder-Free Embryonic Stem Cells

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Embryonic stem (ES) cells can differentiate into many cell types and are expected to be useful for tissue engineering. Recent reports have shown that ES cells can differentiate into insulin-producing cells in response to the transient expression of the pdx-1 gene, after the removal of feeder cells. To investigate the lineage of insulin-producing cells and their in vitro differentiation, we introduced the βgeo gene, encoding a β-galactosidase-neomycin phosphotransferase fusion protein under the control of the mouse insulin 2 promoter, into ES cells that had been adapted to feeder-free culture, and analyzed insulin gene expression during their in vitro differentiation. We also examined the expression of transcription factors that are related to the differentiation of the pancreas. X-gal staining analysis revealed β-galactosidase–positive cells on the surface and in the center of the embryoid body that proliferated during differentiation. Glucose-responsive insulin-producing cells, derived from our feeder-free ES cells, expressed insulin 2, pdx-1, Pax4, and Isl1 and also the glucagon, somatostatin, and PP genes. Moreover, the genes encoding p48, amylase, and carboxypeptidase A were also expressed. These results suggest that ES cells can differentiate not only into endocrine cells but also into exocrine cells of the pancreas, without the initiation of pdx-1 expression. *Diabetes* 52:1163–1168, 2003

Mouse embryonic stem (ES) cells are continuous cell lines derived directly from the fetal founder tissue of the preimplantation embryo. They can be expanded in culture while retaining the functional attributes of pluripotent early embryo cells. Their capacity for multilineage differentiation is reproduced in culture, as ES cells can differentiate into a wide range of well-defined cell types. Cell transplantation to restore tissue function after disease or injury is in theory applicable to a huge variety of human diseases. Thus, the use of lineage-restricted differentiation techniques developed for ES cells will promote future cell therapy. Moreover, an in vitro ES cell differentiation system will be valuable for research into early embryonic cell lineage. We and other groups have shown that ES cells can differentiate into the endoderm cell lineage (1–5). Recently, several groups have reported that ES cells can be induced to differentiate into insulin-producing cells. The pioneer work by Soria et al. (1,2) derived insulin-producing cells from mouse ES cells transfected with the βgeo gene driven by the human insulin promoter, which allowed them to purify insulin-producing cells, and Assady et al. (3) reported that human ES cells spontaneously differentiate into insulin-producing cells via embryoid body (EB) formation. Recently Lumelsky et al. (4) reported the successful differentiation of mouse ES cells into insulin-secreting structures similar to pancreatic islets with a sophisticated five-stage method.

These reports indicate that ES cells can differentiate into insulin-producing cells, but the cell lineage of these insulin-producing cells is still unknown. Indeed, insulin gene expression has been observed not only in the pancreas but also in the yolk sac and embryonic brain in vivo (6,7). To assess the cell lineage of insulin-producing cells during their differentiation from ES cells, we have developed ES cell lines carrying a lacZ-neomycin phosphotransferase fusion gene (βgeo) under the mouse insulin 2 gene promoter and analyzed the lacZ expression pattern of clusters of differentiating ES cells. Moreover, we have examined the expression of a series of transcription factors related to the development of the pancreas in vivo (8–10) to analyze the cell lineage of the insulin-producing cells.

A previous report suggested that the removal of feeder cells is a trigger for the expression of pdx-1, which in turn induces the differentiation of ES cells into insulin-producing cells (4). If so, then feeder-free ES cells may not be suitable for differentiation into insulin-producing cells. For using human ES cells for cell therapy, however, a feeder-free culture system is desirable. Moreover, a feeder-free culture system permits us to simplify the analysis of the environmental factors that are involved in the differentiation of ES cells. Thus, to examine the significance of the initiation of pdx-1 expression in ES cells, we investigated whether ES cells that were maintained under feeder-free conditions could differentiate into insulin-producing cells.

RESEARCH DESIGN AND METHODS

Cell culture and transfection. EB3 ES cells, which are derived from E14tg2a ES cells and carry the blasticidin S-resistant selection marker gene driven by the Oct-3/4 promoter, were maintained in the absence of feeder cells in Glasgow Minimum Essential Medium (GMEM) supplemented with 10% fetal calf serum (Biowhittaker, Walkersville, MD) and leukemia inhibitory factor (LIF). The insulin 2-βgeo reporter gene was constructed by inserting the βgeo gene into exon 1 of the mouse insulin 2 gene, connected to mouse phospho-

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β-gal, β-galactosidase; EB, embryoid body; ES, embryonic stem; IAPP, islet amyloid polypeptide; LIF, leukemia inhibitory factor; pgk, phosphoglycerate-kinase; PP, pancreatic polypeptide.

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TABLE 1
List of gene-specific primers

Genes	Forward primer	Reverse primer	Size of product (bp)	No. of cycles	GenBank accession no.
Insulin 1	ccagctataatcagagacca	gtgtagaagaagccacgct	197	35	AK007345
Insulin 2	tccgctacaatcaaaaaccat	gctgggtagtggtgggtcta	411	35	AK007612
Glucagon	actcacagggcacattcacc	ccagttgatgaagtccctgg	353	40	Z46845
Somatostatin	tcgctgctgcctgaggacct	gccaagaagtacttggccagttc	232	30	AK003014
PP	actagctcagcacacaggat	agacaagagaggctgcaagt	364	30	M18208
p48	tgcagtccatcaacgacgc	ggacagagttcttccagttc	708	35	AK007922
Amylase	caggcaatcctgcaggaacaa	cacttgcggataactgtgcca	484	30	X02579
Carboxypeptidase A	gcatccattctagggagtgg	gaaagagtacttgatgccctg	601	30	AK003088
pdx-1	accatgaacagtgaggagca	tcctcttgttttcctcgggt	451	40	NM_008814
Pax4	aaatggcgcaggcaagagaa	atgaggaggaagccacagga	280	40	XM_133023
Isl1	agatatgggagacatgggcgat	acacagcggaaacactcgatg	327	40	NM_021459
ngn3	tggcactcagcaaacagcga	acccagagccagacaggtct	444	30	AF364300
Beta2	cttggccaagaactacatctgg	ggagtagggatgcaccgggaa	222	30	AK018781
Pax6	cagtcacagcggagtgaatc	cgcttcagctgaagtcgcat	658	30	NM_013627
Nkx2.2	aaccgtgccacgcgctcaaa	agggcctaaggcctccagtct	220	30	NM_010919
Nkx6.1	tcttctggcccggggtgatg	agccgcgtgcttcttcctcca	283	40	NM_144955
Glut2	cggtgggacttgtgctgctgg	ctctgaagacgccaggaattccat	416	30	AK005068
Glucokinase	tggatgacagagccaggatgg	acttctgagccttctggggtg	208	30	L38990
Kir6.2	ggctcctagtgacctgcacca	ccacagccacactgcgcttgcg	317	30	U73626
PC2	agagattccattgtgtggga	caaaatggacttggtgccca	215	30	NM_008792
Oct-3/4	accttccccatggc	acttgatcttttgcccttctg	855	30	M34381
Gapdh	accacagtccatgccatcac	tccaccaccctgttgctgta	452	25	NM_008084

glycerate-kinase (pgk) promoter-driven puromycin-resistance gene. Linearized plasmid was introduced into ES cells by electroporation (800 V, 3 μF) using a Bio-Rad gene pulser. ES cells were then selected using 1.5 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO) to establish ES clones harboring the insulin 2-βgeo gene.

In vitro differentiation procedure. ES cells (stage 1) were dissociated into a single-cell suspension with 0.25% trypsin/0.04% EDTA in PBS and plated onto bacterial culture plates at a density of 2–2.5 × 10^4 cells/cm^2 in DMEM containing 10% FCS. The stage 2 cell clusters, which are also known as EBs, were allowed to form for 4–5 days, then replated onto gelatinized plates in DMEM containing 10% FCS. After incubation for 2 days to allow the EBs to attach to the plate, the medium was replaced with serum-free ITSFn medium (11), and the cells were incubated for 4–7 days. Most of the cells died in this medium, and the surviving cells (stage 3 cell clusters) were dissociated using 0.25% trypsin/0.04% EDTA and replated on gelatinized plates at a concentration of 0.5–2 × 10^5 cells/cm^2 in DMEM/F-12 (1:1; Invitrogen, Carlsbad, CA) containing 25 μg/ml insulin (Sigma-Aldrich), 100 μg/ml transferrin (Sigma-Aldrich), 20 nmol/l progesterone (Sigma-Aldrich), 60 μmol/l putrescine (Sigma-Aldrich), 30 nmol/l sodium selenite (Sigma-Aldrich), 10 ng/ml human keratinocyte growth factor (Peprotech EC, London, U.K.), 20 ng/ml epidermal growth factor (Sigma-Aldrich), and 25 ng/ml basic fibroblast growth factor (Strathmann Biotech, Hamburg, Germany), in the presence of B27 supplement (Invitrogen) and 10 ng/ml nicotinamide, and cultured for 6–8 days (stage 4 cell clusters).

RNA extraction and RT-PCR analysis. Total RNA was prepared from the undifferentiated and differentiating parental ES cells and the βgeo-expressing cells at each culture stage (stages 1–4) by the acid guanidinium thiocyanate-phenol-chloroform method followed by DNase treatment (12). One entire plate of cells was used for each isolation of total RNA. For cDNA synthesis, oligo dT primers were used to prime the RT reactions. The primers for RT-PCR were designed according to the published mouse sequences, and the PCR products were confirmed by sequence analysis. The primer sequences and PCR conditions used for the RT-PCR are shown in Table 1.

X-gal staining and immunohistochemical analysis. For the X-gal staining, the cells were fixed with 1% glutaraldehyde, and β-galactosidase (β-gal) enzyme activity was detected using X-gal. For analyzing cross-sections of EBs, the EBs were stained with X-gal, cross-sections were cut, and the sections were counterstained with eosin. For immunohistochemical analysis, cells were fixed with 4% paraformaldehyde in PBS. After blocking with 5% normal goat serum (DAKO, Kyoto, Japan) for 30 min at room temperature, the cells were incubated overnight at 4°C with primary antibodies: guinea pig anti-insulin (DAKO), rabbit anti-glucagon (DAKO), rabbit anti–pdx-1 (gift from Dr. Kajimoto, Osaka University Medical School, Osaka, Japan), or mouse anti–β-gal (Promega, Madison, WI). The cells were then incubated with the appropriate second antibodies and labeled with Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR) for 1 h at room temperature. Alexa 488 was excited at 488 nm and Alexa 568 at 543 nm, using the Leica confocal microscope system TCS SP2 (Leica Microsystems AG, Wetzlar, Germany).

Insulin secretion assays. For static incubation, stage 4 cell clusters were cultured in a 60-mm dish and allowed to grow for 5 days. The cell clusters from one dish were harvested, washed five times, and incubated in 500 μl of Krebs buffer containing 3.3 mmol/l glucose for 1 h. The culture supernatants were collected after 1 h of incubation. The cells were then washed three times and incubated in Krebs buffer containing 25 mmol/l glucose for 1 h. At the end of the incubation period, the supernatants were collected. The insulin concentration of the culture supernatants was assayed using an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). All values were determined against a standard curve prepared with rat insulin. The total protein of the cells in each experiment was measured by the Bradford method to normalize the amount of insulin secretion. Each experiment was performed at least in triplicate.

RESULTS

Establishment of the ins2-βgeo ES cell line. The insulin 2 reporter transgene was engineered to express a fusion protein between β-gal and neomycin phosphotransferase (βgeo) under the control of the mouse insulin 2 promoter. The vector contained a pgk-puromycin unit, which allowed positive selection of transfected ES cells in the presence of puromycin. After the vector DNA was introduced into EB3 ES cells by electroporation, puromycin-resistant clones were isolated. Two ES cell lines were established (ins2-βgeo5, ins2-βgeo11). Integration of the entire expression cassette was confirmed by PCR (data not shown). These ES cell lines could be maintained in a morphologically undifferentiated state after several passages and had a growth rate similar to the parental EB3 ES cells without feeder cells and in the presence of LIF. These two cell lines showed the same gene expression pattern as the parental cells during differentiation (see below). At stage 4, the cell clusters formed either adherent colonies or floating spheres similar to those reported by Soria et al. (2).

Gene expression pattern during differentiation. We

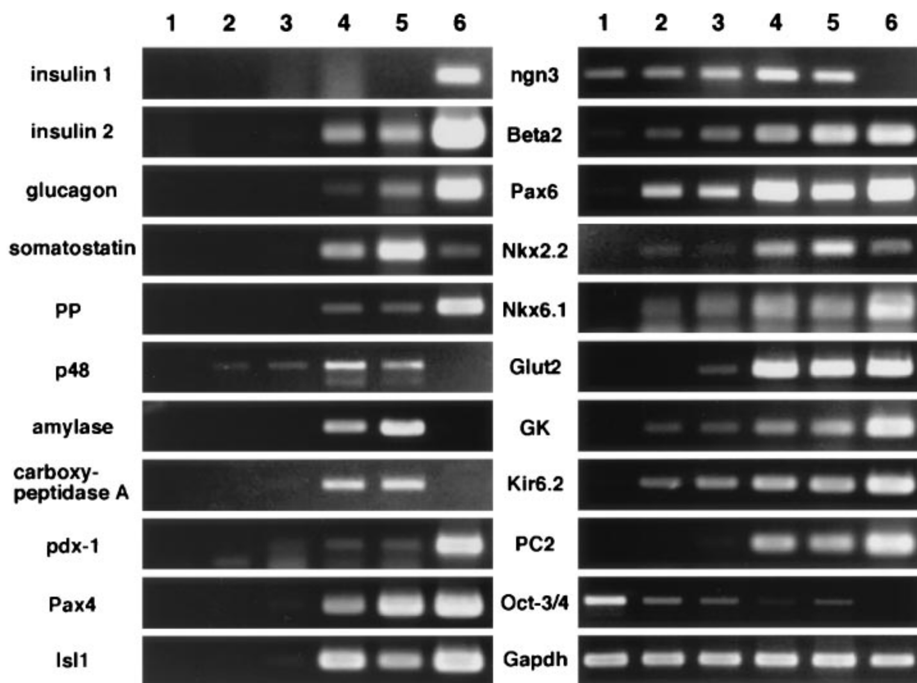

FIG. 1. RT-PCR analysis of gene expression in ins2-βgeo ES cells during in vitro differentiation. Total RNA was extracted from ES cells and differentiated cell clusters at stages 1, 2, 3, and 4 and subjected to RT-PCR analysis. Stage 1 cells (*lane 1*) are undifferentiated ES cells. Stage 2 cell clusters (*lane 2*) are EBs cultured in the absence of LIF. Stage 3 cell clusters (*lane 3*) are cells surviving under serum-free conditions. Stage 4 cell clusters were formed from dissociated stage 3 clusters that were cultured in the presence of growth factors. These clusters had two different morphologies: floating spheres (*lane 4*) or adherent colonies (*lane 5*). There was no significant difference in the gene expression pattern between adherent colonies and floating spheres. Lineage-specific gene expression was observed along with differentiation. Gapdh was used for the internal standard. RT-PCR results from the MIN6 insulinoma cell line (28) served as a positive control (*lane 6*).

performed an RT-PCR analysis of gene expression in ins2-βgeo ES cells during in vitro differentiation (Fig. 1). Although X-gal staining showed that the insulin 2 promoter became active as early as stage 2, insulin 2 gene expression could not be detected by RT-PCR until stage 4. Even at stage 4, insulin 1 gene expression was not detected. However, the mRNAs for other genes, including the other islet hormones, glucagon, somatostatin, pancreatic polypeptide (PP), islet amyloid polypeptide (IAPP; data not shown) (8–10), Pax4 (13), and Isl1 (14), as well as amylase and carboxypeptidase A, which are pancreatic exocrine enzymes, were first detected at stage 4. The expression of the p48 gene (15), a regulatory gene for pancreatic exocrine differentiation, was first observed at stage 2 and had increased at stage 4. Gene expression of pdx-1, a key gene for pancreatic differentiation (16,17), was not observed from stages 1 to 3 but started to be expressed at stage 4 in our feeder-free ES cells. In contrast to previous reports (4), our results showed that pdx-1 gene expression at the ES stage (stage 1) was not necessary for the subsequent expression of the insulin gene. The neurogenin3 (18), Beta2 (19), Pax6 (20), Nkx2.2 (21), and Nkx6.1 (22) genes, which are expressed in both neuronal cells and pancreatic β-cells, were expressed as early as stage 2, and their expression levels had increased at stage 4.

In contrast, the expression of the Oct-3/4 gene, which is required for the maintenance of the undifferentiated state (23), was expressed in stage 1 ES cells, and its level gradually decreased, inversely with the progress of differentiation. Previously, the removal of growth factors was shown to induce the further differentiation in insulin-producing cells (4). However, our results showed that the removal of the growth factors from stage 4 cell clusters did not increase the insulin gene expression or insulin synthesis (data not shown). Stage 4 cell clusters could be maintained for at least 2 weeks with limited growth by

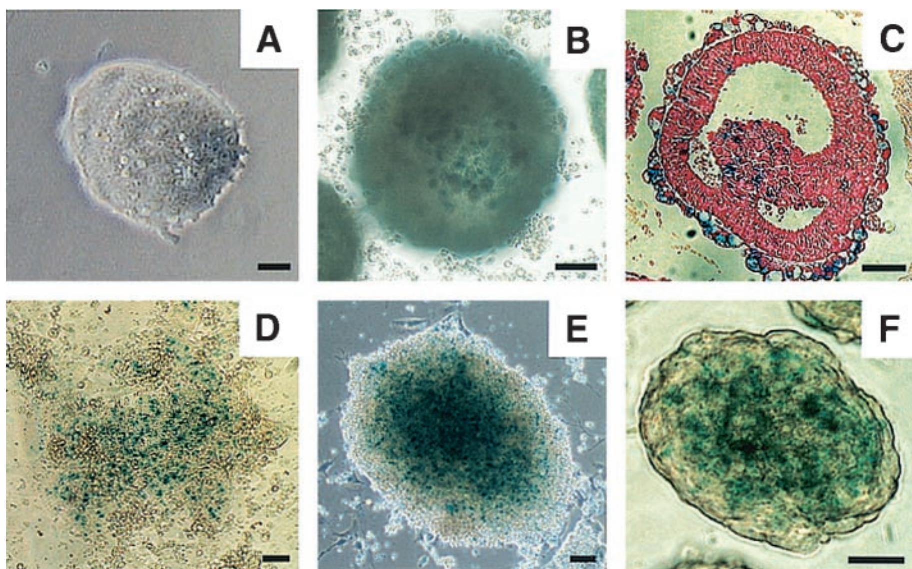

FIG. 2. Insulin gene expression in the ins2-βgeo ES cells during differentiation. Ins2-βgeo cells at each stage were fixed and stained for β-gal activity (stage 1). *A*: X-gal staining of ins2-βgeo ES cells showed no β-gal activity. *B*: EBs derived from ins2-βgeo ES cells. *C*: Cross-sectional view of EBs derived from ins2-βgeo ES cells (stage 2). Scattered cells expressing the insulin 2 gene were observed among the primitive ectoderm-like cells in the inner layer and visceral endoderm-like cells in the outer layer of the EBs. *D*: Scattered cells that survived the serum-free culture conditions at stage 3 expressed β-gal. *E*: Stage 4 cell clusters with the adherent colony morphology derived from ins2-βgeo ES cells. *F*: Stage 4 floating spheres derived from ins2-βgeo ES cells. Bars, 20 μm.

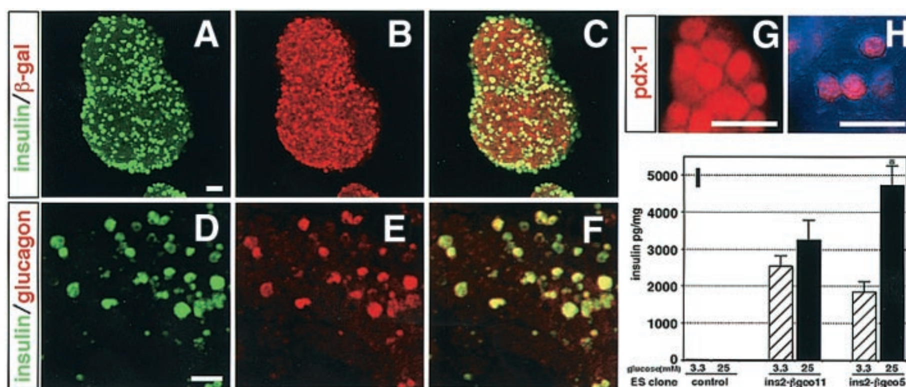

FIG. 3. Expression of insulin, β-gal, glucagon, and pdx-1, and glucose-induced insulin secretion in stage 4 cell clusters. *A* and *F*: Stage 4 cell clusters were stained for insulin, β-gal, or glucagon. Scattered insulin-producing cells (*A*) and β-gal–expressing cells (*B*) were observed. Completely overlapping expression (*yellow*) of insulin and β-gal was observed (*C*). Co-staining of insulin (*D*) and glucagon (*E*) showed that insulin-producing cells also expressed glucagon (*F*). Pdx-1 was expressed in the nucleus of the insulinoma cells (*G*) and was also expressed in the nucleus of the stage 4 cells at lower levels (*H*). Insulin release from stage 4 cell clusters (*I*). Both the ins2-βgeo5 and the ins2-βgeo11 ES cells at stage 4 secreted insulin in response to glucose, whereas undifferentiated ES cells, which were incubated in the same medium used for the culture of stage 4 cell clusters in the presence of 10% FCS, LIF, and 2-ME for 1 day, did not secret any insulin. Error bars represent SD. *P < 0.01 compared with 3.3 mmol/l glucose stimulation. Bars, 20 μm.

changing medium every 2 days. There was no significant difference in the gene expression pattern between adherent colonies and floating spheres in the stage 4 cell clusters.

Insulin 2 gene expression during in vitro differentiation. For investigating the distribution of the cells expressing the insulin gene, X-gal staining analysis of the differentiated cells at each stage was performed (Fig. 2). At stage 1, the undifferentiated ES cells did not show any β-gal activity in either the parental ES cells or the ins2-βgeo cells (Fig. 2*A*). As early as stage 2, β-gal–expressing cells were observed, mainly on the surface of the EBs: the EB surface is recognized as a model for the primitive endoderm of the embryo in vivo (Fig. 2*B* and *C*). The β-gal gene–expressing cells were also observed scattered among the cells of the inner layer of the EBs: the inner layer is recognized as a model for the primitive ectoderm of the embryo in vivo (Fig. 2*C*). At stage 3, after selection culture in serum-free culture medium, cells expressing β-gal were observed scattered among nonexpressing cells in the clusters (Fig. 2*D*). At stage 4, the number of cells expressing β-gal had increased in both the adherent colonies and floating spheres (Fig. 2*E* and *F*).

Immunohistochemical analysis of stage 4 cell clusters. Differentiated ES cell clusters at stage 4 were analyzed immunohistochemically. Staining for insulin revealed that a large percentage of the cells produced insulin at stage 4. To confirm whether β-gal was correctly expressed in newly developed ins2-βgeo ES cells, we performed double immunostaining for insulin and β-gal protein (Fig. 3*A*–*C*). In stage 4 cell clusters, the insulin-positive cells coincided exactly with the β-gal–positive cells. Thus, insulin gene expression was correctly detected in our ins2-βgeo ES cells. The immunohistochemical study showed that the pdx-1 protein was localized to the nucleus of the insulin-producing cells (Fig. 3*G* and *H*). Moreover, Glut2 protein was detected in the differentiated cells (data not shown). All of these data support the idea that the insulin-positive cells were very similar to normal pancreatic β-cells. However, our insulin-positive cells also stained with glucagon (Fig. 3*D*–*F*), suggesting that these cells were still developmentally immature (24,25).

Glucose-induced insulin secretion from stage 4 cell clusters. Stage 4 cell clusters were examined for glucose-induced insulin secretion (Fig. 3*I*). In the presence of 3.3 mmol/l glucose, stage 4 cell clusters secreted insulin at 1,851 pg · mg protein^{-1} · h^{-1} (ins2-βgeo5) and 2,538 pg · mg protein^{-1} · h^{-1} (ins2-βgeo11) in independent clones. The addition of 25 mmol/l glucose stimulated the insulin release to 4,732 pg · mg protein^{-1} · h^{-1} and 3,255 pg · mg protein^{-1} · h^{-1}, respectively. Thus, our feeder-free ES cells could differentiate into glucose-responsive insulin-secreting cells, as previously reported for feeder-dependent ES cells.

DISCUSSION

In this study, we investigated the profile of insulin gene expression during the differentiation of ES cells, using cells expressing lacZ under the mouse insulin 2 promoter. The gene expression pattern of the ES cells during differentiation indicated that ES cells have the potential to differentiate not only into endocrine but also into exocrine cells. It is interesting that our ES cells, which can be maintained in a feeder-free condition and do not express pdx-1 in the undifferentiated status, differentiated into glucose-responsive insulin-producing cells.

A previous report showed that the removal of feeder cells induced the expression of pdx-1 in undifferentiated ES cells, leading to the progression of differentiation into insulin-producing cells (4). Human ES cells have usually been maintained with mouse feeder cells (26), but recently it was reported that human ES cells can be maintained without feeder cells (27). Feeder-free ES cell culture systems are essential to avoid the possible contamination of nonhuman cells if human ES cells are to be used for transplantation into humans, and such systems are suitable for the large-scale culture of ES cells for cell replacement. Thus, it is important to know whether ES cells can differentiate into insulin-producing cells without the initiation of pdx-1 gene expression being induced by the removal of feeder cells at a very early stage of differentiation.

Our feeder-free ES cells did not express the pdx-1 gene from stages 1 to 3. Along with the expression of pancreatic

hormones, the pdx-1 gene started to be expressed at stage 4. In addition, the Glut2, glucokinase, Kir6.2, and prohormone-converting enzyme 2 genes, which are necessary for glucose-induced insulin secretion, were also first expressed at stage 4. Moreover, the pdx-1 protein was localized to the nucleus of insulin-positive differentiated cells, leading to the proper physiological function of insulin-secreting cells, although the pdx-1 protein level was low compared with that of the MIN6 cells, an insulinoma cell line (28). Although the insulin secretory response to glucose differed among the cell lines, insulin-producing cells could be differentiated successfully without the initial drive of pdx-1 gene expression in undifferentiated ES cells. Our results encourage the use of feeder-free ES cells for replacement therapy for diabetes.

The newly established ins2-βgeo ES cell lines enabled us to trace the insulin-promoter active cells during the in vitro differentiation of ES cells. We observed β-gal expression as early as stage 2, the EB stage. β-gal–expressing cells were detected mainly on the surface of the EBs and scattered throughout their inner layer. Considering that the epiblast gives rise to definitive endoderm during gastrulation, the EBs arising from in vitro differentiation might not contain endodermal cells mature enough to express the insulin gene. Thus, the β-gal gene expression, observed at the stage 2 during in vitro differentiation, might not be accounted for by the presence of definitive endoderm-derived cells. Further in vivo investigation is needed to learn the specific cell fate of the β-gal–positive cells observed in EBs during the in vitro differentiation of ES cells.

Gene expression analysis by RT-PCR showed that the insulin 2, glucagon, PP, somatostatin, and IAPP genes were expressed in stage 4 cell clusters, whereas insulin 1 gene expression could not be detected at any stage. Two nonallelic insulin genes are expressed in rodents (7). The insulin 2 gene is expressed in the developing brain and yolk sac as well as in the pancreatic β-cells (6–8), whereas insulin 1 gene expression is restricted to the pancreatic β-cells (7), suggesting that our ES cell–derived insulin-positive cells might not be fully mature, as compared with adult pancreatic β-cells.

The pattern of transcription factor gene expression during in vitro differentiation revealed another aspect of the cell lineage of our insulin-positive cells. The neurogenin3, Beta2, Pax6, Nkx2.2, and Nkx6.1 genes, which have pivotal roles in the development of pancreatic endocrine cells, were expressed as early as at stage 2 (the EB stage) and were also expressed in the cells selected in serum-free medium (stage 3). These transcription factors are also expressed in the developing brain and are associated with the differentiation of neurons (8,10). Considering that pancreatic β-cells are known to share some characteristics of neurons, it cannot be excluded that the differentiated insulin-producing cells were derived from neuronal progenitors. Although Isl1 is one of the transcription factors that is expressed in both neurons and pancreatic endocrine cells (8,10,14), the expression of isl1 was restricted to stage 4. Similarly, Pax4, one of the islet-specific cell-type determination factors (10,13), was not expressed until stage 4. It has been reported that Pax4 disappears when the β-cell type has been set (10), but Pax4 gene expression never disappeared in the stage 4 cells. In our study, the carboxypeptidase A and amylase genes were also expressed in stage 4 ES cells in accordance with the gene expression of p48, a determinant transcription factor for exocrine development (15).

Although we showed that ES cells could differentiate into insulin-secreting cells without the priming of pdx-1 gene expression, the resulting insulin-producing cells co-produced glucagon and expressed only the insulin 2 gene but not the insulin 1 gene, suggesting that these insulin-producing cells were not mature enough to cure diabetes. In fact, we transplanted stage 4 cell clusters into streptozotocin-induced diabetic mice, but blood glucose levels were not lowered. To improve the insulin production, we tried to purify insulin-positive cells by neomycin selection, but we found it difficult, probably because of low resistance to neomycin or limited proliferation capacity of insulin-positive cells. However, Soria et al. (1) showed that implantation of ES-derived insulin-secreting cells into streptozotocin-induced diabetic mice led to correction of hyperglycemia. They also observed the β-gal gene expression driven by human insulin promoter and insulin production even 4 months after the engraftment (2). The reason for the difference in the capability of reversal of diabetes in vivo between these and our ES cell–derived insulin-producing cells is not clear, but it should be essential to improve further the methods of differentiation and purification of insulin-producing cells.

The growth and differentiation of the pancreas also requires a series of initial and secondary inductive signals emanating from the surrounding mesenchymal tissues (8–10,29). Thus, it is possible that certain mesenchymal factors are required for the full maturation of insulin-positive cells differentiated from the ES cells. Immunostaining and RT-PCR analysis showed that there was a relatively low level of pdx-1 expression in differentiated ES cells when compared with the mature insulin-producing cells. Thus, the elevation of pdx-1 gene expression in the differentiated ES cells may be another strategy for obtaining the well-differentiated insulin-producing cells for future cell therapy for diabetes.

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REFERENCES

1. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F: Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 49:157–162, 2000
2. Soria B, Skoudy A, Martin F: From stem cells to beta cells: new strategies in cell therapy of diabetes mellitus. *Diabetologia* 44:407–415, 2001
3. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M: Insulin production by human embryonic stem cells. *Diabetes* 50:1691–1697, 2001
4. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R: Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292:1389–1394, 2001
5. Fujikura J, Yamato E, Yonemura S, Hosoda K, Masui S, Nakao K, Miyazaki

J, Niwa H: Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev* 16:784–789, 2002
6. Giddings SJ, Carnaghi L: Rat insulin II gene expression by extraplacental membranes. A non-pancreatic source for fetal insulin. *J Biol Chem* 264:9462–9469, 1989
7. Deltour L, Leduque P, Blume N, Madsen O, Dubois P, Jami J, Bucchini D: Differential expression of the two nonallelic proinsulin genes in the developing mouse embryo. *Proc Natl Acad Sci U S A* 90:527–531, 1993
8. Slack JM: Developmental biology of the pancreas. *Development* 121:1569–1580, 1995
9. Kim SK, Hebrok M: Intercellular signals regulating pancreas development and function. *Genes Dev* 15:111–127, 2001
10. Edlund H: Pancreatic organogenesis-developmental mechanisms and implications for therapy. *Nat Rev Genet* 3:524–532, 2002
11. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD: Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 18:675–679, 2000
12. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
13. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P: The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature* 386:399–402, 1997
14. Ahlgren U, Pfaff SL, Jessell TM, Edlund T, Edlund H: Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature* 385:257–260, 1997
15. Krapp A, Knofler M, Ledermann B, Burki K, Berney C, Zoerkler N, Hagenbuchle O, Wellauer PK: The bHLH protein PTF1–p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev* 12:3752–3763, 1998
16. Guz Y, Montminy MR, Stein R, Leonard J, Gamer LW, Wright CV, Teitelman G: Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. *Development* 121:11–18, 1995
17. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
18. Gradwohl G, Dierich A, LeMeur M, Guillemot F: Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* 97:1607–1611, 2000
19. Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ: Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* 11:2323–2334, 1997
20. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P: Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* 387:406–409, 1997
21. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS: Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* 125:2213–2221, 1998
22. Sander M, Sussel L, Conners J, Scheel D, Kalamaras J, Dela Cruz F, Schwitzgebel V, Hayes-Jordan A, German M: Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Development* 127:5533–5540, 2000
23. Niwa H, Miyazaki J, Smith AG: Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24:372–376, 2000
24. Teitelman G, Alpert S, Polak JM, Martinez A, Hanahan D: Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. *Development* 118:1031–1039, 1993
25. Gu D, Lee MS, Krahl T, Sarvetnick N: Transitional cells in the regenerating pancreas. *Development* 120:1873–1881, 1994
26. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147, 1998
27. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK: Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19:971–974, 2001
28. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K: Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
29. Scharfmann R: Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. *Diabetologia* 43:1083–1092, 2000