Stem Cells, Tissue Engineering and Hematopoietic Elements

Teratoma Formation Leads to Failure of Treatment for Type I Diabetes Using Embryonic Stem Cell-Derived Insulin-Producing Cells

Takahisa Fujikawa, Seh-Hoon Oh, Liya Pi,
Heather M. Hatch, Tom Shupe, and
Bryon E. Petersen

From the Department of Pathology, Immunology, and Laboratory Medicine, and Program in Stem Cell Biology and Regenerative Medicine, College of Medicine, University of Florida, Gainesville, Florida

Embryonic stem (ES) cells have been proposed to be a powerful tool in the study of pancreatic disease, as well as a potential source for cell replacement therapy in the treatment of diabetes. However, data demonstrating the feasibility of using pancreatic islet-like cells differentiated from ES cells remain controversial. In this study we characterized ES cell-derived insulin-expressing cells and assessed their suitability for the treatment of type I diabetes. ES cell-derived insulin-stained cell clusters expressed insulin mRNA and transcription factors associated with pancreatic development. The majority of insulin-positive cells in the clusters also showed immunoreactivity for C-peptide. Insulin was stored in the cytoplasm and released into the culture medium in a glucose-dependent manner. When the cultured cells were transplanted into diabetic mice, they reversed the hyperglycemic state for ~3 weeks, but the rescue failed due to immature teratoma formation. Our studies demonstrate that reversal of hyperglycemia by transplantation of ES cell-derived insulin-producing cells is possible. However, the risk of teratoma formation would need to be eliminated before ES cell-based therapies for the treatment of diabetes are considered. (*Am J Pathol* 2005, 166:1781–1791)

Diabetes mellitus is one of the major causes of death in advanced countries, and has been shown to adversely affect health and quality of life. It is associated with various severe or fatal complications, including blindness, kidney failure, heart disease, stroke, neuropathy, and amputations. Type I diabetes, or insulin-dependent dia-

betes, results from the cellular-mediated autoimmune destruction of pancreatic islet cells that are known to produce insulin. Type I diabetic patients experience high blood glucose levels as a result of insulin deficiency. There is no cure for this form of diabetes to date. Several approaches have been used in attempts to reverse the disease process for type I diabetes, including whole organ pancreas transplants and islet transplants.^{1,2} In addition, options such as the potential use of pancreatic stem and progenitor cells are being investigated.^{3,4} Currently, the only clinically approved treatment for type I diabetes, with the exception of insulin injection, is islet cell transplantation in combination with immunosuppressive therapy.⁵ Unfortunately, this option is only available to a very limited number of patients because of a severe shortage of donor tissue sources. This shortage has focused interest in developing renewable sources of insulin-producing cells appropriate for transplant.

Embryonic stem (ES) cells have been proposed as a potential source of pancreatic β cells because they are self-renewing elements that can generate the many cell types of the body.^{6–12} Recent studies suggest that mouse ES cells can be manipulated to express and secrete insulin.^{13–16} However, insulin-producing grafts derived from ES cells in these initial reports have a high degree of cellular heterogeneity and proliferation, uncharacterized growth and tumor-forming potential, as well as low insulin levels compared to pancreatic islets. Additionally, some researchers claim that the insulin-positive cells derived from ES cells may not be real insulin-producing β -like cells.^{17,18} In one study, contrary to previous reports, no message for insulin was detectable in culture, which suggested that the cells may be concentrating the hormone from the medium rather than producing.¹⁷ Another study showed that the main producers of insulin in culture were neurons and neuronal precursors

Supported by the National Institutes of Health (grants DK60015 and DK58614 to B.E.P.).

Accepted for publication March 7, 2005.

Address reprint requests to Bryon E. Petersen, Department of Pathology, P.O. Box 100275, University of Florida, Gainesville, FL, 32610. E-mail: petersen@pathology.ufl.edu.

and a reporter gene under the control of the insulin I promoter was activated in cells with a neuronal phenotype.¹⁸ Therefore, it is now a matter of controversy whether true pancreatic β cells can be derived from ES cells with the protocols so far developed. The issue whether ES cells can be used clinically for the treatment of diabetes also needs to be addressed.

The original protocol adapted a strategy used to generate neurons to derive endocrine pancreatic cells from ES cells.¹⁷ It involves sequential *in vitro* differentiation steps during which cultures were highly enriched in cells expressing nestin, an intermediate filament present in neural stem cells and possible islet precursors.^{19–21} We reproduced and modified the original protocol for the differentiation of islet-like structures and further characterized the system and its potential suitability for the amelioration of a diabetic condition.

Materials and Methods

Cell Culture

The ES cell lines R1 and green fluorescent protein (GFP)-labeled B5²² were maintained undifferentiated in gelatin-coated dishes in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) containing 15% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 mmol/L HEPES (Life Technologies, Inc.), 300 μ mol/L monothioglycerol (Sigma, St. Louis, MO), and 250 U/ml recombinant mouse LIF (Esgro; Chemicon, Temecula, CA). Differentiation into pancreatic islet-like cell clusters was accomplished according to the original protocol¹³ with slight modification made. Briefly, ES cells were grown in the absence of feeder layer on gelatin-coated dishes for two passages (stage 1). Embryoid bodies were grown in suspension for 4 days in the absence of LIF (stage 2), then transferred to collagen-coated tissue culture dishes and incubated for 7 to 8 days in serum-free ITSFn medium¹⁰ (stage 3). Cultures were then trypsinized and passed onto collagen-coated dishes or coverslips, and grown in N2 medium supplemented with B27 (Life Technologies, Inc.), 20 μ g/ml insulin, 1 μ g/ml basic fibroblast growth factor, and 10 μ g/ml epidermal growth factor (R&D Systems, Minneapolis, MN) (stage 4). After 7 days, basic fibroblast growth factor and epidermal growth factor were withdrawn and 10 mmol/L nicotinamide (Sigma) was added to the medium for the next 14 days (stage 5). Alternatively, to achieve better enrichment of islet-like cell clusters, cells were grown in high-glucose (25 mmol/L) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 21 days (stage 4 and 5), with modification of the method used for enrichment of insulin-producing cells from hepatic stem cells or bone marrow-derived cells.^{23,24} Collagen-coated dishes used were coated with 0.3% type I collagen, which was extracted from the rat-tail tendon by the method described by Michalopoulos and Pitot.²⁵

Detection of Transcription Factors, *Glut-2*, and Pancreatic Endocrine mRNAs

Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was prepared by reverse transcription, using 0.1 μ g of DNA-free total RNA, 0.5 μ g of oligo-d(T) primer, and SuperScript (Invitrogen, Carlsbad, CA). Of the total reverse transcriptase (RT) reaction volume, 1 μ l was amplified using specific primer pairs and *Taq*DNA polymerase (Eppendorf, Westbury, NY). Primer sequences for all cDNAs analyzed are shown in Table 1. Polymerase chain reaction (PCR) cycles were as follows; initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and final extension at 72°C for 10 minutes. PCR products were separated by 1.5% agarose gel electrophoresis.

Analysis of Insulin Gene Expression by Northern Blot

Total RNA was extracted from cultured cells using RNeasy mini kit (Qiagen). RNA concentration and purity were determined by routine spectrophotometry. Samples of RNA (40 μ g/lane) were size fractionated on 1% agarose gels and transferred to nylon membranes (Amersham, Arlington Heights, IL) by capillary action. After cross-linking under UV light, membranes were prehybridized and then hybridized overnight with specific cDNA of both mouse *Ins1* and *Ins2* genes, that had been labeled with [α -³²P]dCTP using an Amersham random primer kit. The membranes were subsequently washed under high-stringency conditions and exposed to X-omat film (Kodak, Rochester, NY).

Immunohistochemistry

Immunoactivity was detected using the cytochemical method described by Oh and colleagues.²⁶ Cultured ES-derived cells were grown on collagen-coated coverslips until spheroid colonies formed at ~21 days of step 4 and 5 cultures. Individual clusters were retrieved, frozen in OCT at -70°C, cut into 6- μ m sections, and placed on Superfrost Plus slides. Sections were then fixed in a 4% paraformaldehyde/phosphate-buffered saline (PBS) solution at room temperature for 15 minutes. The slides were further treated with a 5% w/v skim milk in Tris-buffered saline-Ca blocking medium for 1 hour. These slides were incubated for 1 hour at 4°C with one of the following primary antibodies: anti-human insulin (DAKO, Carpinteria, CA), anti-rat insulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-rat C-peptide (Linco Research Inc., St. Charles, MI), or anti-mouse OCT-4 (Santa Cruz Biotechnology, Inc.) diluted at 1:100. After sections were washed with Tris-buffered saline-Ca, samples were incubated at 4°C for 3 hours with secondary antibodies: anti-rabbit IgG Texas Red-conjugated, anti-guinea pig IgG fluorescein isothiocyanate (FITC)-conjugated, anti-guinea pig IgG Texas Red-conjugated, or anti-mouse

Table 1. RT-PCR Primer Sequences

Gene name	Primer sequences
Insulin I	5'-TAG TGA CCA GCT ATA ATC AGA G-3' 5'-ACG CCA AGG TCT GAA GGT CC-3'
Insulin II	5'-CCC TGC TGG CCC TGC TCT T-3' 5'-AGG TCT GAA GGT CAC CTG CT-3'
Glut-2	5'-CTT ACA GTC ACA CCA GCA TAC A-3' 5'-TGA GGC CAG CAA TCT GAC TAA-3'
Glucagon	5'-AAC AAC ATT GCC AAA CGT CA-3' 5'-TCC GCA GAG ATG TTG TGA AG-3'
Alpha-amylase-2A	5'-TTC TGC TGC TTT CCC TCA TT-3' 5'-CAT TGT TGC ACC TTG TCA CC-3'
Islet amyloid polypeptide (IAPP)	5'-CTC TCT GTG GCA CTG AAC CA-3' 5'-GAT TCC CTA TTT GGA TCC CC-3'
GATA-4	5'-CGC CGC CTG TCC GCT TCC-3' 5'-TTG GGC TTC CGT TTT CTG GTT TGA-3'
HNF-3beta	5'-ACC TGA GTC CGA GTC TGA CC-3' 5'-GGC ACC TTG AGA AAG CAG TC-3'
Pdx-1	5'-CCA CCC CAG TTT ACA AGC TC-3' 5'-TGT AGG CAG TAC GGG TCC TC-3'
Nestin	5'-CTG AGA GCA AGC TAG AGA A-3' 5'-GTG ACA CTA GTG ACT CTT GA-3'
Nkx6.1	5'-CCA CGT TCT CTG GAC AGC AA-3' 5'-GCT TTT TCA GCA GCT GAG TGA-3'
Pax4	5'-GAA GCA GCT GGA GGC TGT TA-3' 5'-AGA TAC CTT AAG GCT CCG TGA A-3'
Pax6	5'-TAA CGG AGA AGA CTC GGA TGA A-3' 5'-CCA ACA TGG AAC CTG ATG TGA A-3'
Oct-4	5'-GGC GTT CTC TTT GGA AAG GTG TTC-3' 5'-CTC GAA CCA CAT CCT TCT CT-3'
Beta-actin	5'-TCC TAT GTG GGT GAC GAG GC-3' 5'-TAC ATG GCT GGG GTG TTG AA-3'

IgG FITC-conjugated (Vector Laboratories, Burlingame, CA), at a 1:100 dilution. Slides were coverslipped using fluorescence mounting media containing 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories). The sections were then photographed using an Olympus microscope and Optronics digital camera (Olympus, Melville, NY).

Flow Cytometric Analysis

For insulin staining, anti-human insulin (DAKO) and FITC-conjugated anti-guinea pig IgG antibodies (Vector Laboratories) were used as primary and secondary, respectively. For stage-specific embryonic antigen-1 (SSEA-1) staining, anti-mouse SSEA-1 (Developmental Studies Hybridoma Bank, Iowa City, IA) and FITC-conjugated anti-mouse IgG (Vector Laboratories) were used. Cells were harvested from cultures by gentle digestion with 0.25% trypsin/0.21 mmol/L ethylenediamine tetraacetic acid solution (Sigma), centrifuged, and resuspended in PBS, and analyzed by flow cytometry. For staining of intracellular insulin, cells were fixed and permeabilized by 70% ethanol overnight at -20°C before antibody reaction. Cells were incubated at 4°C for 30 minutes with each antibody, washed three times, and resuspended in PBS. All samples were analyzed on a FACSort flow cytometer (Becton-Dickinson, San Jose, CA). Gating was determined based on negative control samples in which the primary antibody was omitted.

Measurement of Insulin Content and Secretion by Western Blotting and Enzyme-Linked Immunosorbent Assay (ELISA)

ES cell cultures that had achieved confluent growth were cultured for 1 month (each dish contained ~ 90 to 100 clusters). Before measuring insulin content, the medium was changed to serum-free medium containing 0.5% bovine serum albumin and 5.5 mmol/L glucose to enable the detection of insulin secretion without interference from the fetal serum. The cells were incubated under these conditions for 5 hours at 37°C followed by washing twice with additional serum-free medium. High-glucose challenge of the cells was achieved by the addition of 0.5 ml of serum-free media containing low (5.5 mmol/L)- or high (23 mmol/L)-glucose for 2 hours at 37°C . The conditioned media was collected and frozen at -20°C until assayed for insulin content. Intracellular insulin was detected through cell extraction with lysis buffer and Western blotting, as described by Yang and colleagues.²⁴ Specifically, the presence of insulin was determined by separation of the precipitated material by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 18% gels, transferred to nylon membranes, and detected with rabbit polyclonal anti-insulin antibody (Santa Cruz Biotechnology). Insulin protein was visualized by chemiluminescence. ELISA was performed on the conditioned media to determine insulin content using the 1-2-3 Ultra-

Sensitive rat insulin ELISA kit following the manufacturer's instructions (ALPCO Diagnostics, Windham, NH).

Transplantation of ES Cell-Derived Cell Clusters and Cytochemical Tests

Hyperglycemia was induced in 10-week-old male NOD/scid mice (Jackson Laboratory, Bar Harbor, ME) through an intraperitoneal injection of 40 mg/kg of streptozotocin (STZ) once a day for 5 consecutive days as described by Oh and colleagues.²³ Blood glucose level was determined using a standard blood glucose meter (One Touch Profile; Johnson and Johnson Co., Milpitas, CA). Stable hyperglycemia (blood glucose levels ranging between 350 and 500 mg/dl) developed 5 to 6 days after the last STZ injection. Under general anesthesia, mice received a renal subcapsular transplant of 100 to 150 ES cell-derived clusters from GFP-labeled B5 ES cells ($n = 10$) or a sham transplant of saline solution ($n = 5$) in the right subcapsular renal space. Blood glucose levels were monitored every 2 days after transplantation for 5 weeks. After death, graft and other organ tissues were extracted and examined by hematoxylin and eosin (H&E) staining, immunohistochemistry, and/or RT-PCR. For direct GFP detection,²⁷ GFP was fixed by 10% neutralized buffered formaldehyde for 16 hours followed by 18% sucrose for an additional 16 hours. The tissues were then embedded in OCT compound and frozen at -80°C until examined.

Statistical Analysis

Values are expressed as the mean $\pm$ SE. Statistical differences were determined by analysis of variance. Values at $P < 0.05$ were considered to be statistically significant.

Results

ES Cell-Derived Cells Differentiated into Pancreatic Islet-Like Cell Clusters

To facilitate ES cells to differentiate into islet-like cell clusters, we used a multistep protocol described previously¹³ with some modifications. After culturing ES cell-derived cells in ITSFn medium, they were dissociated and replated onto polyornithine/laminin-coated or collagen-coated dishes for further differentiation. After this step, terminally differentiated cells did not attach, and nestin-positive cells became the most abundant cell type representing up to 85% of the cultures. Alternatively, we used high-glucose media to accelerate the differentiation and production of islet-like cell clusters. Dense clusters of cells appeared after 7 to 10 days of the differentiation culture step (Figure 1A). Insulin staining was already present as early as 7 days after changing to high-glucose media (data not shown). By the end of this stage most of the nestin staining significantly decreased and islet-like clusters were often expanded in number and became the most predominant cell type, representing up to 40% of

the cultures (Figure 1B). A majority of the cells within clusters ($86.1 \pm 2.1\%$ by flow cytometry) were positive for insulin (Figure 1, C and D).

ES Cell-Derived Clusters Expressed Insulin mRNA and Transcriptional Factors Associated with Pancreatic Development

To determine whether the islet-like clusters appearing in the ES cell cultures may have differentiated into endocrine hormone-producing cells, the expression of pancreatic cell genes was examined by RT-PCR. Glut-2 gene and other pancreatic mRNAs including insulin I and II, glucagon, α -amylase-2A, and islet amyloid polypeptide were expressed after ES cells were cultured using our culturing system (Figure 2A). Also, messages for all of the transcriptional factors found in committed endocrine and pancreatic lineages (Pdx1, Nkx2.2, Nkx6.1, Pax6, and nestin), some of which are not detected in the original ES cells, became expressed (Figure 2B). Of note, expression of OCT-4, a marker of undifferentiated ES cells,²⁸ was first seen in the cells in stage 1, but not detected in the cultures just before transplantation (Figure 2B). This implies that sufficient elimination of undifferentiated ES cells was achieved during the culture.

In addition, we also quantitatively examined mRNA expression for insulin by Northern blotting of ES cell-derived clusters. Figure 2C shows the presence of both insulin I and II mRNAs in cultured ES-derived cell clusters, as well as in INS-1/ β -TC-6 cells (rat/mouse insulinoma cell lines used as positive controls). Original undifferentiated ES cells did not express insulin message (data not shown). These results indicate that ES cell-derived clusters not only express the insulin protein in their cytoplasm but also possess the insulin productivity at the gene level.

ES Cell-Derived Clusters Express Both Insulin and C-peptide and Have Glucose-Dependent Insulin Secretory Activity

To specifically address the presence of endogenously produced insulin in our culture, immunohistochemistry was performed for both insulin and C-peptide in ES cell-derived cell clusters. C-peptide antibody can recognize the mature C-peptide and proinsulin, but not insulin. Because only the mature form of insulin is added to the medium, immunoreactivity for C-peptide or proinsulin would indicate that the precursor proinsulin is synthesized by the cells. The islets of Langerhans in normal pancreas are shown to strongly express both insulin and C-peptide (Figure 3, A and D). The ES cell-derived clusters also expressed both insulin and C-peptide proteins ubiquitously in the cytoplasm (Figure 3, B and E). A number of insulin- and C-peptide-positive cells were observed, corresponding to $\sim 85\%$ and 75% of the total cell cluster population, respectively. To specify whether the insulin-positive cell really contains C-peptide or proinsulin, double immunohistochemistry was done by using

Figure 1. ES cells differentiated into pancreatic islet-like cell clusters via *in vitro* culture system. **A:** Dense clusters of cells appeared after 7 to 10 days of the differentiation culture step and they proliferated and grew during the following periods. **B:** By the end of this stage the number of nestin-positive cells significantly decreased and islet-like clusters were often expanded in number and became the most predominant cell type with up to 40% of the cultures. **C:** Most of cells among the clusters expressed insulin (red) by immunohistochemistry (blue, DAPI). **D:** Of the cells in the clusters, $86.1 \pm 2.1\%$ were insulin-positive by flow cytometry (green area, clusters before transplantation; dotted curve, negative control). Scale bars, 100 μm .

both C-peptide and insulin antibodies, the latter of which does not recognize the precursor proinsulin. Most cells in the clusters showed co-expression of C-peptide/proinsulin and mature insulin (Figure 3; G to I).

An essential feature of pancreatic β cells is the ability to release insulin in response to high-glucose concentrations. In Western blotting analysis, the differentiated ES cell-derived cells synthesize and store sufficient amounts of insulin during the exposure to high-glucose media, whereas very little insulin is detected in the cell lysates from cells cultured in low-glucose media (Figure 3J). In addition, we performed an ELISA assay to quantify the insulin being released into the media. Challenge of ES cell-derived cell clusters (~ 90 to 100 clusters per experiment) with high-glucose concentration for 2 hours resulted in secretion of $283.1 \pm 41.2 \mu\text{U} \cdot \text{islets}^{-1} \cdot 60 \text{ min}^{-1}$ of insulin into the media, which is significantly higher than that in low-glucose media ($35.2 \pm 2.2 \mu\text{U} \cdot \text{islets}^{-1} \cdot 60 \text{ min}^{-1}$, $P < 0.05$) or in media alone ($0 \mu\text{U} \cdot \text{islets}^{-1} \cdot 60 \text{ min}^{-1}$, $P < 0.05$) (Figure 3K). These re-

sults suggest that ES cell-derived clusters not only display morphology similar to pancreatic islets, but also possess the function of insulin secretion during glucose challenges.

Transplantation of ES Cell-Derived Clusters for Reversal of Hyperglycemia Failed because of Teratoma Formation

The ability of insulin-producing cells to reverse hyperglycemia was examined *in vivo* using a STZ-induced diabetes NOD/scid mouse model. Multiple treatments of STZ chemically induced a stable diabetic state in NOD/scid mice with the blood glucose concentrations greater than 350 mg/dl. Ten diabetic mice received ~ 100 to 150 GFP-labeled ES cell-derived insulin-producing clusters (each cluster contained 100 to 150 insulin-positive cells) transplanted into the renal subcapsular space. The mice receiving the transplant began to reverse their high blood

Figure 2. Pancreatic gene expression in undifferentiated ES cells and ES cell-derived cell clusters. **A** and **B**: RT-PCR analyses for expression of Glut-2 and pancreatic endocrine proteins and transcription factors. Panc, normal mouse pancreas tissue. **A**: Glut-2 gene and other endocrine pancreatic mRNAs were expressed in the ES cell-derived differentiated cell clusters. **B**: The cell clusters also expressed messages of all of the transcriptional factors found in committed endocrine and pancreatic lineages, but not OCT-4. **C**: Northern blot of the cell clusters to examine the expression of insulin messages. RNAs extracted from INS-1 cells and β -TC-6 cells were used as positive controls. Those from WB-F344 cells were used as a negative control. The results show the presence of both insulin I and II mRNAs at quantitatively sufficient amounts in cultured ES-derived cell clusters.

glucose levels within 2 to 3 days and maintained reversal of hyperglycemia up to 3 weeks after transplantation (Figure 4). Two of ten transplanted mice died without hyperglycemia, whereas all other mice ($n = 8$) reverted to a hyperglycemic state 2.5 to 3 weeks after transplantation and died because of hyperglycemia by day 30. Control animals ($n = 5$) that did not receive implant exhibited persistent hyperglycemia and died by day 20 (Figure 4).

The kidneys that received graft and other organs were excised and further analyzed by conventional H&E staining, immunohistochemistry, and RT-PCR for insulin. Macroscopically, an encapsulated solid tumor was found at each transplant site in 6 of 10 transplanted mice (Figure 5A). When the graft was examined histologically, tumors contained immature epithelial cells with glandular tissues, foci of squamous epithelial cells, and immature muscle in the background stroma (Figure 5, B and C), and were diagnosed as teratoma. There was no invasion into the kidney's parenchyma or surrounding tissues. There were no tumors in other organs including brain, lung, heart, liver, pancreas, spleen, intestine, and muscle (data not shown). A very small number of the cells in the tumor were positive for insulin by immunohistochemistry (Figure 5, D and E), and insulin gene expression, confirmed by RT-PCR, was detected but down-regulated dramatically after transplantation (Figure 5F).

To assess whether undifferentiated ES cells contaminated the insulin-producing clusters, we performed flow cytometric analysis for SSEA-1, another surface marker of undifferentiated ES cells,²⁹ as well as RT-PCR for OCT-4. Surprisingly, OCT-4 was detected in the graft tissues even though the transplanted cells used had no OCT-4 expression before transplantation (Figure 6A). By flow

cytometry, only $0.23 \pm 0.03\%$ of the cells in clusters were SSEA-1-positive (Figure 6B). To further confirm the presence of undifferentiated ES cells, we examined the grafts by immunohistochemistry for OCT-4 and SSEA-1. Most of the undifferentiated ES cells express both markers (Figure 6, C and F), although neither OCT-4 nor SSEA-1 were positive in the clusters before transplantation (Figure 6, D and G). In assessing the grafts, some of the colonized OCT-4- and SSEA-1-positive cells were observed (Figure 6, E and H). These results indicate that immature teratomas containing OCT-4/SSEA-1-positive cells were formed during the treatment of type I diabetes even though highly purified differentiated cells were used for transplantation.

Discussion

Recently the ability to produce insulin-producing cells described by some previous studies^{13–16,30,31} has been challenged. It has since been shown that insulin immunoreactivity was due to insulin uptake from the medium or due to contamination of other types of insulin-positive cells (ie, neuronal cells) rather than endogenous synthesis of the hormone.^{17,18} Thus, more defined and efficient procedures to produce functioning insulin-producing cells from ES cells and direct evidence of feasibility with regard to clinical application is needed. The current study demonstrates that ES cells can differentiate into functional pancreatic β -like cells, capable of producing and secreting insulin, with some modification of Lumelsky and colleagues¹³ original protocol. After transplantation of insulin-producing clusters derived from ES cells into STZ-treated NOD/scid mice, it was demonstrated that hyperglycemia could be reversed for ~ 3 weeks. However, long-term control of hyperglycemia failed.

It became apparent that insulin production is lost as teratoma formation moved forward in the current study. Some previous reports, in which the same Lumelsky and colleagues¹³ protocol with minor modification was used, have not mentioned teratoma formation as the cause of treatment failure.^{13–15,30,31} However, two recent studies reported that when ES cell-derived clusters were transplanted in diabetic mice, they did not reverse the hyperglycemic state and formed teratomas in several cases.^{16,18} The main problem with this strategy may be the purification method for insulin-producing cells. In this system, classical nestin-positive cell selection using multiple culture media was adopted.¹³ Actually, it is very hard to achieve high reproducibility from this system because the number of transplanted cells is hard to calculate due to manually picking up clusters. One study from Kim and colleagues,³¹ for example, showed that the transplant of 1000 ES cell-derived islet-like clusters to diabetic mice (six times more than that of our study), resulting in reversal of hyperglycemia for a week without teratoma formation. We cannot compare the details of each study's methods, but there might be some differences in techniques between their study and ours. On the other hand, some groups have achieved high purification of ES cell-derived insulin-producing cells without tera-

Figure 3. **A to I:** Insulin and C-peptide immunohistochemistry analyses using ES cell-derived islet-like cell clusters. **A–F:** Immunohistochemistry using each single antibody: insulin staining (red) with DAPI nuclear staining (blue) (**A–C**), C-peptide staining (red) with DAPI (blue) (**D–F**). **A, D:** Tissues from normal mouse pancreas were used as positive control. **C, F:** Negative controls were obtained from the samples without primary antibodies. **B, E:** The ES cell-derived clusters did express both insulin and C-peptide proteins ubiquitously in the cytoplasm. **G–I:** Double staining using both insulin (red) and C-peptide (green) antibodies (blue, DAPI). **G:** Merged image of insulin and DAPI, **H:** C-peptide and DAPI, and **I:** insulin and C-peptide. Yellow-colored cells indicate insulin and C-peptide double-positive cells. Most cells in the clusters showed co-expression of C-peptide and mature insulin. **J:** Western blot analysis of insulin storage after collection of cell lysates after a 2-hour culture using low (5.5 mmol/L)- or high (25 mmol/L)-glucose conditions. The differentiated ES cell-derived cell clusters synthesize and store sufficient amounts of insulin during exposure to high-glucose media, whereas only small amounts of insulin were detected in the cell lysate from low-glucose media. **K:** ELISA assay for insulin in conditioned media from ES cell-derived cell clusters exposed to low- or high-glucose conditions for 2 hours. Challenge of ES cell-derived cell clusters (~ 90 to 100 clusters per experiment) with high-glucose concentration resulted in secretion of $283.1 \pm 41.2 \mu\text{U} \cdot \text{islets}^{-1} \cdot 60 \text{ minutes}^{-1}$ of insulin into the media, which is significantly higher than that in low-glucose media ($35.2 \pm 2.2 \mu\text{U} \cdot \text{islets}^{-1} \cdot 60 \text{ minutes}^{-1}$, $P < 0.05$) or in media alone ($0 \mu\text{U} \cdot \text{islets}^{-1} \cdot 60 \text{ minutes}^{-1}$, $P < 0.05$). Scale bars, $100 \mu\text{m}$.

Figure 4. Transplantation of ES cell-derived insulin-producing cell clusters into STZ-treated diabetic NOD/scid mice. Shown in the graph are control STZ-treated mice with no cell transplantation (**dotted line**, $n = 5$) and mice transplanted with 100 to 150 ES cell-derived insulin-producing cell clusters into the renal subcapsular space (**bold solid line** and **solid line**, $n = 10$). Control mice exhibited persistent hyperglycemia and all died by day 20. The mice receiving the transplant began to reverse their high blood glucose levels within 2 to 3 days and these levels were maintained up to 3 weeks after transplantation. Two of ten mice died without hyperglycemia (**solid line**), whereas all other mice eventually became hyperglycemic and died by day 30 (**bold solid line**, $n = 8$).

toma formation by gene insertion (ie, Pax4³⁰ or Pdx-1³²). However, it is still questionable whether it is proper to use genetic manipulation during preparing transplanted cells, because long-term studies have not been performed to determine their efficacy. To eliminate these problems, alternative protocols (ie, development of more efficient and defined purification system using combina-

tion of multiple β -cell-specific markers) should be developed.

In our study, when the graft tissues were examined by immunohistochemistry, RT-PCR, and flow cytometry, the number of insulin-positive cells in the grafts was very low and insulin mRNA expression decreased dramatically after transplantation. Surprisingly, expression of OCT-4, a candidate regulator of pluripotent and germline cells and a marker of undifferentiated ES cells,²⁸ was detected in the graft tissue, even though its expression was not detected after 1 month of differentiation in culture. Similarly, only 0.2% of the cells within the transplanted clusters were positive for another ES cell marker SSEA-1²⁹ by flow cytometry, while abundant expression of this marker was detected in the graft tissue by immunohistochemistry. One possible reason for formation of teratomas despite using OCT-4-negative cell clusters is dedifferentiation of the ES cell-derived differentiated cells. Some reports show the potential for dedifferentiation of germline cells or related tumors.^{33,34} If differentiated ES cells possess the potential for dedifferentiation even after differentiation in culture, this phenomenon must be controlled before the future clinical applications in humans may be considered.

Another possible reason for forming teratoma may be the failure of the cultures to produce a pure cell population. At the end of the final stage of culture, the dish was confluent with differentiated cells including nestin-positive cells and pancreatic islet-like clusters. By flow cytometry, more than 86% of the cells among clusters ap-

Figure 5. Analysis of graft after kidney excision via H&E staining and immunohistochemistry and RT-PCR for insulin expression. **A:** Macroscopically, an encapsulated solid tumor was found at each transplanted site in 6 of 10 transplanted mice. **B, C:** H&E staining of tumor shows immature epithelial cells including mature gland tissues (**B, arrows**), foci of squamous epithelial cells (**B, circle**), and immature muscle in the background stroma (**C, arrows**), which confirmed it as teratoma. **D, E:** Insulin immunohistochemistry of the graft tissues (green, GFP). **E** is a highly magnified image of the boxed area in **D**. Very small numbers of the cells in the tumor were positive for insulin (red). **F:** RT-PCR analysis of the graft tissues for expression of insulin I and II. Both insulin I and II gene expressions were detected but down-regulated dramatically after transplantation. Scale bars, 100 μ m.

Figure 6. Analysis of OCT-4 and SSEA-1 expressions before and after transplantation. **A:** By RT-PCR, the expression of OCT-4 was detected in the graft tissues even though the transplanted cells used had no OCT-4 expression before transplantation. **B:** By flow cytometry, $0.23 \pm 0.03\%$ of the cells in clusters were SSEA-1-positive (green area, clusters before transplantation; **dotted curve**, negative control; **solid curve** with no filling, positive control using undifferentiated ES cells). **C-H:** OCT-4 and SSEA-1 immunohistochemistry analyses. **C-E:** OCT-4 staining (green) with DAPI (blue). **F-H:** SSEA-1 staining (green) with DAPI (blue). **C, F:** Undifferentiated ES cells were used as positive control. **D, G:** Neither OCT-4 nor SSEA-1 were positive in the clusters before transplantation. **E, H:** Some of the colonized OCT-4- and SSEA-1-positive cells were observed in the whole grafted tissues after transplantation. Scale bars, 100 μ m.

peared to be insulin-positive and only 0.2% of the cells were SSEA-1-positive. By immunohistochemistry, neither cells with ES cell-like morphological characteristics nor OCT-4 or SSEA-1 expression were observed in the clusters. In addition, RT-PCR of the cell clusters just before transplantation shows no OCT-4 expression. When the cultured cells were analyzed for OCT-4 using RT-PCR, as low as 1% contamination by undifferentiated ES cells can be detected (data not shown). This result suggests that very few undifferentiated ES cells remained at the time of transplantation. Teratoma formation is usually observed *in vivo* through injection of undifferentiated ES cells or the cells of embryoid bodies in sufficient numbers (1 to 2×10^6 /body). One report showed that the direct injection of ES cells to the brain with the minimal numbers of 1 to 2×10^3 cells/body can restore the function of the damaged brain without formation of teratoma or any other tumors.³⁵ In our studies, we injected 100 to 150 clusters, containing ~ 1 to 2.5×10^4 insulin-positive cells, into the renal sub-capsular space of each recipient, among which we speculate that there may have been as few as 40 OCT-4/

SSEA-1-positive cells, as evidenced by both flow cytometry and RT-PCR. Accordingly, it would seem to be very difficult to avoid teratoma formation using the current techniques and protocols. It is imperative to develop alternative protocols, or consider the use of alternative cell sources, such as adult stem cells, for the purpose of obtaining clinically useful insulin-producing cells for the treatment of diabetes.

In the current study, the glucose level could be reversed to ~ 150 to 250 mg/dl, but not normalized. We estimate that the number of insulin-positive cells we transplanted was $\sim 2.5 \times 10^4$ cells. The number of the transplanted cells is rather low compared to that of conventionally isolated transplanted islets. It would seem logical that to fully normalize the glucose levels of diabetic mice and bring them into euglycemic state, more clusters would be required. However, two recent investigations indicate that substantially lower islet mass can revert diabetes in transplant diabetic recipient,^{36,37} from which the marginal mass islet transplant theory was developed. This theory states that as few as 100 to 125

functional islets (marginal mass) are needed to reverse hyperglycemia, so long as these islets can be functionally maintained within the *in vivo* setting. It is also possible that ES cell-derived insulin-producing cell clusters within these experiments are more efficient at producing insulin than conventionally isolated islets, based on the cells *in vitro* high-glucose environment. Accordingly, the clusters in our system may be applied to the marginal mass transplant theory. Long and relatively high-glucose levels in the culture media may have stimulated a greater insulin response than would be expected from normal islet cells. In addition, the donor cells were prepared for transplantation without trypsin digestion, which could negatively effect the production of insulin. Another explanation may be that the clusters contain not only insulin-producing cells but also cells of progenitor phenotype. RT-PCR showed that ES cell-derived insulin-producing cell clusters express messages for some early pancreas markers such as Nkx2.2, Nkx6.1, or Pdx-1, as well as for various pancreatic hormones and enzymes (insulin, glucagon, amylase, and so forth). The progenitor cells may have the potential to proliferate within the transplanted site and possibly generating substantial islet-like cell mass *in vivo*. Two recent reports support this hypothesis: Oh and colleagues²³ transplanted ~150 bone marrow-derived islet-like clusters into diabetic mice, which could reverse hyperglycemia for an extended period of time. A report by Ramiya and colleagues³⁸ showed that after transplantation of a small number of purified islet progenitor cells into the renal subcapsular space of diabetic mice, the graft grew and distended macroscopically and reversed the hyperglycemia for more than 3 months. These reports suggest that within the *in vivo* environment, additional stimuli may be provided for the transplanted cells to further mature/differentiate amplifying the islet cell number for maximal insulin production capacity.

In our study, two recipient mice died after transplantation without hyperglycemia. These two mice experienced a sudden drop in blood glucose levels at day 24, and continued to have glucose levels below normal until death. We speculate that they may have died because of severe infection or, more probably, because of severe hypoglycemia. It is possible that hypoglycemia could be the result of insulinoma formation, although none was apparent by histopathological analysis. Further investigation is needed if the same or similar situation occurred in the future transplantation.

Some researchers argue that insulin immunoreactivity is due to insulin uptake from the medium or contamination by other types of insulin-positive cells rather than endogenous synthesis of the hormone.^{17,18} It is not yet clear whether this phenomenon could have been responsible for all reported insulin immunoreactivity. It is still controversial whether insulin-producing cells can be generated by the original or modified protocol of Lumelsky and colleagues.¹³ The breakthrough of this argument is in showing conclusive data that these cells work functionally and express insulin message quantitatively in culture. Our studies show that differentiated ES cell-derived cell clusters synthesize, store, and secrete detectable amounts of insulin during glucose challenge as deter-

mined by Western blot analysis and ELISA assay. These results indicate that ES cell-derived clusters truly possess the ability to functionally produce and release active insulin. Also, we performed Northern blot of ES cell-derived clusters to quantitatively examine the mRNA expression of insulin and showed the presence of both insulin I and II mRNAs in the clusters. This indicates that using the modified protocol we can produce cells that generate sufficient amounts of insulin mRNA for the detection by Northern blot.

Some previous studies reported that ES cell-derived differentiated clusters can reverse hyperglycemia or contrarily that they have no effect on normalization of hyperglycemia.^{13–16,30} The discrepancies between our result and the others may be due to differences in the procedure used for preparation of the cell grafts and recipient mice, as well as the methods for transplantation. In the *in vivo* cell transplantation used in the current study, we performed multiple treatments of STZ injection to chemically induce a stable diabetic state in NOD/scid mice and only included mice with definite achievement of blood glucose concentrations greater than 350 mg/dl. We repeatedly confirmed the blood glucose concentrations before transplantation to guarantee that the recipient mice had achieved an irreversible diabetic state without further treatment. This was also confirmed by the fact that control mice that did not receive cell transplantation continuously maintain high-glucose concentration levels more than 400 mg/dl and died due to hyperglycemia. Using these recipient diabetic mice, we achieved the reversal of hyperglycemia after ES cell-derived cell clusters were transplanted, although long-term maintenance could not be achieved. These results show that insulin-producing cells retain their ability to secrete insulin *in vivo* and respond to high-blood glucose levels in the diabetic mice until the grafts were impaired by teratoma formation.

In conclusion, we have generated insulin-producing cells from ES cells using a modified protocol of nestin-positive cell enrichment. These cells functionally produce, store, and release insulin and these functions are glucose-dependent. However, reversal of hyperglycemia using the ES cell-derived insulin-producing clusters failed because of teratoma formation, which suggests that alternative protocols and/or cell sources must be developed to generate insulin-producing cells that are truly useful for the treatment of diabetes mellitus. Our study also indicates that to use ES cells as a clinical tool, 100% purity will have to be achieved. Our data reveals that 99.8% purity is not going to be sufficient. Clearly much more work is going to be needed to elucidate these answers.

Acknowledgments

We thank Dr. Naohiro Terada, Dr. Tadashi Hamazaki, and Dr. Masanori Oka for expert help in manipulating ES cells; Dr. Marda Jorgensen for her excellent expertise and assistance in preparing and performing immunohistochemistry; Dr. Chen Liu for his kind help for observing and diagnosing the transplanted graft tissues; and

Dr. Bhavna Bhardwaj for her kind help in using the flow cytometric analyzer.

References

1. Sutherland DE, Gruessner RW, Dunn DL, Matas AJ, Humar A, Kandaswamy R, Mauer SM, Kennedy WR, Goetz FC, Robertson RP, Gruessner AC, Najarian JS: Lessons learned from more than 1,000 pancreas transplants at a single institution. *Ann Surg* 2001, 233:463–501
2. Ricordi C, Strom TB: Clinical islet transplantation: advances and immunological challenges. *Nat Rev Immunol* 2004, 4:259–268
3. Lechner A, Leech CA, Abraham EJ, Nolan AL, Habener JF: Nestin-positive progenitor cells derived from adult human pancreatic islets of Langerhans contain side population (SP) cells defined by expression of the ABCG2 (BCRP1) ATP-binding cassette transporter. *Biochem Biophys Res Commun* 2002, 293:670–674
4. Suzuki A, Nakauchi H, Taniguchi H: Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. *Diabetes* 2004, 53:2143–2152
5. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000, 343:230–238
6. Wiles MV, Keller G: Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* 1991, 111:259–267
7. Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J, Wobus AM: Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic termination genes and functional expression of ionic currents. *Dev Biol* 1994, 164:87–101
8. Wobus AM, Wallukat G, Hescheler J: Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca²⁺ channel blockers. *Differentiation* 1991, 48:173–182
9. Dani C, Smith AG, Dessolin S, Leroy P, Staccini L, Villageois P, Darimont C, Ailhaud G: Differentiation of embryonic stem cells into adipocytes in vitro. *J Cell Sci* 1997, 110:1279–1285
10. Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD: Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 1996, 59:89–102
11. Keller GM: In vitro differentiation of embryonic stem cells. *Curr Opin Cell Biol* 1995, 7:862–869
12. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S: Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 2000, 408:92–96
13. 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* 2001, 292:1389–1394
14. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M: Insulin production by human embryonic stem cells. *Diabetes* 2001, 50:1691–1697
15. 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* 2000, 49:157–162
16. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD, Kim SK: Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc Natl Acad Sci USA* 2002, 99:16105–16110
17. Rajagopal J, Anderson WJ, Kume S, Martinez OI, Melton DA: Insulin staining of ES cell progeny from insulin uptake. *Science* 2003, 299:363
18. Sipione S, Eshpeter A, Lyon JG, Korbutt GS, Bleackley RC: Insulin expressing cells from differentiated embryonic stem cells are not beta cells. *Diabetologia* 2004, 47:499–508
19. Lendahl U, Zimmerman LB, McKay RD: CNS stem cells express a new class of intermediate filament protein. *Cell* 1990, 60:585–595
20. Hunziker E, Stein M: Nestin-expressing cells in the pancreatic islets of Langerhans. *Biochem Biophys Res Commun* 2000, 271:116–119
21. Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF: Multipotent nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 2001, 50:521–533
22. Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A: Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 1998, 76:79–90
23. Oh SH, Muzzonigro TM, Bae SH, LaPlante JM, Hatch HM, Petersen BE: Adult bone marrow-derived cells trans-differentiating into insulin-producing cells for the treatment of type I diabetes. *Lab Invest* 2004, 84:607–617
24. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB: In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. *Proc Natl Acad Sci USA* 2002, 99:8078–8083
25. Michalopoulos G, Pitot HC: Primary culture of parenchymal liver cells on collagen membranes. Morphological and biochemical observations. *Exp Cell Res* 1975, 94:70–78
26. Oh SH, Miyazaki M, Kouchi H, Inoue Y, Sakaguchi M, Tsuji T, Shima N, Higashio K, Namba M: Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. *Biochem Biophys Res Commun* 2000, 279:500–504
27. Kusser KL, Randall TD: Simultaneous detection of EGFP and cell surface markers by fluorescence microscopy in lymphoid tissues. *J Histochem Cytochem* 2003, 51:5–14
28. Niwa H, Miyazaki J, Smith AG: Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 2000, 24:372–376
29. Cheng L, Hammond H, Ye Z, Zhan X, Dravid G: Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells* 2003, 21:131–142
30. Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM: Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc Natl Acad Sci USA* 2003, 100:998–1003
31. Kim D, Gu Y, Ishii M, Fujimiyama M, Qi M, Nakamura N, Yoshikawa T, Sumi S, Inoue K: In vivo functioning and transplantable mature pancreatic islet-like cell clusters differentiated from embryonic stem cell. *Pancreas* 2003, 27:e34–e41
32. Miyazaki S, Yamato E, Miyazaki J: Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells. *Diabetes* 2004, 53:1030–1037
33. Subramaniam K, Seydoux G: Dedifferentiation of primary spermatocytes into germ cell tumors in *C. elegans* lacking the pumilio-like protein PUF-8. *Curr Biol* 2003, 13:134–139
34. Pais V, Leav I, Lau KM, Jiang Z, Ho SM: Estrogen receptor-beta expression in human testicular germ cell tumors. *Clin Cancer Res* 2003, 9:4475–4482
35. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O: Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci USA* 2002, 99:2344–2349
36. Pileggi A, Molano RD, Berney T, Cattani P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, Inverardi L: Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. *Diabetes* 2001, 50:1983–1991
37. Garcia-Ocana A, Vasavada RC, Cebrian A, Reddy V, Takane KK, Lopez-Talavera JC, Stewart AF: Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. *Diabetes* 2001, 50:2752–2762
38. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG: Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med* 2000, 6:278–282