The glucose challenge

To remove insulin added to the culture medium, cultured cells were washed 10 times with phosphate-buffered saline (PBS). The RPMI medium with a low glucose concentration (2.8 mM) was added, and the cells were cultured at 37°C for 30 min as preincubation. After the preincubation, RPMI with a low glucose concentration (2.8 mM) was added, and the cells were cultured at 37°C for 60 min. This low-glucose medium was then collected. The RPMI medium with a high glucose concentration (20 mM) was then added, and the cells were cultured at 37°C for 60 min. This high-glucose medium was also collected. The collected media were assayed for insulin concentration using an enzyme-linked immunosorbent assay (ELISA; SRL, Tokyo, Japan).

An immunofluorescence assay

Cells were fixed in 4% paraformaldehyde for 20 min. Subsequently, the cells were blocked with 10% serum and 0.2% Triton X-100 in PBS, and then incubated with a primary antibody against mouse insulin (guinea pig polyclonal antibody to mouse insulin, 1:100; Abcam, Tokyo, Japan) overnight at 4°C. The cells were next incubated with a secondary antibody (goat polyclonal antibody against guinea pig IgG, H&L labeled with fluorescein isothiocyanate [FITC], 1:100; Abcam). A medium for fluorescence microscopy that contained 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) was used for mounting onto slides.

Results

Directed pancreatic differentiation of fibroblast-derived iPS cells

To generate pancreatic stem cells from iPS cells, we applied the D'Amour's protocol [12]. At stage 1, iPS cells differentiated into definitive endoderm using high concentrations of activin A with Wnt3a (day 1) and FBS supplementation (days 2–3). At stage 2, the definitive endoderm cells differentiated into gut tube endoderm through removal of activin A and addition of FGF10 and KAAD-cyclopamine. After the cells were passaged once and cultured for 4 days, the clones were manually picked under a dissecting microscope (Figure 1A). These clones were then cultured on STO feeder cells in the ES culture medium. The 52 clones were cultured and passaged for 30 days (Figure 1B). After the 30-day culture of these clones, 12 clones were still viable (Figure 2A),

Establishment of pancreatic stem cells

Among the 12 clones, 6 were eliminated because they did not proliferate as actively as did the iPS cells. Expression of pancreatic and duodenal homeobox factor 1 (Pdx1) mRNA, a transcription factor required for pancreatic development and β-cell maturation, in the remaining 6 clones is shown in Figure 2B. The proliferative activity of 4 of the 6 clones significantly decreased during a 60-day culture after the isolation. Therefore, we selected the 2 clones (C#15 and D#22) that maintained a constant cell proliferation rate as candidate clones for differentiation into pancreatic stem cells. Clone C#15 was ascertained to have a tumorigenic potential. Finally, clone D#22 was selected (Figure 2C). D#22 cells did not express green fluorescent protein (GFP), whereas the

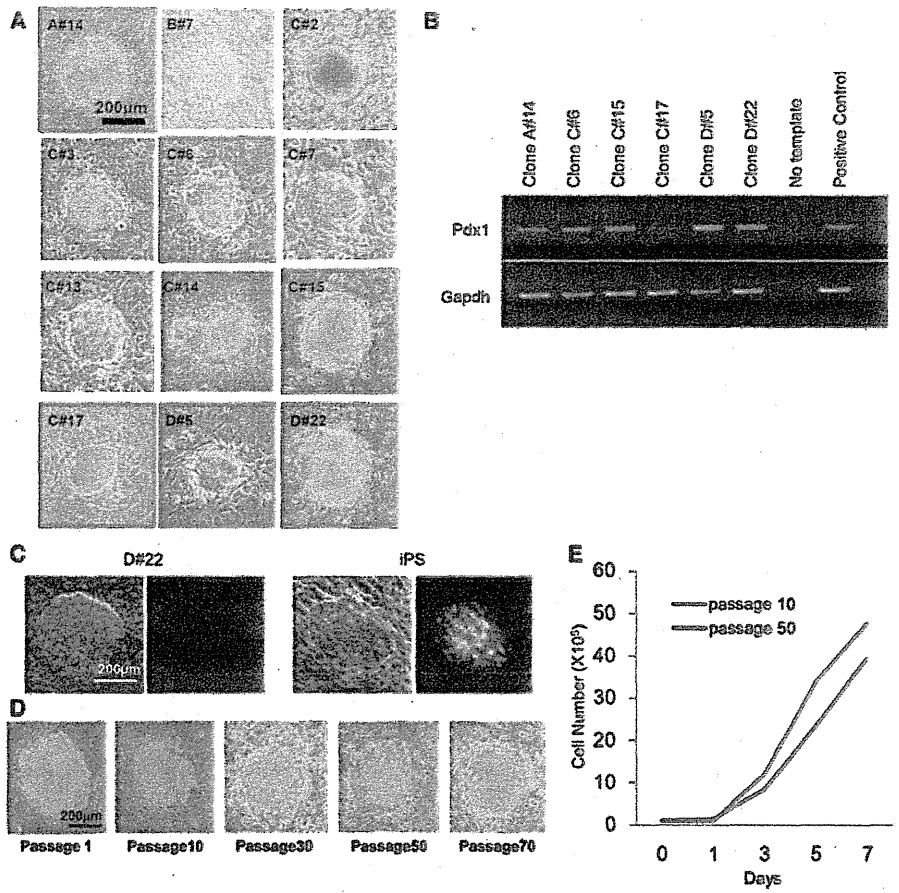

Figure 2 Morphology and expression of Pdx1 and Nanog, and growth activity. A. Morphology of 12 clones after 30-day culture (passage 7). Scale bars = 200 μm. **B.** Pdx1 gene expression of 6 candidate clones. These 6 clones were selected based on their ability to grow in culture for over 30 days. Pancreatic stem cells from pancreatic tissue (data not shown) were used as a positive control. **C.** Nanog (GFP) expression of D#22 and iPS cells. The iPS cells but not D#22 cells expressed GFP. **D.** Morphology of D#22 cells at several passages. Scale bars = 200 μm. **E.** Growth activity of D#22 cells at passages 10 and 50.

iPS cells in this study expressed GFP according to Nanog expression [18]; this result suggests that D#22 cells do not express Nanog and are therefore different from iPS cells (Figure 2C).

Morphology, gene expression, and growth activity of the pancreatic stem cell line D#22

Clone D#22 formed a flat "cobblestone" monolayer, which is characteristic of cultured duct cells [20]. D#22 cells continued to divide actively beyond passage 80 (over 6 months) without changes in morphology (Figure 2D) or in growth activity (Figure 2E). We performed normal and quantitative RT-PCR testing of clone D#22 for ES cell markers, endodermal/pancreatic progenitor cell markers, and pancreatic cell markers. Mouse pancreas cDNA and iPS cells were used as controls. Representative genes of the posterior foregut (Pdx1) and pancreatic endoderm (Ngn3, NeuroD) were expressed by clone D#22, whereas genes typically expressed in undifferentiated iPS cells (Nanog) and in pancreatic tissue (insulin, glucagon, amylase, and somatostatin) were not expressed in D#22 (Figure 3A, B).

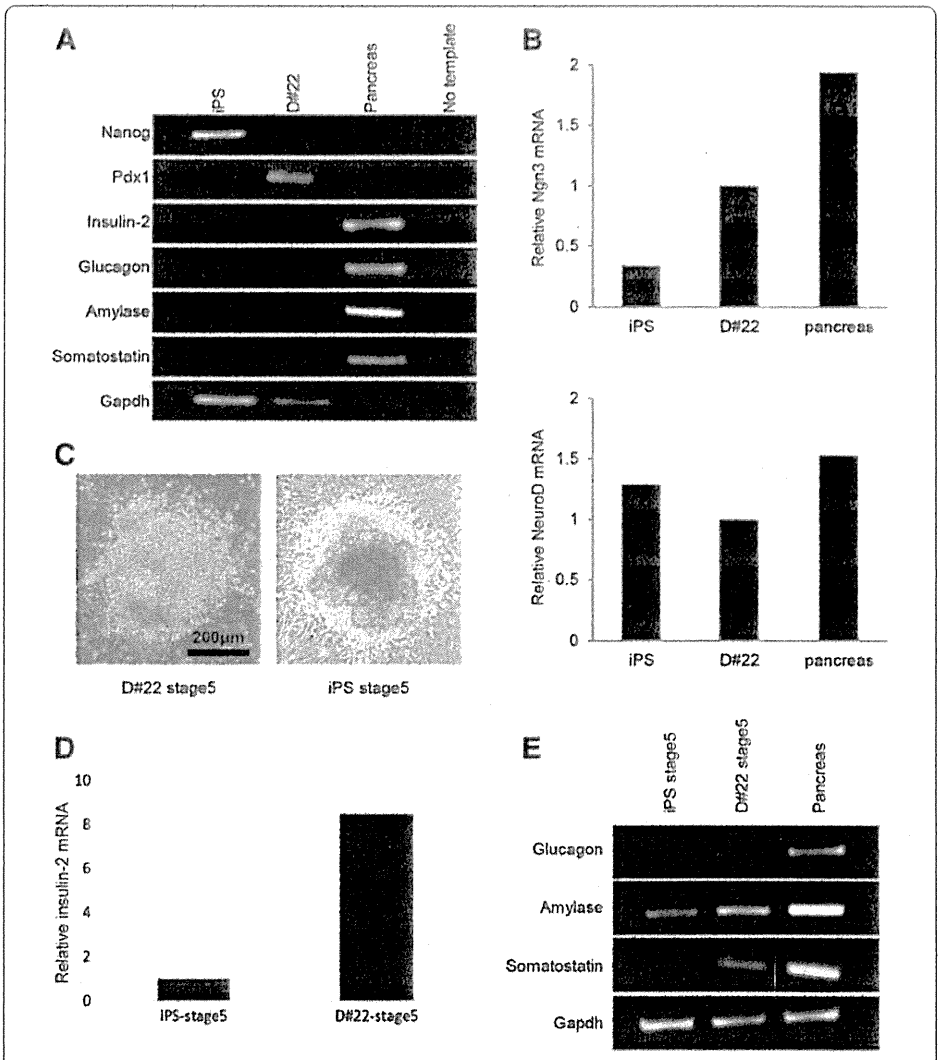

Figure 3 Characterization of the pancreatic stem cell line D#22. A. RT-PCR analysis of expression of an ES cell marker (Nanog), pancreatic progenitor markers (Pdx1), and mature pancreatic markers (insulin-2, glucagon, amylase, and somatostatin) in D#22 cells. Pancreatic cells and iPS cells were used as controls. The primers used for RT-PCR are shown in Table 1. **B**. Quantitative RT-PCR analysis of the Ngn3 and NeuroD genes in iPS, D#22, and pancreatic cells. The data are expressed as the Ngn3 and NeuroD to β-actin ratio, with the expression in D#22 cells arbitrarily set at 1.0. **C**. Morphology of D#22 stage 5 cells and iPS stage 5 cells. Scale bars = 200 μm. **D**. Quantitative RT-PCR analysis of the insulin-2 gene in D#22 stage 5 cells and iPS stage 5 cells. Differentiated cells derived from D#22 cells by stages 3–5 or iPS cells by stages 1–5 were analyzed using quantitative RT-PCR. The data are expressed as the insulin-2 to β-actin ratio, with the expression in iPS stage 5 cells arbitrarily set at 1.0. **E**. RT-PCR testing for pancreatic markers of maturity (glucagon, amylase, and somatostatin) in D#22 stage 5 and iPS stage 5 cells. Pancreatic cells were used as a control. The primers used for RT-PCR are shown in Table 1.

The differentiation capacity of the pancreatic stem cell line D#22

For differentiation of D#22 cells into insulin-producing cells, the following protocols were used (stages 3–5 in Figure 1A). At the final stage of differentiation, D#22 cells underwent a morphological transition (Figure 3C). The differentiated cells derived from D#22 cells, named D#22 stage 5 cells, were tested for pancreas-specific gene expression and insulin production. Mouse iPS cells were also induced to differentiate using the 5-step protocol (Figure 1); the pancreatic potential of the final product (iPS stage 5

cells) was compared with that of D#22 stage 5 cells. Quantitative PCR demonstrated that levels of insulin 2 mRNA were 8.46-fold higher in D#22 stage 5 cells than in iPS stage 5 cells (Figure 3D). D#22 stage 5 cells were positive for gene expression of hormones produced in the endocrine pancreas (e.g., insulin, glucagon, and somatostatin) in addition to a representative enzyme produced in the exocrine pancreas (amylase; Figure 3E).

Insulin expression and functional analysis of D#22 stage 5 cells

To determine whether D#22 cells could differentiate into insulin-producing cells, immunofluorescence analysis was performed. Some D#22 stage 5 cells were positive for insulin, and the insulin-positive cells were C-peptide positive, thus excluding the possibility of insulin uptake from the medium (Figure 4A).

To evaluate the insulin production of D#22 stage 5 cells, a glucose challenge test was performed *in vitro*. The amount of insulin secreted by D#22 stage 5 cells tended to increase when the glucose concentration of the medium was high, whereas iPS stage 5 cells showed little or no response to the glucose challenge (Figure 4B). These data suggested that D#22 stage 5 cells had the ability to secrete insulin in response to the pathological glucose concentrations similar to those in diabetic patients.

Tumorigenicity of the pancreatic stem cell line D#22

To rule out the possibility of teratoma formation as a result of contamination with iPS cells or spontaneous transformation of D#22 cells, the tumorigenic potential was tested in vivo. D#22 cells (1×10^7) at passage 50 were transplanted into nude mice (n = 3), and no tumors developed after 2 months. In contrast, injection of 1×10^7 iPS cells resulted in tumor development after 2 weeks. Tumors derived from the iPS cells became larger over time and had a size of 40 mm at 2 months after transplantation (Figure 4C).

Discussion

In this study, we established a pancreatic stem cell line, D#22, from mouse fibroblast-derived iPS cells. Generally, stem cells are defined as cells capable of self-renewal that can develop into various lineages in the body. D#22 cells were maintained by repeated passaging for more than 6 months without growth inhibition, which indicates that D#22 cells have the potential for self-renewal. The differentiated D#22 stage 5 cells express insulin, glucagon, amylase, and somatostatin mRNA; these data indicate that D#22 cells can generate both endocrine and exocrine pancreatic lineages. Consequently, we consider the clone D#22 a pancreatic stem cell line. As shown in Figure 3A, D#22 cells express Pdx1, Ngn3, and NeuroD. Pdx1-expressing epithelial progenitors are involved in the development of organs during embryogenesis, especially the cells giving rise to the endocrine, exocrine, and ductal cells of the pancreas [21]. Ngn3 is expressed in all endocrine progenitors [21], initiating a cascade of expression of transcription factors that control endocrine cell differentiation. NeuroD, a basic helix-loop-helix (bHLH) transcription factor, is also a key regulator of pancreatic islet morphogenesis and insulin gene transcription [22,23]. However, D#22 cells did not express key transcription factors of ES cells (Nanog) or mature pancreatic cells (insulin, glucagon,

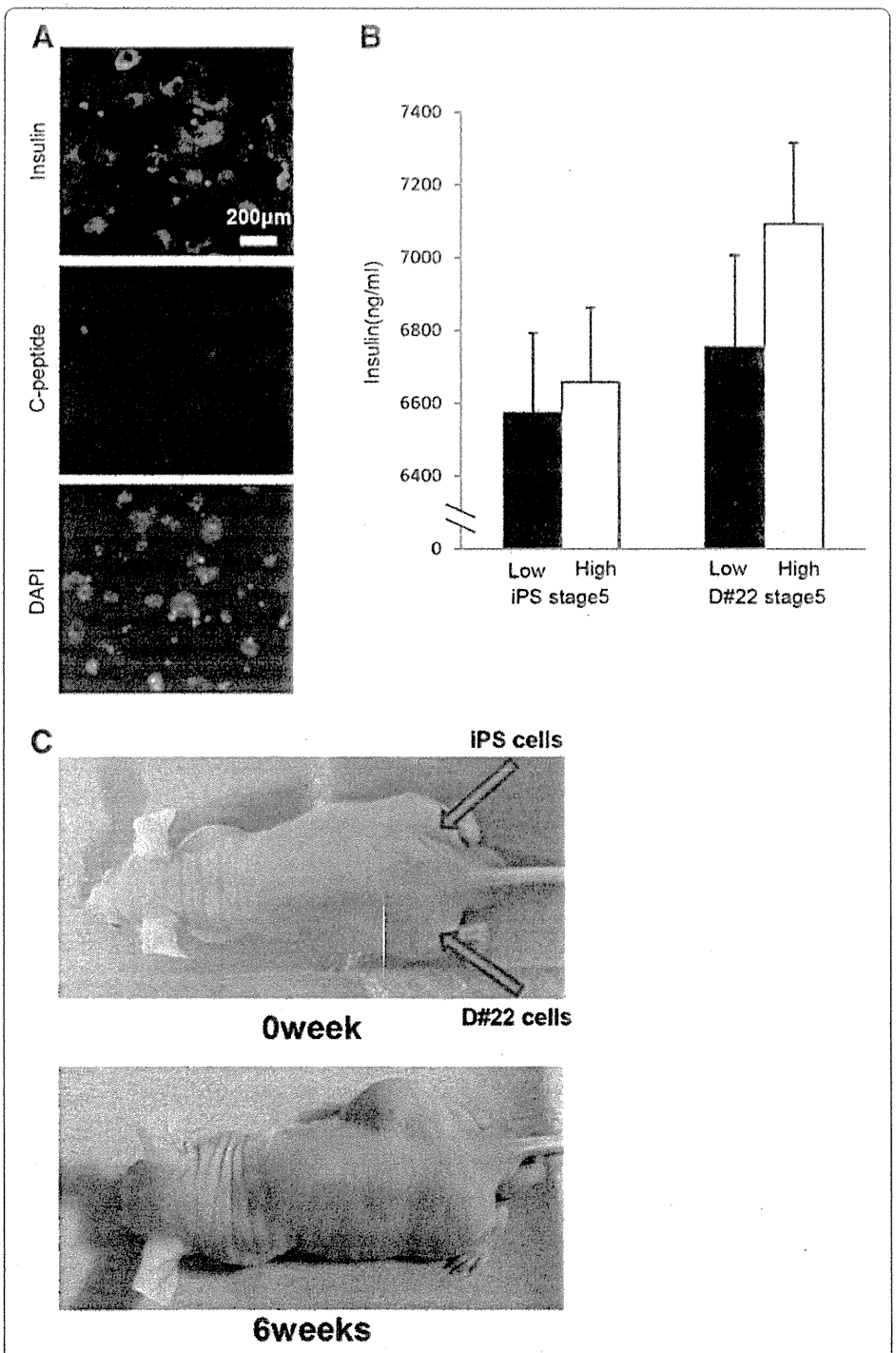

Figure 4 Insulin expression and teratoma formation/tumorigenicity assay of the pancreatic stem cell line D#22. A. Immunofluorescence analysis of differentiated cells derived from D#22 cells. Insulin and C-peptide staining of D#22 stage 5 cells was performed. Scale bars = 200 μm. **B**. Insulin release assay. D#22 stage 5 cells and iPS stage 5 cells were stimulated with D-glucose at 2.8 mM and 20 mM, and the amount of insulin released into the culture supernatant was measured using ELISA. Error bars represent standard error. **C**. In total, 1×10^7 of D#22 cells were injected into the thigh of nude mice. As a positive control, we transplanted 1×10^7 iPS cells into the contralateral thigh.

amylase, and somatostatin). These data indicate that D#22 cells have the characteristics of pancreatic endoderm-committed intermediates or pancreatic progenitors.

D#22 stage 5 cells secreted insulin in response to glucose stimulation, and immunofluorescent analysis of the differentiated cells showed production of insulin and C-peptide. Moreover, in the real-time PCR analysis, mRNA expression of insulin 2 was 8.46-fold higher in D#22 stage 5 cells than in iPS stage 5 cells generated using the same stepwise differentiation protocol. In addition, clone D#22 is not tumorigenic, whereas transplanted iPS cells rapidly form tumors. In general, tumorigenesis is a major concern in the clinical application of stem cell therapy. Therefore, we can conclude that D#22 cells are potentially a more useful cell source for β-cell replacement therapy in diabetes than iPS cells because the former are safer and capable of generating greater numbers of functional insulin-producing cells. Nevertheless, the level of insulin expression was still low; suggesting that the induced cells were still immature. Therefore, it is necessary to develop a more efficient differentiation protocol for production of insulin-producing cells.

The advantages of D#22 cells compared with iPS cells are 1) more efficient differentiation, and 2) no teratoma formation. Recently, a self-renewing endodermal progenitor (EP) cell line generated from human ES and iPS cells was reported [24]. The self-renewing EP cells display morphological properties and a gene expression pattern characteristic of a definitive endoderm. These EP cells differentiate into pancreatic cells, hepatocytes, and intestinal epithelial cells. Moreover, EP cells are not tumorigenic in vivo. D#22 cells may be similar to EP cells, but are likely to be differentiated further into the pancreatic linage because they express the Pdx1, Ngn3, and NeuroD transcription factors.

Conclusions

We established a mouse pancreatic stem cell line from iPS cells derived from mouse embryonic fibroblasts. This clonal cell line has the ability of self-renewal and efficiently differentiates into insulin-producing cells without any signs of tumorigenesis. Therefore, this strategy provides a new approach for generation of insulin-producing cells more efficiently and safely compared to iPS cells. We believe that this approach will help implement a patient-specific cell transplantation therapy for diabetic patients in the near future.

Abbreviations

DAPI: 4′,6-diamidino-2-phenylindole; DAPT: N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMEM: Dulbecco's modified Eagle medium; ES: Embryonic stem, FBS, fetal bovine serum; FGF10: Fibroblast growth factor 10; FITC: Fluorescein isothiocyanate; Foxa2: Forkhead box protein a2; GFP: Green fluorescent protein; HGF: Hepatocyte growth factor; IGF-1: Insulin-like growth factor 1; iPS: Induced pluripotent stem; LIF: Leukemia inhibitory factor; MEXT: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Pdx1: Pancreatic and duodenal homeobox factor 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TK carried out most of the experimental work with the help of. HT, TK, MI. HN designed the experiments and analyzed the data. IS, HUK, MW, YN and TF provided materials and discussion. TK and HN wrote the manuscript. All authors discussed and commented on the manuscript. All authors read and approved the final manuscript.

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A New Method for Generating Insulin-Secreting Cells from Human Pancreatic Epithelial Cells After Islet Isolation Transformed by *NeuroD1*

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Abstract

The generation of insulin-secreting cells from nonendocrine pancreatic epithelial cells (NEPEC) has been demonstrated for potential clinical use in the treatment of diabetes. However, previous methods either had limited efficacy or required viral vectors, which hinder clinical application. In this study, we aimed to establish an efficient method of insulin-secreting cell generation from NEPEC without viral vectors. We used nonislet fractions from both research-grade human pancreata from brain-dead donors and clinical pancreata after total pancreatectomy with autologous islet transplantation to treat chronic pancreatitis. It is of note that a few islets could be mingled in the nonislet fractions, but their influence could be limited. The *NeuroD1* gene was induced into NEPEC using an effective triple lipofection method without viral vectors to generate insulin-secreting cells. The differentiation was promoted by adding a growth factor cocktail into the culture medium. Using the research-grade human pancreata, the effective method showed high efficacy in the differentiation of NEPEC into insulin-positive cells that secreted insulin in response to a glucose challenge and improved diabetes after being transplanted into diabetic athymic mice. Using the clinical pancreata, similar efficacy was obtained, even though those pancreata suffered chronic pancreatitis. In conclusion, our effective differentiation protocol with triple lipofection method enabled us to achieve very efficient insulin-secreting cell generation from human NEPEC without viral vectors. This method offers the potential for supplemental insulin-secreting cell transplantation for both allogeneic and autologous islet transplantation.

Introduction

CELL THERAPY AS A TREATMENT for diabetes requires a source of human insulin-secreting cells that can respond to glucose in a physiologic manner. Allogeneic islet cell transplantation has been performed for the treatment of type 1 diabetes with promising results (Shapiro *et al.*, 2000, 2006; Matsumoto *et al.*, 2005, 2011; Matsumoto, 2010); however, it often needs multiple transplantations to achieve insulin independence. Therefore, increasing the functional transplanted beta cell mass should improve the clinical outcomes. On the other hand, autologous islet transplantation after total pancreatectomy has been performed for the treatment of chronic pancreatitis with severe abdominal pain (Sutherland *et al.*, 2008). All isolated islets were transplanted into the patient; however, less than half of the patients could maintain insulin independence (Sutherland *et al.*, 2008; Matsumoto, 2011; Takita *et al.*, 2011). To improve the efficacy of autologous islet transplantation, it is necessary to increase the transplanted beta cell mass. Thus, the generation and transplantation of insulin-secreting cells from nonendocrine pancreatic epithelial cells (NEPEC) derived from the remaining pancreatic tissues after islet isolation has the potential to improve the efficacy of both allogeneic and autologous islet transplantation. It has been demonstrated that insulin-secreting cells can be generated from nonendocrine pancreatic

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cells under certain conditions (Bonner-Weir *et al.*, 2000; Ramiya *et al.*, 2000; Seaberg *et al.*, 2004; Suzuki *et al.*, 2004; Zhao *et al.*, 2005; Hao *et al.*, 2006; Noguchi *et al.*, 2006; Yamamoto *et al.*, 2006; Inada *et al.*, 2008). However, previous methods had limited efficacy or required viral vectors, which hinder clinical application. In addition, so far there is no report to generate them from pancreata with chronic pancreatitis. In this study, we introduced the human *NeuroD1* gene into human NEPEC from both cadaveric donors and removed pancreata with chronic pancreatitis using an effective nonviral gene transfection protocol, since a previous study suggested that *NeuroD1* could facilitate the differentiation of pancreatic nonendocrine cells *in vitro* (Noguchi *et al.*, 2006). This method promoted their differentiation into glucose-responsive insulin-secreting cells that were able to ameliorate diabetes *in vivo* after transplantation.

Materials and Methods

Plasmid constructs

The plasmid encoding human *NeuroD1* under human cytokeratin19 promoter (pCK19-hND) was produced as shown previously (Kagaya *et al.*, 2001; Shimoda *et al.*, 2010a). After transfection of this plasmid into human islets, the NeuroD1 expression did not increase (data not shown). We produced and tested the plasmids encoding some reporter genes such as *GFP* and *DsRed* under the human CK19 promoter and transfected them to some cell lines expressing or not expressing CK19 and confirmed that it functioned in only CK19-expressing cells. Briefly, we used Panc-1 cell line for CK19+ cells and HFL-1 cell line for CK19− cells. The efficacy of the transfection of pCK19-GFP, pCK19-DsRed, and pCK19-hND into Panc-1 by single lipofection was about 50–70%. In contrast, no transfected gene expression was detected in HFL-1 cells.

Disease-free human pancreata from brain-dead donors

Fifteen donor pancreata were procured from deceased multiorgan donors after obtaining consent for research through local organ procurement organizations (Southwest Transplant Alliance, Dallas, TX, and LifeGift, Fort Worth, TX) (Matsumoto *et al.*, 2010, 2011). This study was approved by the institutional review board of Baylor Research Institute (Dallas, TX). Islets were isolated using the semi-automated method described previously with our modifications (Matsumoto *et al.*, 2006; Ikemoto *et al.*, 2009; Noguchi *et al.*, 2009). The islets used in this study were handpicked for further purification. The donor and isolation variables were as follows: donor age 46.0 ± 3.6 years; 8 men and 7 women; body mass index (BMI) 30.3 ± 1.3 kg/m^2; cold ischemic time 183 ± 10 min.

Culturing and development of NEPEC

The pancreatic nonislet fraction obtained from the remnant of islet purification was used. We preliminarily examined the remnant cells with quantitative RT-PCR and found that amylase expression was high and the expression levels of NeuroD1, insulin, C-Peptide, Sox9, and Pdx1 were very low as expected by pancreatic cell proportion. CK19 expression was also low compared with normal pancreas. The cells were cultured to generate NEPEC as shown previously (Hao *et al.*, 2006; Shimoda *et al.*, 2010a). Briefly, it was cultured in suspension in RPMI 1640 medium (Cellgro, Manassas, VA) with 5.5 m*M* glucose and 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and antibiotics for 2 days. G418 (40 μg/ml; Invitrogen, Carlsbad, CA) was added in the culture medium for 4 days to deplete fibroblasts. Without G418, the fibroblastic cells rapidly increased and became dominant (Hao *et al.*, 2006; Shimoda *et al.*, 2010a). We counted and calculated the ratio of fibroblastic cell numbers to the total cells at day 7 after plating in the groups with G418 treatment (NEPEC) and without the treatment and found that more than 80% (84.3 ± 4.3%) of cells were fibroblastic in the non-G418 group. On the other hand, less than 1% (0.7 ± 0.2%) of cells were fibroblastic in the G418 treatment group. Samples of the suspension culture cells with G418 at days 0 and 4 were stained with dithizone to check for contamination of islets. The ratio of the area of dithizone-positive cells was calculated using more than 20 randomly captured photomicrographs at a magnification of 100×. Data are presented as mean ± standard error of three independent experiments. Afterward, NEPECs were plated on dishes with the same medium without G418 and spread into a cell monolayer. After 7 days, each preparation from a single pancreas was divided into four groups as described in the following section. We also performed a preliminary experiment to test the transfection efficiency to NEPECs by lipofection using a plasmid encoding GFP reporter gene under CMV promoter (pCMV-EGFP). As a result, the transfection efficacy was about 15%, and there was no remarkable change of cellular appearance.

NEPEC differentiation protocol (triple lipofection method)

Seven days after plating, the pCK19-hND plasmid was transfected with lipofectamine^{2000} (Invitrogen) on days 0, 2, and 4, according to the manufacturer's protocol. At the same time, a set of five growth factors (named F5) was added in the culture medium for 7 days (days 0–7). The supplemented growth factors were 10 m*M* nicotinamide, 1% (v/v) insulin–transferrin–selenium, 10 ng/ml basic fibroblast growth factor, 50 ng/ml exendin-4 (Sigma, St. Louis, MO), and 10 ng/ml bone morphogenetic protein 4 (Pepro Tech, Rocky Hill, NJ). In this study, NEPEC were divided into four groups: (1) nontreated NEPEC (NEPEC group); (2) NEPEC with five growth factors added in culture medium (F5 group); (3) NEPEC with transfection of pCK19-hND plasmid (ND group); (4) NEPEC with both pCK19-hND and the growth factors (ND + F5 group). The cells were evaluated at day 7 with the following assays.

Quantitative real-time PCR

For the four groups and human islets, the whole cells in each culture plate were collected, and the total RNA was prepared from TRIzol (Invitrogen) according to the manufacturer's instructions and was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Then, 1 μl of cDNA was used as a template and analyzed by RT^2 qPCR Primer Assays (SABiosciences, Frederick, MD) on Mx 3000P (Stratagene, La Jolla, CA). The number of

amplification cycles was normalized to the endogenous control GAPDH and displayed as fold change. Then, the relative quantification value to a reference group (NEPEC group or human islets) was calculated.

Immunohistochemistry

The samples were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 20% Aquablock (East Coast Biologics, North Berwick, ME). The following antibodies were used for immunohistochemistry: rabbit anti-NeuroD1 (#AB15580), mouse anti-proinsulin C-peptide (#C-PEP-01), rabbit anti-somatostatin (#AB5494), rabbit anti-neurogenin-3 (#AB5684) (Millipore, Billerica, MA), guinea pig anti-insulin (#ab7842), rabbit anti-Ki67 (#ab15580) (Abcam, Cambridge, MA), mouse anti-cytokeratin 19 (#RCK108; Dako, Glostrup, Denmark), mouse anti-glucagon (#K79bB10; Sigma), goat anti-PDX1 (#A-17), goat anti-Nkx6.1 (#C-14), mouse anti-amylase (#G-10), and rabbit anti-Sox9 (#H-90) (Santa Cruz, Santa Cruz, CA). The antigens were visualized using appropriate secondary antibodies conjugated with fluorescein isothiocyanate, Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa-fluor-488, and Alexa-fluor-568 (Invitrogen). Then, 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) was added to the sections for nuclear staining. Images were captured on an epifluorescent microscope and analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Insulin/C-peptide release assay and cellular C-peptide content

In this study, secreted and cellular C-peptide was measured as well as insulin to eliminate the possibility that cells cultured with insulin did not truly differentiate and could slowly release absorbed insulin (Hansson *et al.*, 2004). The human insulin and C-peptide levels in culture supernatants were measured by enzyme-linked immunosorbent assay kits (Alpco, Salem, NH). The cells were incubated in the fresh RPMI 1640 with 10% FBS. The supernatants were collected 24 hr after incubation and analyzed. The cellular C-peptide content was examined by acid–ethanol extraction. The values were normalized by the cell number.

Glucose stimulation test

After two washes and preincubation in the low glucose solution (RPMI 1640 containing 2.8 m*M* glucose without serum) for 1 hr, the differentiated cells were incubated with RPMI 1640 containing 2.8 m*M* glucose at 37°C for 1 hr followed by incubation with the same medium containing 25 m*M* glucose at 37°C for 1 hr. The conditioned supernatant was collected and analyzed. Human islets were used as a control. The values were normalized by the cell number.

In vivo *transplantation assay*

Diabetes was induced in 10–13-week-old male nude mice (Athymic Nude-*Foxn1*^{nu}; Harlan, Houston, TX) using a single tail vein injection of streptozotocin (STZ; 150 mg/kg; Sigma). Animals were considered to be diabetic after two consecutive blood glucose measurements ≥350 mg/dl. NEPEC, F5, ND, and ND+F5 cells at day 7 were detached from the culture dishes using Accutase (Innovative Cell Technologies, San Diego, CA), and grafts containing 10,000,000 cells of each group were transplanted under the left kidney capsule in the diabetic nude mice. Each mouse received the cells derived from a single pancreas without mixing cells from different pancreata. The nonfasting blood glucose levels were measured three times a week for 30 days. Before STZ administration, before transplantation, and at day 3, 10, 20, and 30 after transplantation, nonfasting blood was taken for measurement of serum mouse and human C-peptide levels. The intraperitoneal glucose tolerance test was performed at day 31. Nontransplanted diabetic nude mice were used as a control (STZ group). ND+F5 cells were transplanted into normal nude mice and examined as well. The graft-bearing kidney was removed at day 32 to establish that the graft was functional, as determined by the elevation of blood glucose postnephrectomy. To investigate the cell proliferation of the graft in the early period after transplantation, the graft-bearing kidney was taken at day 3 as well. The grafts were fixed in 4% paraformaldehyde at 4°C overnight and equilibrated in 30% sucrose at 4°C overnight and cryopreserved with Tissue Tek optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA) at −80°C. Sections were cut at 8 μm increments and analyzed by hematoxylin–eosin staining and immunohistochemistry as described previously (Shimoda *et al.*, 2010b) and above. For assessment of cell proliferation, the grafts were stained with insulin and Ki67. The ratio of PDX1-positive, NeuroD1-positive, Nkx6.1-positive, and Ki67-positive cells in the insulin-positive cells were measured, respectively, by counting the positive cell and the total cell numbers using more than 20 photomicrographs at a magnification of 200×.

The native pancreas was taken from nontreated mice (wild, $n=5$), STZ-induced diabetic mice (STZ, $n=5$), and the diabetic mice after transplantation of the ND+F5 cells at day 35 (ND+F5, $n=5$), and analyzed by immunohistochemistry. The ratio of insulin-positive cells to the total cell number was measured by counting the cells using 20 photomicrographs at a magnification of 40× for each pancreas.

NEPEC derived from pancreas with chronic pancreatitis

The pancreatic nonendocrine tissues were obtained from the five patients who had been clinically diagnosed with chronic pancreatitis and received total pancreatectomy with autologous islet transplantation from August 2010 to August 2011 at Baylor University Medical Center. This study was approved by the institutional review board at Baylor Research Institute (Dallas, TX). The development, differentiation, and assessment of NEPEC were performed in the same manner as with the brain-dead donors.

Statistical analysis

Data were expressed as mean ± standard error. Statistically significant differences between two groups were determined by Student's *t*-test. For the comparison of more than two experimental groups, analysis of variance (ANOVA) was used and then *t*-test with Bonferroni adjustments was used. The repeated-measures ANOVA (RM-ANOVA) test was used to examine differences in blood glucose values.

Results

Nonendocrine pancreatic cells from disease-free human pancreata

Dithizone-positive contaminated islets in the nonislet fraction were very few (Fig. 1A and B), and they comprised 0.5±0.2% of the sample after isolation and further decreased to 0.08±0.03% after culturing with G418.

Differentiation by human NeuroD1 *gene induction and growth factors*

The NEPECs proliferated well during adhesive culture. The proliferation rates of the cells in all four groups were similar (Fig. 1C), which indicated that there was low toxicity associated with the transfection. The morphology of all four groups of cells was indistinguishable 7 days after induction (Fig. 1D). Almost all NEPEC expressed human CK19, and most ND and ND+F5 cells also stained for human NeuroD1, whereas NEPEC and F5 cells did not (Fig. 1E). None of the cells expressed amylase (data not shown). At the same time, *NeuroD1* gene expression was quantified (Fig. 1F). The ND and ND+F5 group had an approximate 10,000-fold greater expression level of the *NeuroD1* gene than the NEPEC group. The insulin gene expression in the ND+F5 group was significantly higher than in other groups and was approximately 3% that of the human islets (Fig. 1G). Glucokinase gene expression in the ND+F5 group was significantly higher than in the NEPEC and F5 groups. CK19 was still expressed in the ND+F5 group, because about 90% of cells expressed CK19 as strongly as NEPEC (Fig. 1E). The expression of glucagon, somatostatin, and pancreatic polypeptide did not differ among the four groups. Amylase expression was not detectable within 40 cycles in all groups (data not shown).

Number of NeuroD1- and insulin-positive cells

In the ND+F5 group, the number of insulin-positive cells clearly increased (Fig. 2A–D). Many of those cells were NeuroD1 positive as well. The ND group had fewer insulin-positive cells than the ND+F5 group. On the other hand, the NEPEC and F5 groups had very few insulin-positive cells. C-peptide staining showed similar results with respect to insulin. The ratio of NeuroD1-positive cells in the ND and ND+F5 groups was significantly higher than in the NEPEC and F5 groups (NEPEC, 8.1±0.7%; F5, 9.1±0.8%; ND, 87.2±2.3%; ND+F5, 85.3±2.0%; $p<0.00001$ in ND and ND+F5 vs. NEPEC and F5; Fig. 2C). The ratio of insulin-positive cells in the ND+F5 group was significantly higher than in the other three groups (NEPEC, 0.8±0.2%; F5, 0.9±0.2%; ND, 8.3±0.6%; ND+F5, 11.9±0.7%; $p<0.00001$ in ND and ND+F5 vs. NEPEC and F5; $p<0.002$ in ND+F5 vs. other groups; Fig. 2D). In the ND+F5 group, most of insulin-positive cells coexpressed NeuroD1 (92.3±2.4%). The ratio of insulin-positive cells in the NEPEC group was higher than in the suspension culture cells after treatment with G418 (0.08%; Fig. 1B), probably because the culture condition might change the characteristic of the cells even without growth factors. Data were obtained from five different pancreata.

Characteristics of insulin-positive cells

Immunohistochemical analysis showed that insulin-positive cells in the ND+F5 group expressed CK19 very weakly, whereas the cells that were strongly CK19 positive did not express insulin (Fig. 3A). The insulin-positive cells strongly coexpressed C-peptide and PDX1 (Fig. 3B and C). Some insulin-positive cells coexpressed neurogenin-3 (Fig. 3D). A few glucagon-positive cells were found near the insulin-positive cells, but they did not coexpress insulin (Fig. 3E). On the other hand, somatostatin, amylase, and Sox9 were not expressed (Fig. 3F–H).

Insulin secretion potency of the differentiated NEPEC

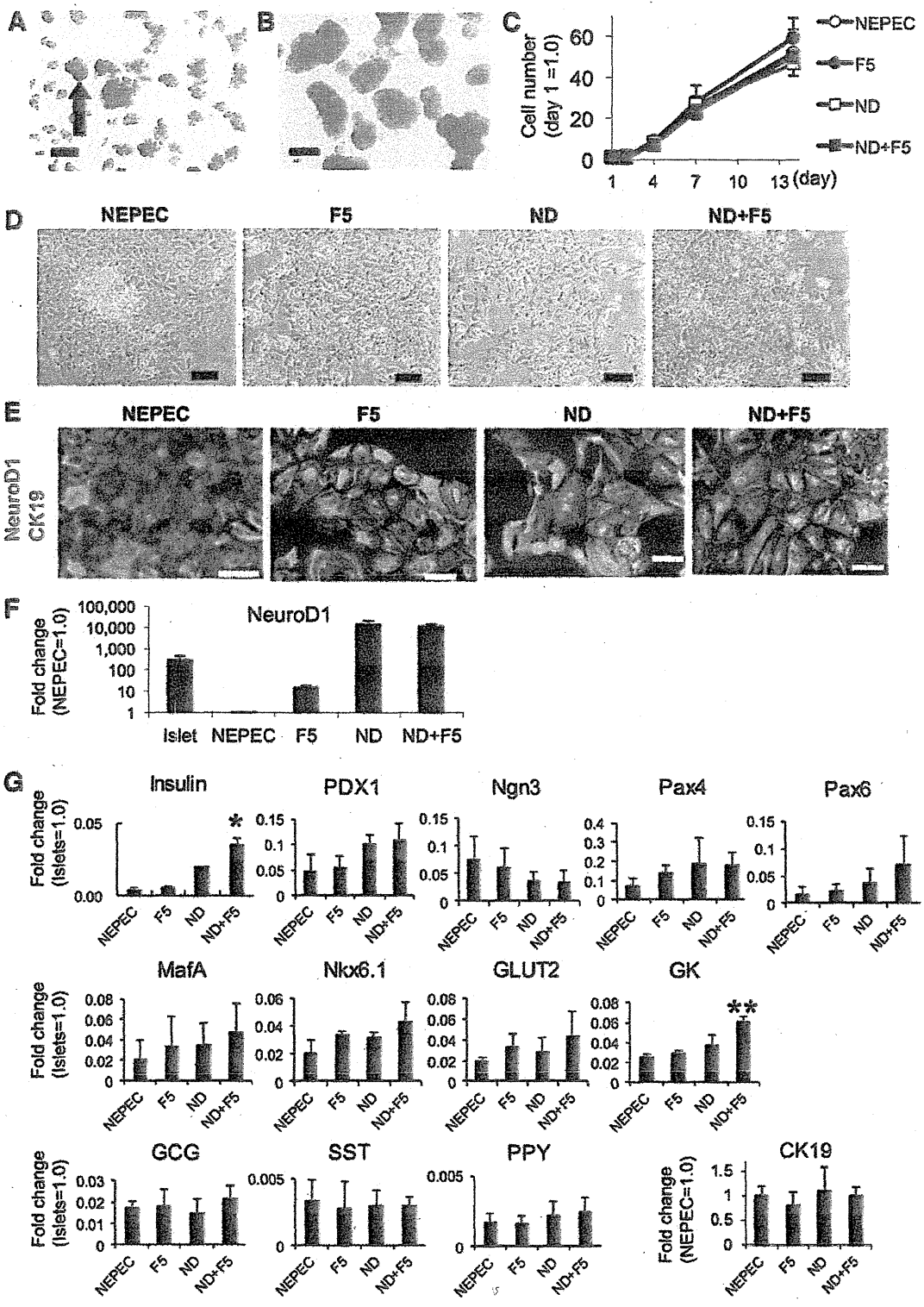
The ND+F5 group had significantly higher cellular C-peptide content than the other groups (NEPEC, 0.8±0.2; F5, 1.6±0.2; ND, 12.1±0.4; ND+F5, 22.8±2.6 pmol/1×10^6 cells; $p<0.001$ in ND and ND+F5 vs. NEPEC and F5 groups; $p<0.01$ in ND+F5 vs. ND; Fig. 3I), and about 1.2% of the content of human islets (Fig. 3J). The ND+F5 group had the highest insulin level in the culture medium among the four groups but a lower level than the islets (NEPEC, 2.5±0.6; F5, 3.6±0.6; ND, 15.7±2.8; ND+F5, 26.6±6.0; islet, 333.3±20.2 pmol/1×10^6 cells; $p<0.003$ in ND+F5 vs. other groups; Fig. 3K). In addition, C-peptide levels in ND+F5 were also the highest among the 4 groups (NEPEC, 3.3±0.5; F5, 5.4±1.0; ND, 14.1±1.5; ND+F5, 27.4±5.0; islet, 317.9±10.3 pmol/1×10^6 cells; $p<0.003$ in ND+F5 vs. other groups; Fig. 3L). The glucose-stimulated C-peptide secretion test showed that there was a significant difference

FIG. 1. Pancreatic nonendocrine cells after isolation and NEPEC after differentiation protocol. **(A** and **B)** Representative figures of the human pancreatic nonislet fraction **(A)** immediately after islet isolation and **(B)** 4 days after suspension culture with G418. The arrow indicates a contaminated islet detected by dithizone staining. Scale bars: 100 μm. **(C)** A ratio of cell proliferation of the four groups. The cell number at day 1 after starting cell attachment culture was used as the starting point (=1.0). At day 7, the differentiation procedure started. There was no significant difference of proliferation among the four groups. **(D)** Morphology of NEPECs of the four groups at day 7 after differentiation. Scale bars: 100 μm. **(E)** Effective induction of human *NeuroD1* into NEPEC, shown through immunohistochemistry of the four groups 7 days after the start of differentiation. Most CK19-positive cells produced NeuroD1 in the ND and ND+F5 groups. Green, CK19; red, human NeuroD1; blue, DAPI. Scale bars: 50 μm. **(F** and **G)** Gene expression profile of the differentiated NEPEC. The human islets and the four groups of cells were harvested 7 days after the initiation of differentiation. A panel of genes was assessed by quantitative real-time PCR. For statistical analysis, ANOVA and *t*-test with Bonferroni adjustments were used. **(F)** Relative quantification represents the fold change of expression between each group and the NEPEC group (NEPEC=1.0). **(G)** Relative quantification represents the fold change of expression between each group and human islets (islets=1.0). For CK19, the relative values of the 4 groups were presented (NEPEC=1.0). Data are presented as mean±standard error of five independent experiments. *$p<0.01$ in ND+F5 group vs. all other groups. **$p<0.01$ in ND+F5 group vs. NEPEC and F5 groups. ANOVA, analysis of variance; GCG, glucagon; GK, glucokinase; NEPEC, nonendocrine pancreatic epithelial cells; Ngn3, neurogenin-3; PPY, pancreatic polypeptide; SST, somatostatin.

between the 2.8 and 25 m*M* glucose conditions in the ND+F5 group and islets (Fig. 3M), and ND+F5 cells had the strongest potency (stimulation index: NEPEC, 0.9±0.1; F5, 1.0±0.0; ND, 1.2±0.1; ND+F5, 2.2±0.7; islet, 7.5±1.0; $p<0.001$ in ND+F5 vs. other groups; Fig. 3M and N).

Function of engrafted differentiated NEPEC

The ND+F5 cells at day 7 after differentiation were transplanted under the kidney capsule of diabetic nude mice ($n=16$). For this experiment, five independent pancreatic



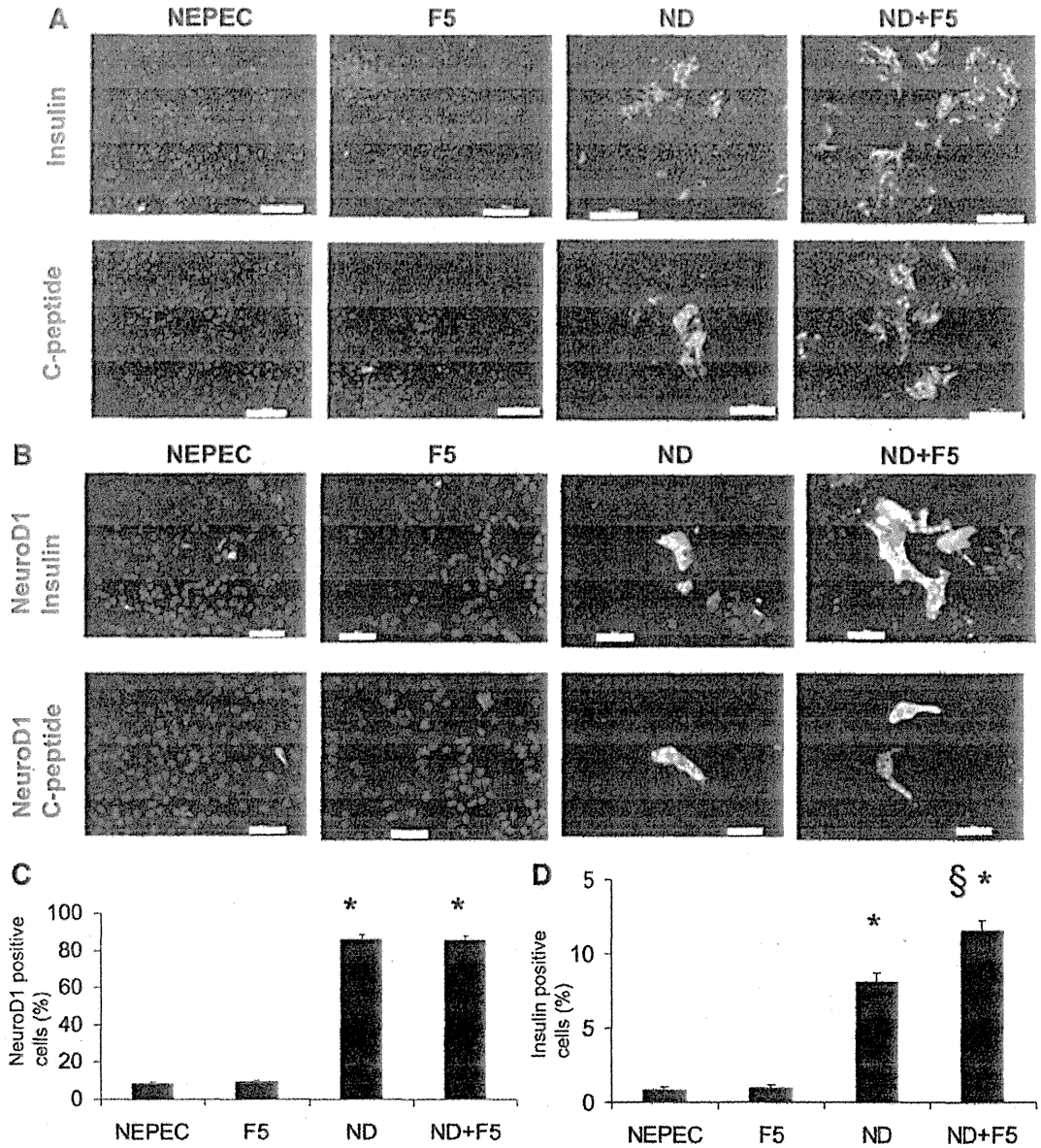


FIG. 2. *NeuroD1* promotes NEPEC differentiation. **(A)** Immunostaining for human insulin (upper panels) and C-peptide (lower panels) of the four groups at day 7 after differentiation. Green, insulin or C-peptide; blue, DAPI. Scale bar: 100 μm. Magnification: 100×. **(B)** Immunostaining for human NeuroD1, insulin (upper panels), and C-peptide (lower panels) of the four groups at day 7 after differentiation. Green, insulin or C-peptide; red, human NeuroD1; blue, DAPI. Scale bar: 50 μm. Magnification: 200×. Percentage of the cells expressing NeuroD1 **(C)** and insulin **(D)** as determined by immunohistochemistry at day 7 after differentiation. The NeuroD1 or insulin-positive cells were counted using more than 20 photomicrographs at a magnification of 200×. $^{*}p < 0.00001$ between the ND group vs. NEPEC and F5 and between ND+F5 vs. NEPEC and F5 **(C** and **D)**. $^{\S}p < 0.002$ between ND+F5 vs. all other groups **(D)**. Values are presented as mean ± standard error of five independent experiments. For statistical analysis, ANOVA and *t*-test with Bonferroni adjustments were used.

preparations were used separately. NEPEC ($n=5$), F5 ($n=5$), and ND cells ($n=8$) were used as a control. After transplantation, nonfasting blood glucose levels decreased to around 200 mg/dl for the ND+F5 group, whereas none of the NEPEC- and F5-transplanted mice showed a decrease in blood glucose, and all of NEPEC mice died by day 15 with diabetes (Fig. 4A). The ND group showed a small effect, but it deteriorated after 30 days. Serum mouse C-peptide was detected at a low level before transplant, and it did not change up to day 30 (Fig. 4B). In contrast, serum human C-peptide was not detectable in nonfasting condition before transplant, whereas it was detected and gradually increased after transplantation (Fig. 4C). After removal of the kidney containing the ND+F5 cells from the mice, blood glucose

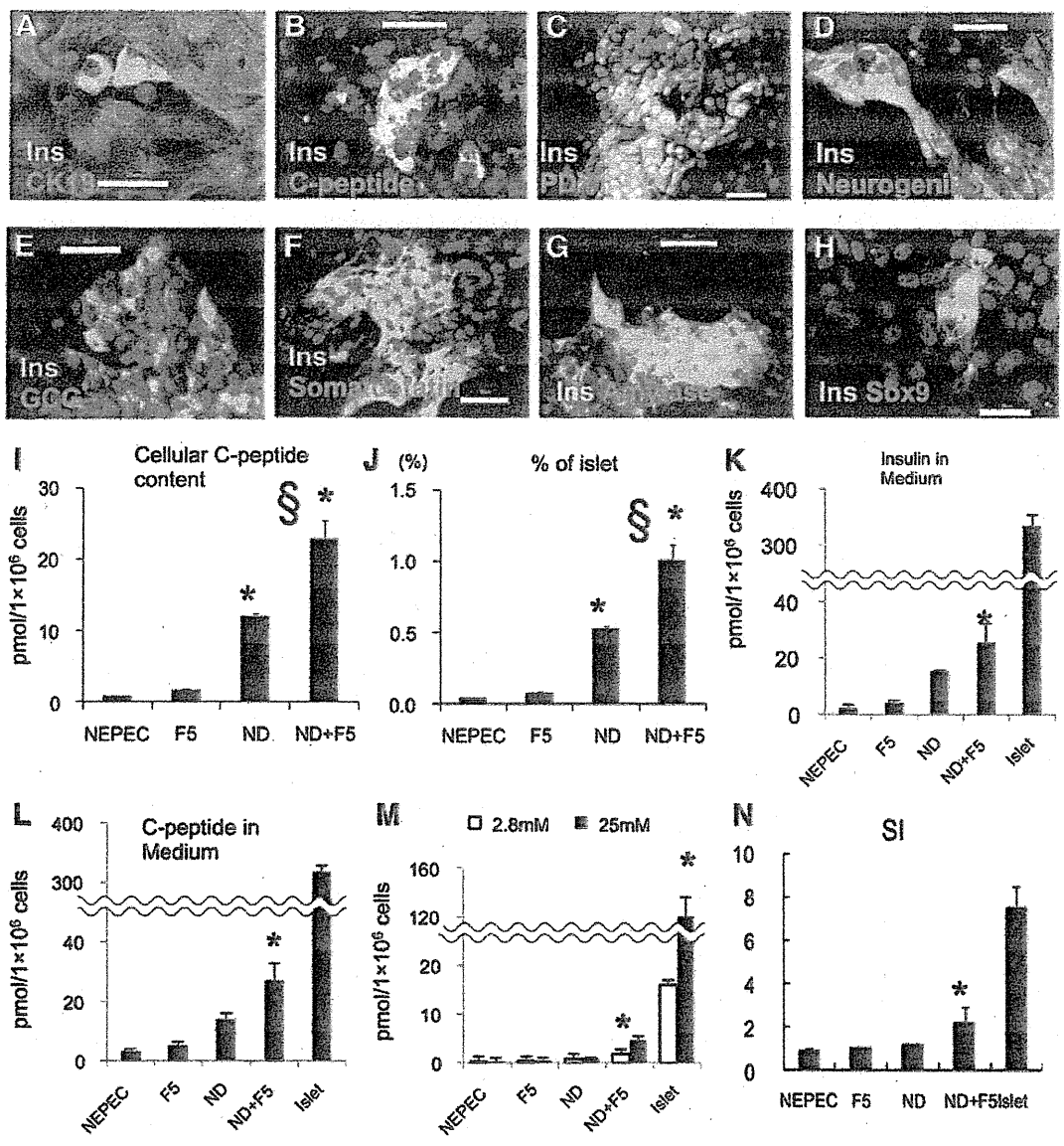


FIG. 3. Characteristics of insulin-positive cells in ND+F5 at day 7 *in vitro*. (**A**) CK19 expression was very low in the insulin-positive cells. The cells strongly coexpressed C-peptide (**B**), PDX1 (**C**), and neurogenin-3 (**D**), whereas glucagon (**E**), somatostatin (**F**), amylase (**G**), and Sox9 (**H**) were negative. Original magnification: 400×. Scale bar: 25 μm. In micrographs, DAPI was used for nuclear staining (blue). (**I–N**) Insulin secretion potency and glucose responsiveness. (**I**) Assessment of cellular C-peptide content in the four groups at day 7 after differentiation ($n=5$). Values were normalized by cell numbers (per 1×10^6 cells). (**J**) The ratio of the C-peptide content of the four groups to human islets. $^*p<0.001$ between the ND group vs. NEPEC and F5, and between ND+F5 vs. NEPEC and F5. $^§p<0.01$ between ND+F5 vs. ND group. (**K** and **L**) Human insulin (**K**) and C-peptide (**L**) levels in the culture media of the four groups at day 7 after differentiation ($n=5$). Values were normalized by cell numbers. $^*p<0.001$ between ND+F5 vs. NEPEC, F5, ND, and islets. (**M** and **N**) C-peptide secretion in response to glucose. (**M**) The culture medium was replaced with a fresh medium with 2.8 m*M* (white bar) or 25 m*M* glucose (black bar) for 1 hr. C-peptide levels were measured and normalized by cell number ($n=5$). Human islets were used as a control. $^*p<0.001$ between 2.8 m*M* vs. 25 m*M*. (**N**) SI of the four groups ($N=5$). SI was calculated by dividing the C-peptide concentration of 25 m*M* glucose by that of 2.8 m*M* glucose. $^*p<0.001$ between ND+F5 vs. NEPEC, F5, ND, and islets. All values are presented as mean ± standard error. For statistical analysis, ANOVA and *t*-test with Bonferroni adjustments were used. SI, stimulation index.

levels returned to pretransplant diabetic levels (~500 mg/dl), confirming that the transplant was responsible for reducing the blood glucose. In addition, the human C-peptide levels returned to be undetectable after removal of the graft (Fig. 4C).

To determine whether ND+F5 cells might cause oversecretion of insulin after transplant, the cells were transplanted into normal nude mice ($n=5$). Their blood glucose stayed in a normal range for 30 days, and no effect was found after removal of the graft (Fig. 4D). This suggests that

the endogenous beta cells maintain the blood glucose within normal range and the insulin secretion by the transplanted cells was not automatic but respondent to the blood glucose level. The glucose tolerance tests performed at day 31 posttransplantation showed that the blood glucose levels in the ND+F5 group were significantly lower than those in the STZ group (Fig. 4E). The F5 and ND groups showed a pattern similar to that of the STZ group. We also performed the test for normal nude mice with or without transplantation (Fig. 4F). There was no difference between the groups. During the glucose challenge, serum human C-peptide was detected in mice transplanted with ND+F5 cells (Fig. 4G and H). When ND+F5 cells were transplanted into normal nude mice, the serum C-peptide levels were lower than those in diabetic mice. The mice in the F5 and ND groups did not have a detectable level of human C-peptide (Fig. 4G and H).

Histological and immunohistochemical analysis of the graft

We examined the grafts of the ND+F5 group at day 32 after transplantation by histology and immunofluorescence. Typical grafts contained epithelial cell clusters that included insulin-positive cells bordered by areas of connective tissue and the surface of renal cells (Fig. 5A and B). Some ductal structures were seen mainly in the thick-stratified part of the graft (Fig. 5C). Within the grafts, insulin-positive cells were detected (Fig. 5D), and they mainly existed as thin-layer cells without CK19 expression. The cells forming ducts were CK19 positive, and there were very few insulin-positive cells in the thick part of the graft (Fig. 5E). These insulin-positive cells were also C-peptide positive (Fig. 5F). The beta cell transcription factors PDX1, Nkx6.1, and NeuroD1 were expressed in the most of insulin-positive cells (Fig. 5G–J). Glucagon-, somatostatin-, and amylase-positive cells were not detected (Fig. 5K–M). Some insulin-positive cells coexpressed Ki67 (Fig. 5N and O). The rate of Ki67-positive cells in the insulin-positive cells at day 3 after transplant was 12.4%, whereas it was 0.47% at day 32 (Fig. 5P), indicating that the transplanted cells could proliferate in the early period after transplant and the proliferating cells decreased afterward.

Effect of transplant of ND+F5 on endogenous pancreas

The β-cell number in the pancreas of STZ-induced diabetic mice substantially decreased. After transplant of the ND+F5 cells, the β-cell number did not significantly change (Supplementary Fig. S1A–D; Supplementary Data are available online at www.liebertpub.com/hgtb). These were compatible with the fact that serum mouse C-peptide level was not different between before and after transplant (Fig. 4B).

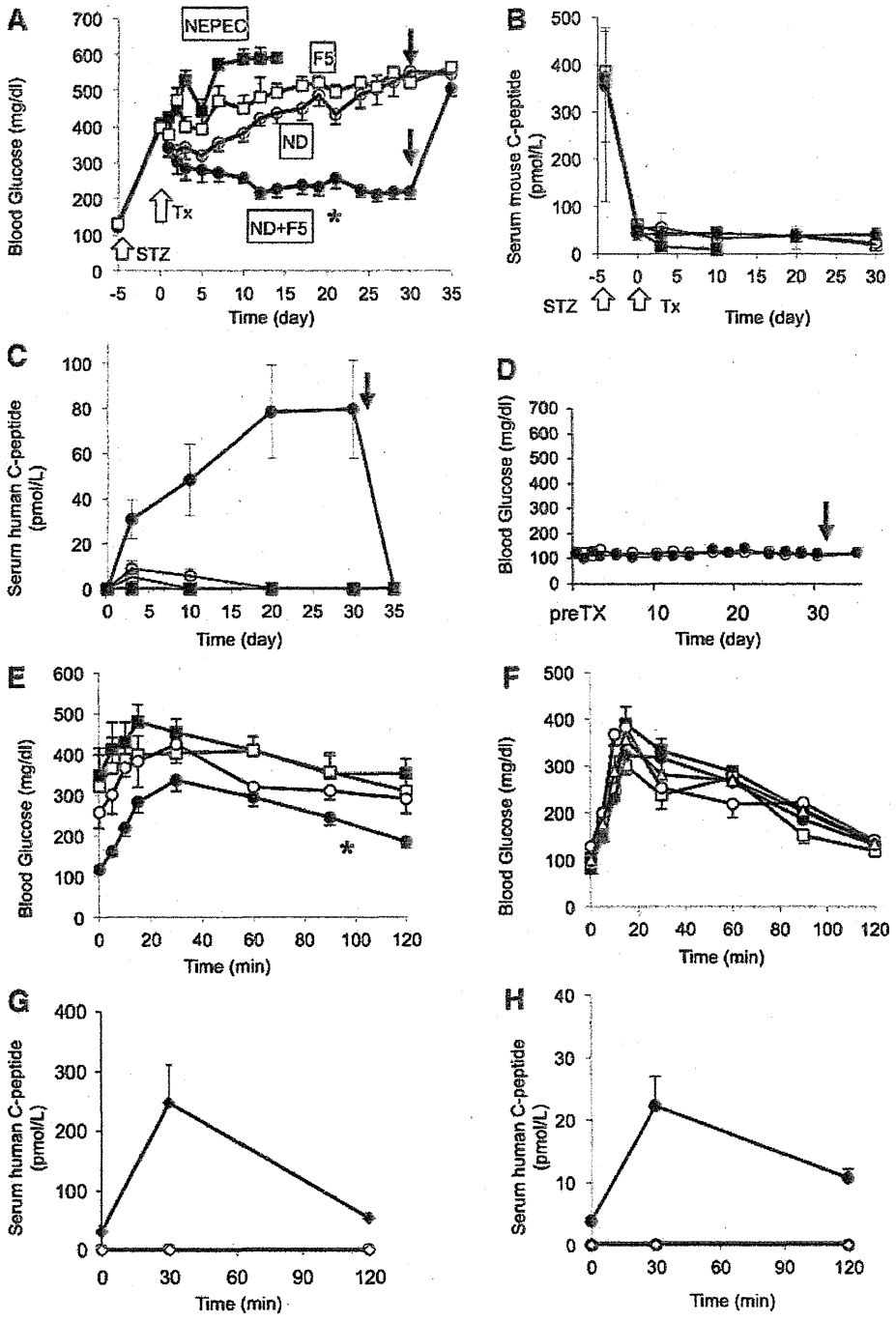
NEPEC derived from pancreas with chronic pancreatitis

We developed NEPEC using the pancreatic nonislet fraction after islet isolation for total pancreatectomy with autologous islet transplantation ($n=5$). The donor and isolation variables were as follows: donor age 42.2±4.1 years; 3 men and 2 women; BMI 24.3±1.7 kg/m^2; duration of symptoms 4.8±1.0 years; cold ischemic time 36±5 min. The cells were differentiated into ND+F5 cells *in vitro* using the same protocol as that for deceased donors and evaluated at day 7 after *in vitro* differentiation. The characteristics of ND+F5 cells from both groups (normal pancreas vs. chronic pancreatitis) were quite similar (Supplementary Table S1). The efficacy of transfection of the *NeuroD1* gene, percentage of insulin-positive cells, cellular insulin content, and insulin secretion potency were comparable in both groups. ND+F5 cells at day 7 after differentiation were transplanted under the kidney capsule

FIG. 4. Improvement of diabetes after transplantation of the cells. **(A)** Nonfasting blood glucose levels in diabetic nude mice with subcapsular kidney transplantation for NEPEC (black squares, $n=5$), F5 (white squares, $n=5$), ND (white circles, $n=8$), and ND+F5 (black circles, $n=16$). At day 32, nephrectomy was performed (black arrow). The blood glucose levels in the ND+F5 group were significantly decreased ($^*p<0.001$ vs. F5 and ND by RM-ANOVA). White arrows indicate the STZ administration and the transplantation of the cells. **(B and C)** Serum mouse and human C-peptide levels in mice transplanted with NEPEC (black squares, $n=5$), F5 (white squares, $n=5$), ND (white circles, $n=8$), and ND+F5 (black circles, $n=16$). Mouse C-peptide **(B)** and human C-peptide **(C)** before STZ injection, before transplant and at days 3, 10, 20, and 30 after transplant. White arrows indicate the STZ administration and the transplantation of the cells. At day 32, nephrectomy was performed (black arrow, **C**). **(D)** Nonfasting blood glucose levels in normal nude mice with ND+F5 transplanted (black circles, $n=5$). Untreated nude mice were used as a control (white circle, $n=5$). The arrow indicates nephrectomy at day 32. The ND+F5 group showed no remarkable change. **(E–H)** Glucose tolerance test at day 31. After the mice had fasted for 12 hr, glucose (2 g/kg) was intraperitoneally injected. The blood glucose levels were measured for 120 min after injection. At 0, 30, and 120 min, blood was taken for measurement of serum human C-peptide levels. **(E)** Blood glucose levels after a glucose tolerance test in diabetic nude mice for STZ control (black squares, $n=5$) and F5 (white squares, $n=5$), ND (white circles, $n=8$), and ND+F5 (black circles, $n=16$). The transplant of ND+F5 cells lowered glucose levels during the test ($^*p<0.02$ vs. STZ by RM-ANOVA). **(F)** Blood glucose levels in normal nude mice for untreated control (white triangles, $n=5$), NEPEC (black squares, $n=5$), F5 (white squares, $n=5$), ND (white circles, $n=5$), and ND+F5 (black circles, $n=5$). There was no significant difference between the five groups. **(G and H)** Serum human C-peptide levels during the glucose tolerance test. All values are presented as mean±standard error. **(G)** F5 (white circle, $n=5$) or ND cells (white diamond, $n=8$) or ND+F5 cells (black circles, $n=16$) were transplanted into diabetic nude mice. Human C-peptide was not detected in the NEPEC group or the ND group. **(H)** ND cells (white diamond, $n=5$) or ND+F5 cells (black circles, $n=5$) were transplanted into normal nude mice. Untreated normal nude mice were used for control (white circle, $n=5$), Human C-peptide was not detected in control and ND group mice. RM-ANOVA, repeated-measures ANOVA; STZ, streptozotocin; Tx, Transplantation.

of diabetic nude mice ($n=12$). Nonfasting blood glucose was measured for 30 days (Fig. 6A) and the levels in most of the mice decreased to around 200 mg/dl. After removal of the kidney containing the ND+F5 cells from the mice, the levels returned to pretransplant diabetic levels (~450 mg/dl). Human C-peptide was detected in the mice (Fig. 6B). Glucose tolerance tests were performed at day 31 posttransplantation. The blood glucose levels showed a pattern similar to that of the ND+F5 groups from normal pancreata (Fig. 6C), and serum human C-peptide was detected in mice during the tests (Fig. 6D). These data indicate that ND+F5 cells from chronic pancreatitis functioned to lower blood glucose after transplantation, as well as those from normal pancreata.

The graft cells were between the connective tissue and the surface of renal cells (Fig. 6E). The insulin-positive cells were seen in the thin layer of the graft, without CK19 expression (Fig. 6F). CK19-positive ductal structures were seen in the thick-stratified part of the graft with a few insulin-positive cells (Fig. 6G). The beta cell transcription



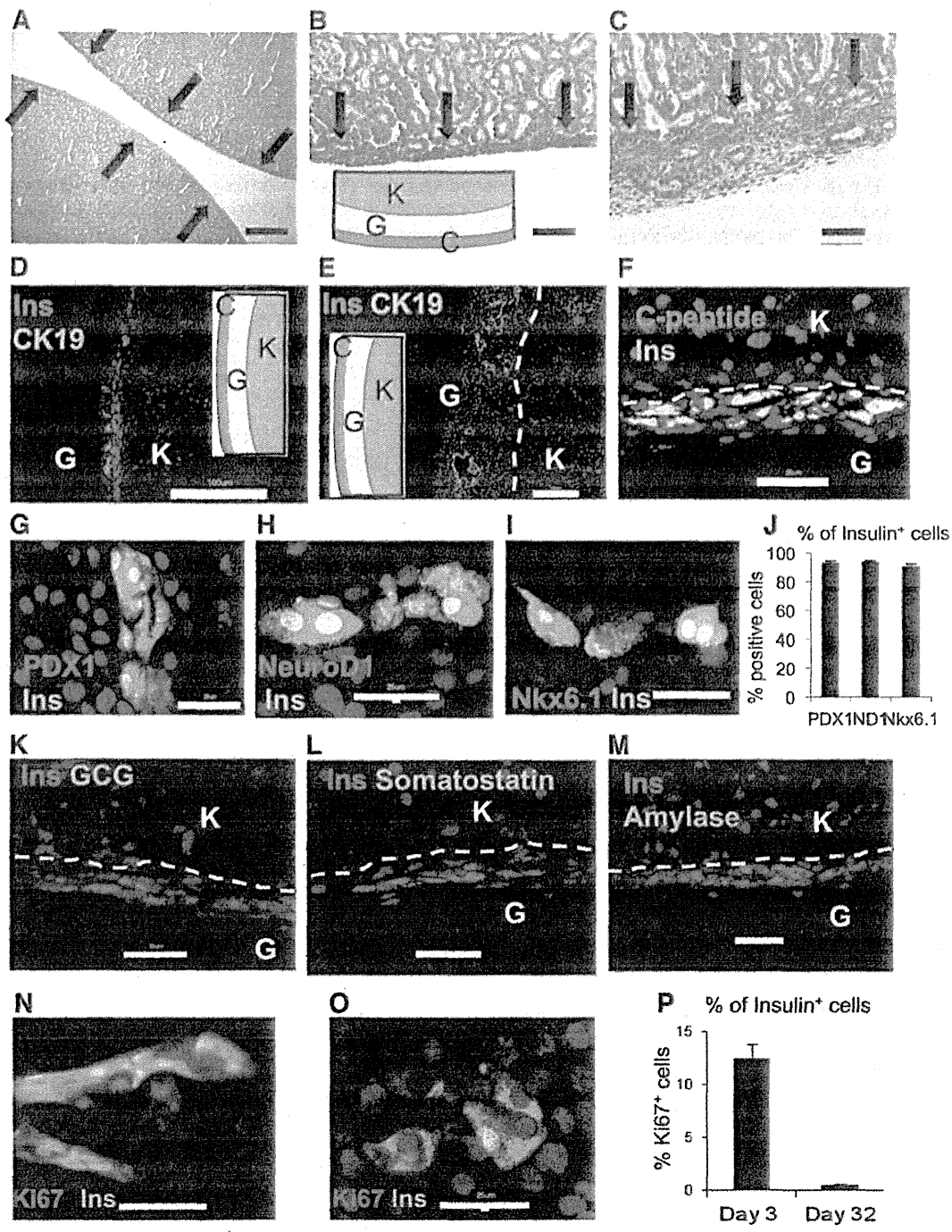


FIG. 5. ND+F5 cells differentiated toward beta cells *in vivo*. Histological and immunohistochemical characteristics of the kidney subcapsular region of ND+F5 transplantation at day 32. (**A–C**) Histological sections of the transplant site. (**A** and **B**) The transplanted cells were detected as a thin layer on the kidney surface. (**C**) Some cells formed ductal structures. Magnification: (**A**) 40×; (**B** and **C**) 200×. Scale bars: (**A**) 250 μm; (**B** and **C**) 50 μm. Arrows indicate the graft. (**D–L**) Immunofluorescence analysis of grafts. (**D–E**) Staining for CK19 (green) and insulin (red). (**F**) Staining for insulin (green) and C-peptide (red). (**G–I**) Staining for insulin (green), PDX1 (red), NeuroD1 (red), and Nkx6.1 (red). (**J**) Ratio of PDX1-, NeuroD1-, Nkx6.1-positive cells in the insulin-positive cells at day 32 after transplant. For statistical analysis, ANOVA and *t*-test with Bonferroni adjustments were used. (**K–M**) Staining for insulin (red), glucagon (green), somatostatin (green), and amylase (green). (**N** and **O**) Cell proliferation of the insulin-positive cells in the graft of the ND+F5 cells at day 3 (**N**) and day 32 (**O**) after transplant. Staining for insulin (green), Ki67 (red), and DAPI (blue). (**P**) Ratio of Ki67-positive cells in the insulin-positive cells at day 3 and day 32 after transplant. In all micrographs, DAPI was used for nuclear staining (blue). Scale bars: (**D** and **E**) 100 μm; others, 25 μm. Original magnification: (**D** and **E**) 100×; others, 400×. White line, border of graft; K, kidney; G, graft; Ins, insulin; GCG, glucagon. The schema of the engrafted cells and kidney surface is illustrated (**B, D,** and **E**). K, kidney; G, graft cells; C, connective tissue.

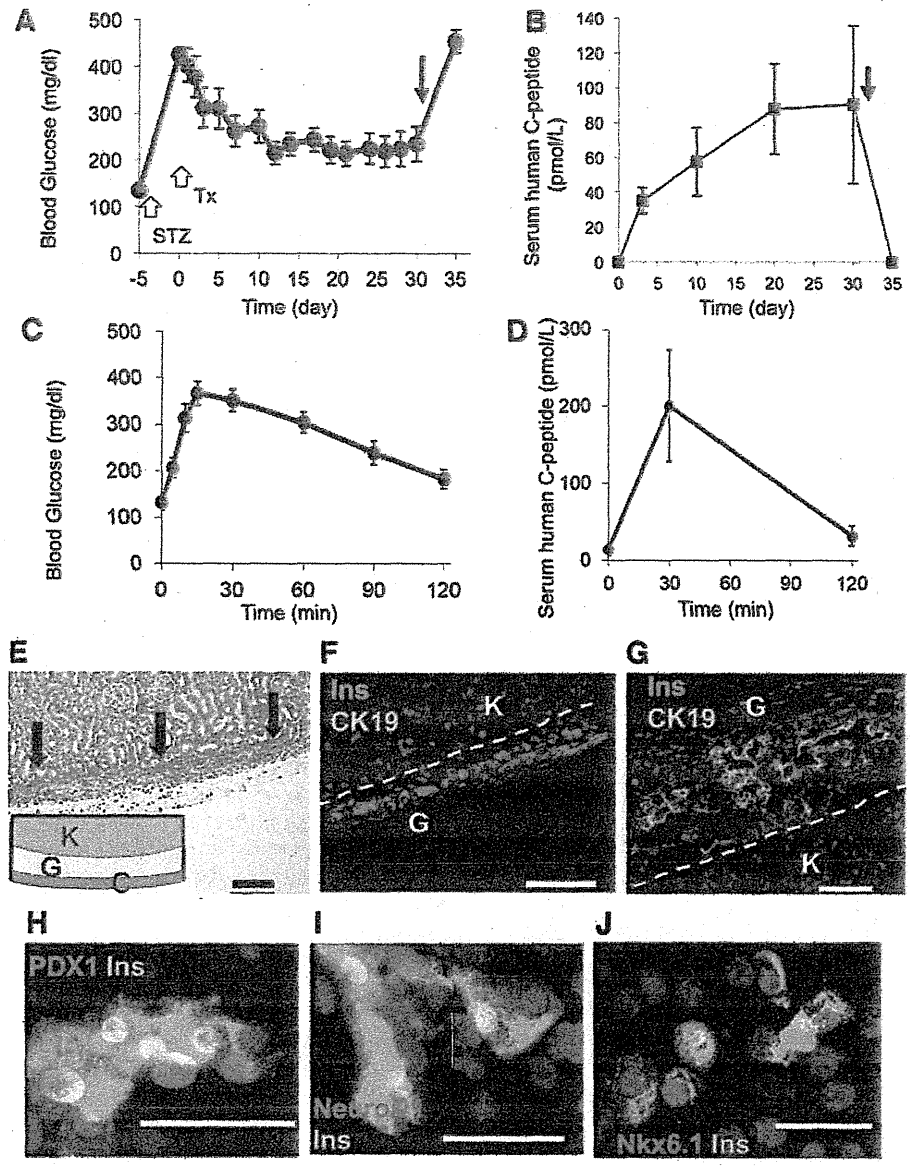


FIG. 6. Effects of transplantation of the ND+F5 cells derived from pancreata with chronic pancreatitis. **(A)** Nonfasting blood glucose levels in diabetic nude mice with subcapsular kidney transplantation for ND+F5 ($n=12$). At day 32, nephrectomy was performed (arrow). The blood glucose increased to pretransplant levels. White arrows indicate the STZ administration and the transplantation of the cells. **(B)** Human C-peptide before transplant and at days 3, 10, 20, and 30 after transplant. At day 32, nephrectomy was performed (black arrow). **(C)** Blood glucose levels after a glucose tolerance test in diabetic nude mice (day 31) for ND+F5 ($n=12$). **(D)** Serum human C-peptide levels during the glucose tolerance test at day 31 ($n=12$). All values are presented as mean ± standard error. **(E–J)** Histological and immunohistochemical characteristics of the kidney subcapsular region of ND+F5 transplantation at day 32. **(E)** A histological section of the transplant site. The transplanted cells were detected as a thin layer on the kidney surface. Magnification: 200×. Scale bar: 50 μm. **(F–J)** Immunofluorescence analysis of grafts. **(F** and **G)** Staining for CK19 (green) and insulin (red). **(H–J)** Staining for insulin (green), PDX1 (red), NeuroD1 (red), and Nkx6.1 (red). In all micrographs, DAPI was used for nuclear staining (blue). Scale bars: **(D** and **E)** 50 μm; **(H–J)** 25 μm. Original magnification: **(F** and **G)** 200×; **(H–J)** 400×. White line, border of graft; K, kidney; G, graft; Ins, insulin. The schema of the engrafted cells and kidney surface is illustrated **(E)**. K, kidney; G, graft cells; C, connective tissue.

factors PDX1, NeuroD1, and Nkx6.1 were expressed in most of insulin-positive cells (Fig. 6H–J). These findings show that the grafts derived from patient pancreata with chronic pancreatitis were comparable to those from the cadaveric donor pancreata.

Discussion

We report here for the first time that insulin-secreting cells can be generated from the pancreatic nonendocrine fraction obtained from human pancreata with chronic

pancreatitis, as compared with those from normal pancreata. In our protocol, an effective triple lipofection method without viral vectors was used to induce insulin production. The triple lipofection method in this study produced high transfection efficacy for NEPECs. Although the mechanism is not clear, to repeat lipofection at a suitable interval might be important. At a rough estimate, 10^7 NEPECs were obtained from 0.25 ml of nonislet tissue after islet isolation. The ND+F5 cells included about 12% insulin-positive cells. In general, 20–40 ml of nonislet tissue was obtained per pancreas. Therefore, approximately $1–2\times10^8$ insulin-positive cells could be obtained per pancreas. Although the current differentiation protocol requires further improvement, it can be said that this method is moving closer to clinical application. Supplemental transplantation of the generated insulin-secreting cells from the autologous pancreas might be an excellent candidate for clinical application to improve the effect of autologous islet transplantation and achieve a high insulin-free rate. In contrast to allotransplantation, the recipients of autologous islet transplants do not require immunosuppressive drugs and are not at risk for autoimmune destruction or alloimmune rejection. A possible problem is that pancreatic tissues with chronic pancreatitis may be damaged and unsuitable as a source of the generated cells. However, it was reported that partial pancreatic ductal obstruction could enhance the islet neogenesis-associated proteins that stimulate beta cell regeneration (Rosenberg, 1988). In addition, chronic inflammation could promote regeneration of islet progenitor cells in nonislet tissues (Phillips *et al.*, 2007). Interestingly, our data indicated that ND+F5 cells derived from pancreata with chronic pancreatitis had a similar potency to those from normal pancreata.

Recent studies have reported the methods to generate insulin-positive cells derived from healthy human pancreatic nonislet cells under specific conditions *in vitro* (Bonner-Weir *et al.*, 2000; Bogdani *et al.*, 2003; Todorov *et al.*, 2006; Gao *et al.*, 2007; Yatoh *et al.*, 2007). However, those cells had a low level of insulin compared with mature beta cells and did not reverse diabetes after implantation. Zhao *et al.* (2005) reported that insulin-producing cells were derived from human pancreatic nonendocrine cells using *PDX1* gene induction and the cells reversed hyperglycemia in mice. Compared with their method, our method has some advantages: high transfection efficacy (85% vs. 30%), shorter time period required to differentiate the insulin-producing cells, no need to transplant the cells inside the renal parenchyma, and the unique CK19 promoter that could function in only CK19-positive cells to eliminate the possibility that the reversal of diabetes was caused by proliferation of residual beta cells. It is difficult to exclude all contaminating islets from the initial pancreatic tissue. However, Russ *et al.* (2009, 2011) studied using genetic cell-lineage tracing and reported that the expanding human islet cells in tissue culture could be induced to re-differentiate into insulin-producing cells and there was no evidence that the insulin-expressing cells were derived from a non-beta-cell origin in their culture condition, which is consistent with our data showing that the NEPEC and F5 group cells did not differentiate into the insulin-producing cells. It indicates that NEPECs are not derived from the endocrine cells. We previously reported that *NeuroD1* gene induction stimulated the differentiation of NEPEC into insulin-producing cells, comprising approximately 8% of NEPEC (Shimoda *et al.*, 2010a). *NeuroD1* is a basic helix–loop–helix transcription factor that is found in the pancreas, intestine, and central nervous system (Naya *et al.*, 1995). In *NeuroD1* gene knockout experiments, the mutant mice developed severe diabetes with ketoacidosis and died after birth (Naya *et al.*, 1997). Previous reports have shown that adenoviral-mediated introduction of *NeuroD1* induced beta cell neogenesis in the liver and reversed diabetes in mice with betacellulin gene therapy (Kojima *et al.*, 2003) or PDX-1/VP16 gene therapy (Kaneto *et al.*, 2005). Another study suggested that *NeuroD1* is not essential for early differentiation but has an important role in later-stage differentiation and maintenance of beta cells (Chao *et al.*, 2007). Recently, we demonstrated that *NeuroD1* gene delivery into pancreas reversed STZ-induced diabetes in rats (Chen *et al.*, 2010). These studies indicate that *NeuroD1* is an important gene for determining the lineage to pancreatic endocrine cells. Furthermore, *NeuroD1* can promote the differentiation of pancreatic progenitor cells into beta cells *in vitro* (Noguchi *et al.*, 2006). Taken together, the overexpression of *NeuroD1* can be a highly effective method to differentiate NEPEC into beta cells. However, the induction of the *NeuroD1* gene without the five growth factors, which were reported to maturate human embryonic stem cells and induce pluripotent stem cell-derived pancreatic progenitor cells into insulin-producing cells (Zhang *et al.*, 2009), was not sufficient to reverse diabetes, as shown in this study (the ND group). Our current protocol allowed us to increase the ratio of insulin-positive cells *in vitro* (the ND+F5 group).

The mechanism to generate fully mature beta cells is still unknown. In our method, the insulin secretion gradually decreased 3 weeks after induction of differentiation, and the induced *NeuroD1* expression became lower 2 weeks after transfection *in vitro* (data not shown). It indicates that the differentiation protocol could not differentiate NEPEC completely and could not maintain it. *In vivo* condition is required for further differentiation and maintenance. Indeed, our results indicate that the transplanted cells proliferated and further differentiated in the early period resulting in improving blood glucose control with a relatively limited number of insulin-positive cells (11.9% of 10,000,000 cells), but the mechanism is unclear. In fact, it was reported that another type of engineered human insulin-producing cells required more cells (Ravassard *et al.*, 2011). In addition, ND+F5 cells were not fully matured beta cells even under *in vivo* conditions. Their phenotype became more similar to that of mature beta cells ($CK19^-$, $PDX1^+$, $Nkx6.1^+$, $NeuroD1^+$, $glucagon^-$, $amylase^-$); however, their morphology was still unlike that of islets, clearly indicating that the cells were immature. Their characteristics should be investigated more by other beta cell markers, including MafA and Nkx2.2, and by microstructure, but they were not performed because of technical difficulties so that further examinations are required. Interestingly, the insulin-positive cells mainly appeared as thin-layer cells in the graft. Although the exact cause has yet to be determined, one possibility is that their survival might require sufficient oxygen and nutrition and thick grafts might lack these factors. It is a dilemma that many functional cells are required to establish normoglycemic control, but the number of transplantable cells is

limited. In the rodent model, cotransplantation of other cells might stimulate graft vascularization by producing some factors such as vascular endothelial growth factor, resulting in improved beta cell graft survival (Baeyens *et al.*, 2009). Promoting vascularization might improve our protocol. However, even though the cell survival would be improved, they would not have three-dimensional structure like islets. In addition, although most of the cells overexpressed *NeuroD1* in ND+F5, only 11.9% of cells became insulin positive *in vitro*. The reason is unknown, but there may be two possibilities. First, *NeuroD1* is an essential but not sufficient to induce full maturation. Other unknown factors might be required. Second, there may be a few "special" cells in the CK19-positive cell population, such as stem or progenitor cells, although they are indistinguishable by morphology, and only these cells could be differentiated into beta cells. There may be other undiscovered mechanisms, and further studies are required.

Another point of uncertainty is the exact origin of the new insulin secretory cells. In this study, they were probably generated from epithelial cells because almost all fibroblasts were eliminated before monolayer culture, and the pCK19-hND plasmid worked in only CK19-positive cells (CK19 is a marker of epithelial cells). There are two major types of pancreatic epithelial cells: ductal cells and acinar cells. It has been reported that acinar cells can be transdifferentiated to have duct cell-like properties (Minami *et al.*, 2005; Zhao *et al.*, 2005). In contrast, others found that the ductal cells are the main source (Bonner-Weir *et al.*, 2000; Leng and Lu, 2005; Yatoh *et al.*, 2007). In addition, it is theoretically possible that a few contaminated beta cells dedifferentiated and somehow expressed CK19, and then redifferentiated to insulin-producing cells by pCK19-hND transfection. Therefore, a part of insulin-positive cells might be derived from original islet cells because it is almost impossible to completely exclude all islets from the pancreatic nonislet tissues before culture. This is a limitation of this study. However, their contributions would be slight because contaminated islets were too few to be dominant during culture. Thus, this question remains to be solved.

Conclusively, our new highly effective method for beta cell generation without viral vectors might help this field move toward clinical application for the cure of diabetes.

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Author Disclosure Statement

The authors declare no conflict of interest.

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