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Pdx1-transfected adipose tissue-derived stem cells differentiate into insulin-producing cells *in vivo* and reduce hyperglycemia in diabetic mice

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ABSTRACT Insulin-dependent diabetes mellitus (IDDM) is characterized by the rapid development of potentially severe metabolic abnormalities resulting from insulin deficiency. The transplantation of insulin-producing cells is a promising approach for the treatment of IDDM. The transcription factor pancreatic duodenal homeobox 1 (Pdx1) plays an important role in the differentiation of pancreatic beta cells. In this study, the human *Pdx1* gene was transduced and expressed in murine adipose tissue-derived stem cells (ASCs). To evaluate pancreatic repair, we used a mouse model of pancreatic damage resulting in hyperglycemia, which involves injection of mice with streptozotocin (STZ). STZ-treated mice transplanted with *Pdx1*-transduced ASCs (Pdx1-ASCs) showed significantly decreased blood glucose levels and increased survival, when compared with control mice. While stable expression of Pdx1 in ASCs did not induce the pancreatic phenotype *in vitro* in our experiment, the transplanted stem cells became engrafted in the pancreas, wherein they expressed insulin and C-peptide, which is a marker of insulin-producing cells. These results suggest that Pdx1-ASCs are stably engrafted in the pancreas, acquire a functional beta-cell phenotype, and partially restore pancreatic function *in vivo*. The ease and safety associated with extirpating high numbers of cells from adipose tissues support the applicability of this system to developing a new cell therapy for IDDM.

KEY WORDS: *ASC, in vivo differentiation, Pdx1, stem cell therapy, type 1 diabetes*

Introduction

Insulin-dependent diabetes mellitus (IDDM) is characterized by the rapid development of potentially severe metabolic abnormalities caused by insulin deficiency (Gepts, 1965, Notkins and Lernmark, 2001, Herold *et al.*, 2005, Keymeulen *et al.*, 2005, Santana *et al.*, 2006, Voltarelli *et al.*, 2007). IDDM results from deficient insulin secretion due to destruction of beta cells in the islets of Langerhans in the pancreas. However, since pancreatic beta cells do not regenerate and xenotransplantation is problematic, it is impossible to cure IDDM by ordinary medical treatments for tissue failure.

Many studies have attempted to generate insulin-producing cells *in vitro* for the treatment of IDDM. Although embryonic stem (ES) cells can be induced to differentiate into insulin-producing cells (Lumelsky *et al.*, 2001, Soria, 2001, Blyszczuk *et al.*, 2004, D'Amour *et al.*, 2006, Jiang *et al.*, 2007, Nakanishi *et al.*, 2007, Marchand *et al.*, 2009), these cells, as well as induced pluripotent stem cells, may form teratomas after transplantation. Therefore, somatic cells represent a safer option for

Abbreviations used in this paper: ASC, adipose tissue-derived stem cell; IDDM, insulin-dependent diabetes mellitus; Pdx1, pancreatic duodenal homeobox-1; STZ, streptozotocin.

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transplantation therapy (Hess *et al.*, 2003). With regard to the establishment of systems for inducing the differentiation of somatic stem cells that can be applied to regenerative medicine, there are two major considerations: 1) the type of stem cell, which should be easy to collect in large quantities from the patient's own body; and 2) the ability to induce with high efficiency the differentiation of these stem cells into pancreatic beta cells.

Adipose tissue-derived stem cells (ASCs) are easily collected in high numbers from adipose tissue that is both abundant and easily accessible in patients. Adipose tissue could therefore also harbor cells with the potential to differentiate into insulin-producing cells (Timper *et al.*, 2006). ASCs differentiate towards osteogenic, adipogenic, myogenic, and chondrogenic lineages *in vitro* (Zuk *et al.*, 2002). ASCs can also differentiate into putative neurogenic cells, exhibiting a neuronal-like morphology and expressing several proteins consistent with the neuronal phenotype (Zuk *et al.*, 2002, Timper *et al.*, 2006). These cells show multipotency, making them a promising new source of tissue for transplantation.

Exogenous expression of the Pdx1 gene, a key regulator of normal pancreatic development and beta cell differentiation (Leonard *et al.*, 1993, Ohlsson *et al.*, 1993, Ahlgren *et al.*, 1996, Mfopou *et al.*, 2005), induces differentiation of both ES cells and bone-marrow-derived mesenchymal stem cells into insulin-producing cells (Miyazaki *et al.*, 2004, Li *et al.*, 2007).

In the present report, we show that while stable expression of Pdx1 in ASCs does not induce a pancreatic phenotype *in vitro* under the culture conditions used in our experiment, the transplantation of these cells into mice with IDDM induced by streptozotocin (STZ) treatment results in engraftment of the transplanted Pdx1-ASCs in the pancreas, leading to the amelioration of hyperglycemia and improved survival.

Results

Generation of adipose tissue-derived stem cells (ASCs) that express Pdx1 (Pdx1-ASCs)

Stable expression of Pdx1 in the Pdx1-ASCs was confirmed by RT-PCR and immunostaining for Pdx1 protein (Fig. 1 A,B). A total of three Pdx1-ASC cell lines were generated in three independent experiments. All expressed Pdx1 at equivalent levels (60-70% of total cells), and there were no apparent morphological differences between the three lines (data not shown). The three cell lines were thus used interchangeably in this study.

RT-PCR analysis of the Pdx1-ASCs showed that they lacked the expression of pancreatic marker genes, including the two non-allelic insulin genes *insulin 1* and *insulin 2* (Fig. 1C). These findings demonstrated that the Pdx1-ASCs had not differentiated into pancreatic beta cells under the culture conditions used here. To investigate the effect of Pdx1 expression on the differentiation of ASCs, the expression of a set of marker genes (*Nkx2.2, Pax6, NeuroD, insulin 1, and insulin 2)* for the pancreatic lineage was examined by RT-PCR at stages 1 and 2 of *in vitro* differentiation (see Materials and Methods for details). *Pax6* and *Nkx2.2* were not detected in normal ASCs, but were detected in Pdx1-ASCs at stage 2 (data not shown). *NeuroD, insulin1 and insulin2* were not detected in Pdx1-ASCs or normal ASCs at stage 2. Nevertheless, we suspected that they might change into a cell lineage with features of pancreatic beta cells. Since Pdx1 continued to be stably expressed in the Pdx1-ASCs, we decided to examine the effect of altering the external environment of the cells by transplanting them into a murine model of diabetes.

Pancreatic damage in mice with STZ-induced IDDM

To evaluate whether Pdx1-ASCs could differentiate into insulin-producing cells and restore pancreatic function, we used a mouse model of pancreatic damage resulting in hyperglycemia generated by injecting the mice with STZ. Pancreatic repair was then assessed in these mice after transplantation of ASCs expressing green fluorescent protein (GFP) (Fig. 2A). The recipients were intraperitoneally injected in advance with STZ at 250 mg/kg body weight to induce pancreatic damage and secondary hyperglycemia (blood glucose > 500 mg/dl; Fig. 2B). Morphological assessment of pancreatic sections from these mice immunostained with anti-insulin antibody showed destruction of the pancreatic islets by day 5 (Fig. 2C).

Pancreatic sections from these mice were immunostained for insulin and blood samples were assayed for glucose levels. The blood glucose levels of the STZ-treated mice were significantly elevated by day 0 (5 days after STZ injection) and above 500 mg/dl at day 20. No spontaneous reversal of the hypergly-

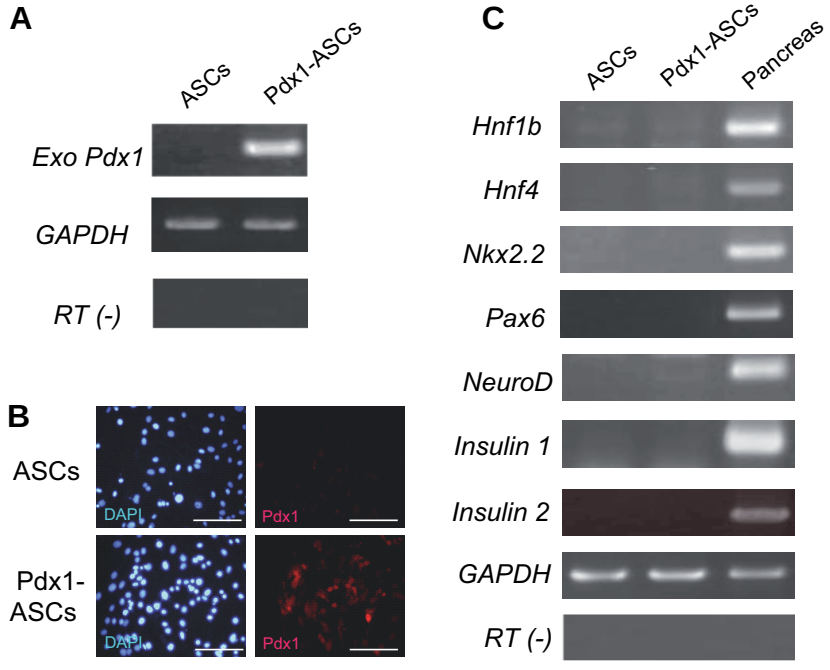

Fig. 1. Characterization of the Pdx1-ASCs. (A) *RT-PCR analysis of* Pdx-1 *expression in ASCs and Pdx1-ASCs. Pdx1-ASCs were cultured for 20 days after viral infection, and the cells were harvested and subjected to RT-PCR analysis.* **(B)** *Immunostaining of ASCs and Pdx1-ASCs for Pdx-1. Pdx-1 expression is shown in red. Nuclei are stained with DAPI (blue). Scale bars, 100 μm.* **(C)** *Expression of pancreas-related genes in ASCs, Pdx1-ASCs, and pancreatic tissues. Pdx1-ASCs were cultured for 20 days after viral infection, and the cells were harvested and subjected to RT-PCR analysis.*

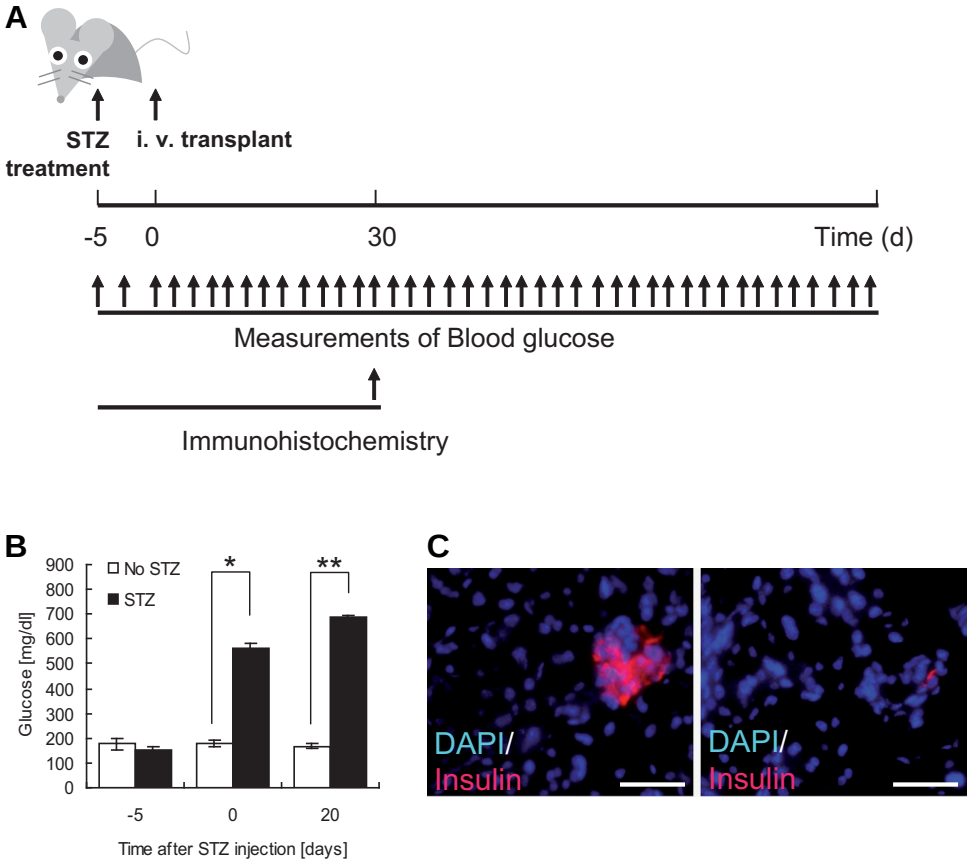

Fig. 2. Induction of hyperglycemia in streptozotocin (STZ)-treated mice. (A) *Schematic representation of the experimental design. C57BL/6J mice were treated with STZ to induce hyperglycemia 5 days prior to intravenous (i.v.) cell transplantation. The blood glucose levels were measured every 2 to 3 days. On day 30 post-transplantation, pancreatic sections were immunostained with anti-insulin antibody.* **(B)** *Blood glucose levels of the STZ-treated mice (closed column, n = 7) and PBS-treated mice (open column, n = 5) just after treatment (-5 days), and at 5 days (day 0) and 25 days (day 20) after STZ treatment. Data are presented as mean ± SEM; * p<0.05 and ** p<0.005 (t-test).* **(C)** *Immunohistochemistry of pancreatic sections of vehicle-treated (left) and STZ-treated mice (right) 5 days after STZ treatment (day 0) with anti-insulin antibody. Insulin-producing cells are stained red. Nuclei are stained blue with DAPI; scale bar, 100 μm.*

cemia was seen in the STZ-treated mice. The transplanted ASCs were distinguishable from the cells of the recipient by the GFP expression.

Effects of Pdx1-ASC transplantation on blood glucose levels in STZ-treated mice

We hypothesized that intravenously transplanted Pdx1-ASCs would improve the impaired pancreatic function as evaluated by blood glucose control. The STZ-treated mice were transplanted with GFP-expressing ASCs or GFP-expressing Pdx1-ASCs at day 0. As a control (vehicle), STZ-treated mice were injected with Hank's balanced saline solution (HBSS) intravenously. Within 12-14 days after transplantation (days 12-14), the blood glucose levels of the hyperglycemic mice that had received Pdx1-ASCs had declined (Fig. 3A), independently of weight loss or food consumption (data not shown). The blood glucose reduction was sustained after 12-14 days, whereas the animals that received HBSS or ASCs remained severely hyperglycemic (Fig. 3A). The beneficial effect of Pdx1-ASC transplantation was also demonstrated by 20% survival of the Pdx1-ASCs-transplanted mice at day 70 compared to 0% (100% morbidity) of mice receiving HBSS or ASCs (Fig. 3B). These results indicated that transplantation of HBSS or ASCs alone was not effective in restoring pancreatic function, but that transplantation of Pdx1-ASCs reduced the blood glucose levels of hyperglycemic mice with STZ-induced pancreatic tissue damage.

Differentiation of Pdx1-ASCs into insulin-producing cells in vivo

To analyze the distribution and differentiation of transplanted Pdx1-ASCs in the STZ-treated mice, pancreatic tissue sections were analyzed immunohistochemically. A small proportion of the transplanted Pdx1-ASCs expressed insulin as well as C-peptide (Fig. 4 C,F). An average of 12.5 ± 0.96 cells per section of pancreas were positive for GFP, and an average of 3 ± 0.35 cells per section of pancreas were positive for both C-peptide and GFP (Fig. 4G). On the other hand, no insulin- or C-peptide-positive cells were found in the pancreas of HBSS or normal ASC-injected mice (Fig. 4 A,B,D and E). GFP-positive cells were analyzed for Pdx1 expression immunohistochemically, but Pdx1 expression was undetectable (data not shown). Liver tissue sections were also analyzed immunohistochemically, but GFP-positive cells were not detected in any sections (data not shown). Tumor formation was not observed in any mice transplanted with Pdx1-ASCs.

These results show that about 25% of the transplanted Pdx1-ASCs had differentiated into insulin-producing cells in the pancreas of the STZ-treated mice. The Pdx1-ASCs thus seemed competent to differentiate into insulin-producing cells *in vivo*.

Discussion

This study found that blood glucose levels in a murine model of IDDM decreased significantly after transplantation of mouse Pdx1-ASCs, with a corresponding increase in survival rate. The amelioration of blood glucose levels was sustained for up to 15 weeks, confirming that Pdx1-ASCs had stably engrafted to the pancreas, acquired a functional beta cell phenotype, and could partially restore pancreatic function. These transplanted Pdx1-ASCs did not form tumors. However the blood glucose levels in mice transplanted with Pdx1-ASCs were still higher than in the normal mice. This may be because the number of transplanted Pdx1-ASCs which differentiated into insulin-producing cells in

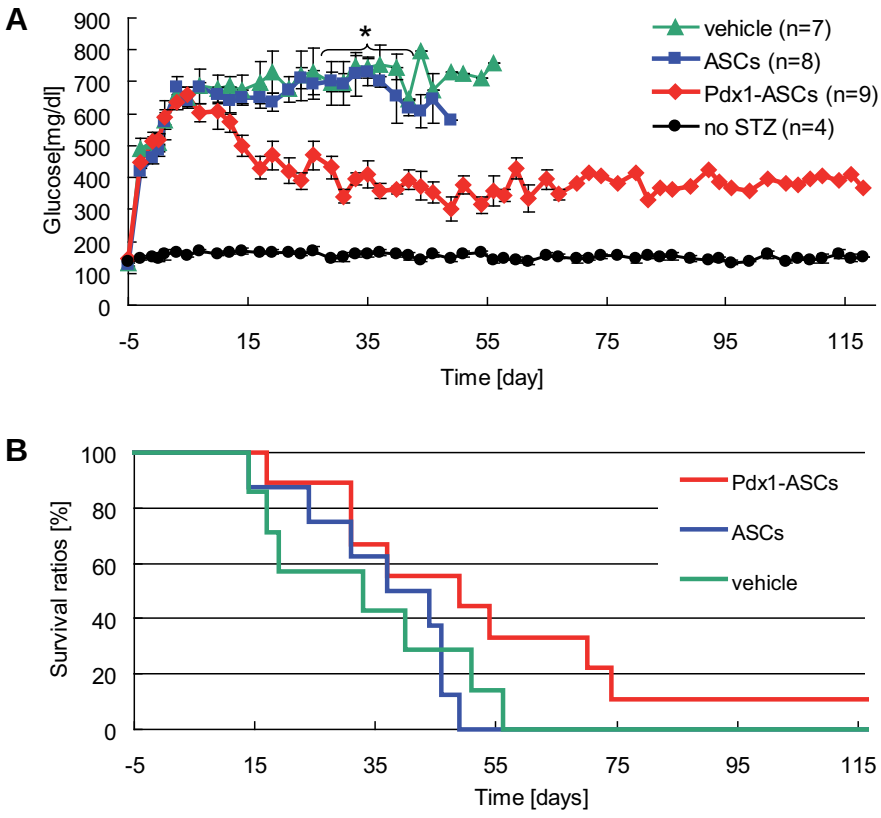

Fig. 3. Blood glucose levels and survival of STZ-treated mice after cell transplantation. **(A)** *Comparison of blood glucose levels in vehicle-treated mice (triangle, green, n = 7), ASC-transplanted mice (square, blue, n = 8), Pdx1-ASC-transplanted mice (diamond, red, n = 9), and non-STZ-treated mice (circle, black, n = 4). Data are presented as mean ± SEM; * p<0.05 (t-test). The n-values indicate the number of mice at -5 days.* **(B)** *Comparison of mouse survival ratios. The colors of the lines correspond to those listed in (A).*

the pancreas of the STZ-treated mice was less than the number of insulin-producing cells in normal mice. Alternatively, the induced insulin-producing cells did not organize into islet structures, remaining as single cells or in small clusters. Signaling between beta cells inhibits basal insulin secretion and enhances glucose-stimulated insulin secretion (Konstantinova *et al.*, 2007), and therefore a lack of organization of induced insulin-producing cells would be expected to impair their function. Strategies that increase the number and promote aggregation of transplanted Pdx1-ASCs in the adult pancreas may help to ameliorate blood glucose levels.

Our results emphasize the importance of the 'soil' when transplanting stem cell 'seeds'. We showed that the ectopic expression of a single factor, Pdx1, in transplanted ASCs is sufficient to drive their differentiation into insulin-producing cells. The success of this strategy likely hinged on two factors; the priming of multipotent stem cells for a beta cell lineage, and the subsequent control of differentiation by the tissue microenvironment. A previous study successfully induced pancreatic beta cells *in vivo* by introducing three genes, *Pdx1, Mafa, and Ngn3*, directly into the pancreas (Zhou *et al.*, 2008). We have shown that more targeted expression of one of these genes specifically in ASCs, relatively undifferentiated somatic stem cells with a similar differentiation potential to mesenchymal stem cells (i.e., adipocyte, chondrocyte and osteoblast lineages) (Zuk *et al.*, 2002), may be sufficient to commit these cells to a beta cell lineage. However, it is likely that additional factors from the surrounding environment in the pancreatic tissue *in vivo* were needed to drive these cells to become beta cells. Consistent with this, one study showed that while nine different transcription factors were necessary to transdifferentiate exocrine cells into endocrine cells *in vitro*, in an *in vivo* environment only three of these factors were needed for this transdifferentiation (Zhou *et al.*, 2008). In addition, together with such transcription factors, the present study indicates that other factors such as environmental substances are also important for the final differentiation. A high glucose condition is possibly one of the important factors needed for differentiation into pancreatic beta cells from other tissue stem cells (Cao *et al.*, 2004).

We have successfully treated IDDM in mice by transplantation with ASCs. These cells are both abundant and safely accessible in subcutaneous body fat, facilitating autotransplantation which averts many of the challenges associated with other stem cell sources for transplantation. One practical limitation of this study was the use of viral-vector gene transfer, but safer methods may be considered for application to humans (Koya *et al.*, 2008, Okita *et al.*, 2008). The results of this study form the basis of a promising new approach to the treatment of IDDM.

Materials and Methods

Isolation and culture of ASCs

ASCs were prepared using a previously described method (Zuk *et al.*, 2002). In brief, GFP-transgenic C57BL/6J mice (8-week-old females) were sacrificed by cervical dislocation and the grayish-white adipose tissue was excised from the inguinal region. The adipose tissue was minced into 2-3-mm pieces in standard culture medium (DMEM low glucose [Invitrogen, USA], 10% fetal bovine serum [FBS; Invitrogen, USA], 1% streptomycin-penicillin [Invitrogen, USA]) and incubated at 37ºC in a 5% CO_2 incubator for at least 60 min. The suspension was then centrifuged at 1300 rpm for 6 min at room temperature, and after the addition of 0.12% collagenase solution to the pellet, it was incubated at 37ºC for 30 min. Then, 20 ml of control medium was added, and the pellet was recovered after centrifugation at 1300 rpm for 6 min at room temperature. The pelleted and resuspended cells were passed through a 40 μm filter, and seeded in a dish for culturing in the standard medium. The medium was replaced every 2-3 days, and the cells that attached to the dish and proliferated were used as GFP-positive murine ASCs in the subsequent experiments.

Transfer of the Pdx1 *gene into ASCs*

The *Pdx1* cDNA was obtained from an RNA sample of human pancreas total RNA (Clontech, USA) by RT-PCR. The ORF of the human

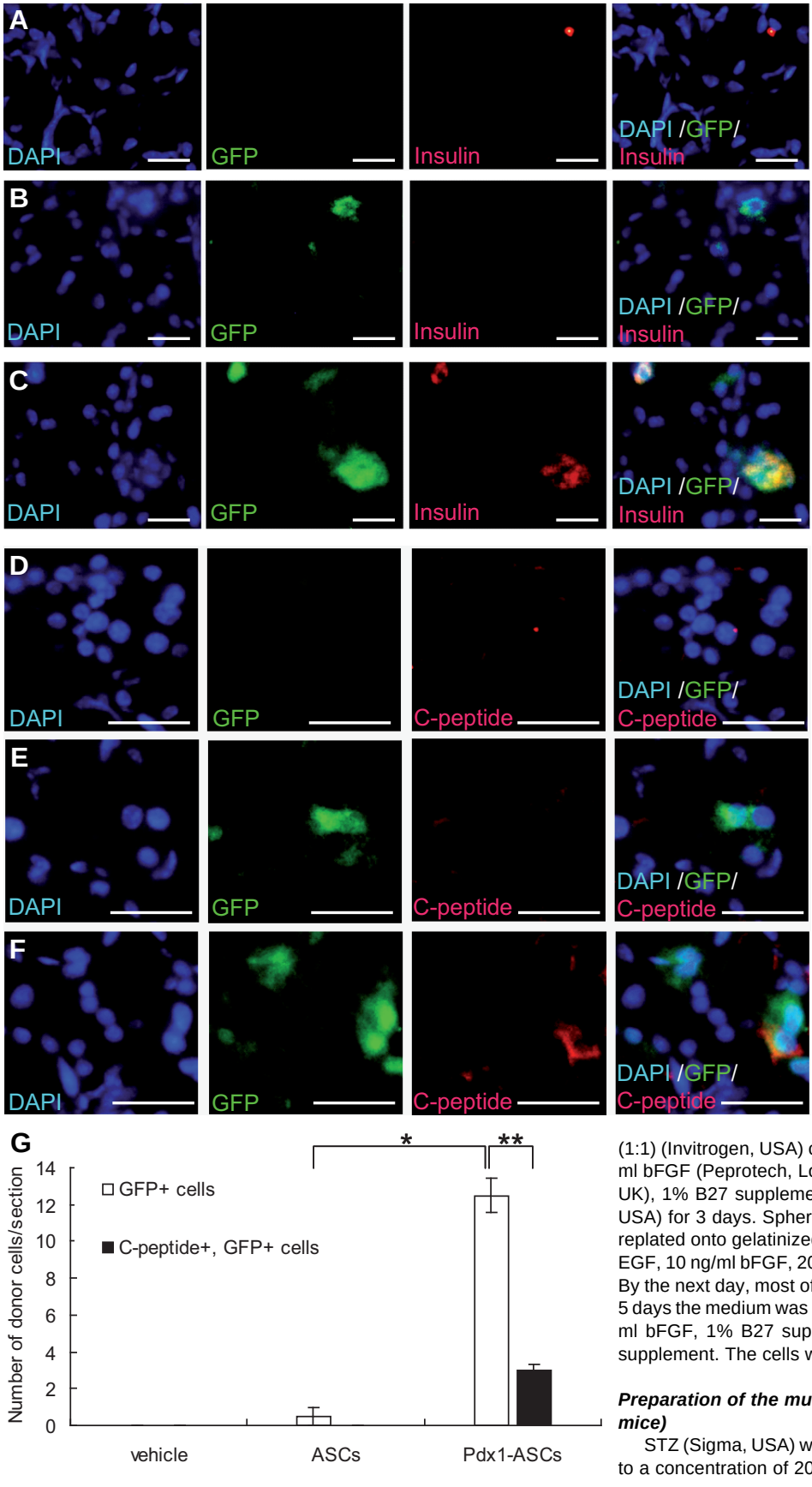

Fig. 4. Transplanted donor Pdx1-ASCs engraft in recipient pancreases of STZ-treated mice and produce donor insulin. (A-F) *Immunohistochemistry for insulin (red, A-C) and C-peptide (red, D-F) in pancreatic sections of STZ-treated vehicle mice* **(A,D)**, *ASC-transplanted mice* **(B,E)**, *and Pdx1-ASC-transplanted mice* **(C,F)** *on day 30. Blue: nuclei (DAPI); green: transplanted GFP-positive cells; scale bars, 20 μm.* **(G)** *Number of C-peptide-positive donor cells in the pancreases of vehicle-treated, ASC-transplanted, and Pdx1-ASC-transplanted mice. Data are presented as mean ± SEM (n = 4); * p<0.05 and ** p<0.005 (t-test).*

Pdx1 gene was integrated into a retrovirus vector (pMSCV-puro). The obtained plasmids were mixed with Fugene 6 (Roche Diagnostics, Switzerland) and added to a culture of PT67 cells (Clontech, USA) for transfection. Two days after introduction of the plasmid, the cells were selectively cultured in high-glucose medium (DMEM high glucose (Invitrogen, USA), 10% FBS, 1% streptomycin-penicillin) that contained puromycin (final concentration, 1 μg/ml). When the surviving (Pdx1-PT67) cells had reached 90% confluence, the culture medium was changed, and 2 days later, the supernatant (virus-containing solution) was recovered. ASCs at 80% confluence were cultured in the supernatant fluid, which after 4 h was replaced with the normal culture medium, and 2 days later with culture medium that contained puromycin (final concentration, 0.75 μg/ml). After selectively culturing the cells for about 14 days, the remaining Pdx1-ASCs were used in the experiments.

In vitro *differentiation procedure*

Pdx1-ASCs were subjected to an *in vitro* differentiation procedure as described previously (Miyazaki *et al.*, 2004). Before differentiation, the Pdx1-ASCs were cultured in standard culture medium (DMEM low glucose, 5% FBS, 1% streptomycin-penicillin) (stage 1). The cells were dissociated with 0.25% trypsin/0.04% EDTA solution (Sigma, USA) and plated onto non-coated culture dishes in DMEM/F12 (1:1) (Invitrogen, USA) containing 20 ng/ml EGF (R and D, USA), 10 ng/ml bFGF (Peprotech, London, UK), 20 ng/ml HGF (Peprotech, London, UK), 1% B27 supplement (Invitrogen, USA), and 0.5% N2 (Invitrogen, USA) for 3 days. Spheres then formed within 1 day. The spheres were replated onto gelatinized plates in DMEM/F12 (1:1) containing 20 ng/ml EGF, 10 ng/ml bFGF, 20 ng/ml HGF, 1% B27 supplement, and 0.5% N2. By the next day, most of the spheres had attached to the plates. After 3-5 days the medium was replaced with DMEM/F12 (1:1) containing 10 ng/ml bFGF, 1% B27 supplement, 10 ng/ml nicotinamide, and 0.5% N2 supplement. The cells were cultured for 5-8 days (stage 2).

Preparation of the murine model of IDDM by injection of STZ (STZ mice)

STZ (Sigma, USA) was dissolved in phosphate-buffered saline (PBS) to a concentration of 20 mg/ml, to which 50 μl of 0.05 M citric acid was

added per 100 mg STZ to create the STZ stock solution. C57BL/6J mice (14- to 15-week-old females) were deprived of food for approximately 4 h, and then injected intraperitoneally with 250 mg/kg STZ. STZ-injected mice with blood glucose levels greater than 500 mg/dl and body weight losses of less than 33% in the fed state 10 days after STZ injection were used experimentally as IDDM mice. Control mice were injected with an equivalent volume of PBS.

Transplantation of Pdx1-ASCs into the IDDM mice

Pdx1-ASCs were suspended in Hank's balanced saline solution (HBSS) to a concentration of 1.25 x 10^3 cells/μl. The 14- to 15-week-old STZ mice were each transplanted with 5 x 10^5 Pdx1-ASCs by tail vein injection.

Blood glucose measurements in the IDDM mice

Blood was collected from the tail veins of IDDM mice that were fed *ad libitum* between 14:00 h and 15:00 h every few days. The blood samples were assayed for glucose using a commercial blood glucose meter (G Checker; Gunze, Japan).

Immunostaining of mouse tissue sections and cultured cells

After fixation of the excised murine pancreases in 10% formalin, the formalin was replaced with distilled deionized water, and the organs were incubated overnight at 4ºC. The liver was then removed and washed with PBS. Tissue samples from each of the organs were embedded in OTC compound (Sakura Seiki, Tokyo, Japan) and frozen at -80ºC. A cryostat was used at -20ºC to cut 5 μm-thick tissue sections. The liver was fixed in 10% formalin. Tissue samples were then subjected to immunostaining with an anti-insulin antibody (no dilution Dako, Denmark), anti-Pdx1 antibody (1:350 dilution; Chemicon, USA) and an anti-C-peptide antibody (1:350 dilution; Linco, USA). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Binding of the anti-Pdx1 antibody and the anti-C-peptide antibody were analyzed using the VECTASTAIN kit (Vector Laboratories, USA) with streptavidin, and an Alexa Fluor 594 conjugate (Molecular Probes, USA) was used to visualize both primary antibodies.

Cultured cells were fixed with 4% paraformaldehyde for 20 min at room temperature. The samples were treated with 0.5% Triton X-100 in PBS for 15 min and then incubated with 3% bovine serum albumin (BSA) for 30 min, to block nonspecific binding. The cells were immunostained with anti-Pdx1 antibody (1:200 dilution) for 1 h. An Alexa 594-conjugated secondary antibody was then added for 1 h at room temperature. The cell nuclei were stained with DAPI for 10 min.

Insulin-positive and/or C-peptide-positive cells were counted in whole cross sections which were randomly selected (n=4 for each staining).

RNA extraction and reverse-transcription polymerase chain reaction

Total RNA was extracted using Isogen (Nippon Gene, Tokyo) according to the manufacturer's protocol. DNase-treated total RNA (500 ng) was used for first-strand cDNA synthesis. Reverse-transcription (RT) reactions were performed using SuperScript II (Invitrogen, USA) and oligo(dT) primer. The primers (forward and reverse, respectively) and the conditions used for PCR were as follows:

Exogenous *Pdx1*,
5'-ctgcctttcccatggatgaa-3' and
5'-cgcttcttgtccctcctccttt-3', 40 cycles;
Hnf1b 5'-gttgaaattccaagagtgacttgctc-3' and
5'-ctttaatgggaggcttcctgagatg-3', 40 cycles;
Hnf4 5'-ctcttctgattataagctgaggatg-3' and
5'-ccacaggaaggtgcagattgatctg-3', 40 cycles;
Nkx2.2 5'-ctaaatatttatggccatgtacacg-3' and
5'-gttccaagctccgatgctcaggag-3', 40 cycles;
Pax6 5'-cagtcacagcggagtgaatc-3' and
5'-cgcttcagctgaagtcgcat-3', 40 cycles;
NeuroD 5'-cttggccaagaactacatctgg-3' and
5'-ggagtagggatgcaccgggaa-3', 40 cycles;
insulin 1 5'-ccagctataatcagagacca-3' and
5'-gtgtagaagaagccacgct-3', 40 cycles;
insulin 2 5'-tccgctacaatcaaaaaccat-3' and
5'-gctgggtagtggtgggtcta-3', 40 cycles;
GAPDH 5'-tgaaggtcggtgtgaacggatttggc-3' and
5'-catgtaggcccatgaggtccaccac-3', 25 cycles.

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