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Insulin-Secreting Cells from Human Eyelid-Derived Stem Cells Alleviate Type I Diabetes in Immunocompetent Mice

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ABSTRACT

Various attempts have been made to develop stem cell-based therapy to alleviate type I diabetes using animal models. However, it has been a question whether human insulin produced from explanted cells is solely responsible for the normoglycemia of diabetic animals. In this study, we isolated neural crest-like stem cells from the human eyelid fat and examined their therapeutic potentials for diabetes. The human eyelid adipose-derived stem cells (HEACs) displayed characteristics of neural crest cells. Using a two-step culture condition combined with nicotinamide, activin, and/or GLP-1, we differentiated HEACs into insulin-secreting cells and examined in vivo effects of differentiated cells by transplantation experiments. Following differentiation in vitro, HEACs released insulin and c-peptide in a glucose-dependent manner. Upon their transplantation under kidney capsules of streptozotocin-treated immunocompetent mice, we observed normalization of hyperglycemia in 10 of 20 recipient mice until sacrifice after 2 months. Only the human, but not the mouse, insulin and c-peptide were detected in the blood of recipient mice. Removal of the kidneys transplanted with HEACs resulted in a sharp increase of blood glucose level. Removed kidney tissues showed distinct expression of various human genes including insulin, and colocalization of the human insulin and the human nuclear protein in many cells. However, they showed diminished or null expression of some immune-related genes. In conclusion, human insulin alone produced from eyelid-derived stem cells following differentiation into insulin-secreting cells and transplantation could normalize type I diabetes in mice. STEM CELLS *2009;27:1999–2008*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Type I diabetes is caused by the autoimmune destruction of pancreatic β cells. Whole pancreas transplantation is an ideal treatment, but the availability of this organ is limited. Alternative sources have been sought in extrapancreatic tissues. Bone marrow cells of mice were shown to differentiate into insulin-secreting cells in vitro and could reduce the blood glucose level of mice with hyperglycemia [1, 2]. Similar normoglycemia was observed when in vitro differentiated human cells were used. Embryonic stem cells could differentiate into insulin-producing cells in vitro and reverse the hyperglycemic state when transplanted into diabetic mice [3, 4]. Liver cells following gene manipulation have shown to secrete insulin in vitro and ameliorate hyperglycemia when transplanted into diabetic mice [5, 6]. Peripheral blood-derived monocytes [7] or umbilical cord-derived mesenchymal stem cells (MSCs) [8], after in vitro differentiation into insulin-secreting cells, could reduce blood glucose levels when transplanted into diabetic murines. Although these studies demonstrated that transplantation of differentiated cells could normalize hyperglycemia of diabetic animals, it is not clear whether the donor cells themselves could synthesize insulin and release it into the blood of recipient animals. In contrast, other studies have shown that cell transplantation might lower the blood glucose level of the recipient via supporting the regeneration of recipient β cells rather than providing insulin synthesized by donor cells. When mouse undifferentiated bone marrow cells [9, 10] or splenocytes [11] were transplanted into diabetic mice, the cells could reduce the hyperglycemia. Transplantation of human marrow

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stromal cells into diabetic mice has also been shown to reduce hyperglycemia by supporting the regeneration of recipient β cells [12]. Since regenerated β cells in a type I diabetic patient can again be damaged by autoimmune attacks, recruitment of therapeutic cells that directly regulate the blood glucose level of the patient following transplantation is an important issue in treating type I diabetes.

Neural crest-derived cells in mammals are peculiar in that although they originate from ectoderm, they can differentiate into many mesodermal lineage-like tissues such as facial muscle, cartilage, bone, adipose, arterial endothelial cells, and neural cells [13]. Indeed, stem cells isolated from neural crest-derived dental tissue can differentiate into mesodermal cells such as muscle, cartilage, ectodermal neurons, and odontoblasts in vitro, and bone cells in vivo [14]. Furthermore, cells from a molar are capable of differentiating into endodermal cells such as hepatocytes in vitro [15]. It is possible that neural crest-derived cells could also differentiate into insulin-secreting cells, a lineage of endodermal cells, similar to hepatocytes.

Many studies have demonstrated that human stem/progenitor cells could survive in the absence of immunosuppressors following xenogeneic transplantation into the bodies of experimental animals [16–18]. Using undifferentiated cells, these studies have shown that transplanted cells could survive as long as 13 months in immunocompetent animal bodies. Since type 1 diabetes is caused by the immune attack against the differentiated β cells, we have questioned whether differentiated cells could also survive and physiologically function in foreign bodies following xenogeneic transplantation.

In the present study, we isolated novel stem cells with neural crest characteristics from human eyelid adipose tissue. These cells could effectively differentiate into insulin-secreting cells under our in vitro culture condition and normalize the hyperglycemia of streptozotocin (STZ)-treated immunocompetent mice upon transplantation. The reversal was observed only when differentiated cells were transplanted, was exclusively due to the human insulin from the grafts, and persisted as long as 1 year without graft rejection.

Materials and Methods

Human Eyelid Adipose-Derived Stem Cell Isolation and Culture

The human eyelid adipose tissue was obtained from nine subjects aged between 11 and 79 years (mean age, 46.8 $\pm$ 6.5 years) undergoing cosmetic surgery with informed consent. Fat tissues were surgically dissected from the subcutaneous zone. All experiments were approved by Institutional Review Boards of Seoul Women's University and Yonsei University. Adipose tissue was treated with 0.075% type I collagenase (Gibco, Grand Island, NY, http://www.invitrogen.com) in phosphate-buffered saline (PBS) for 30 minutes at 37°C with gentle stirring. Cell suspensions were cultured in Dulbecco's modified Eagle's medium low glucose (DMEM-LG) (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, http://www.hyclone.com), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 3.7 mg/ml sodium bicarbonate at 5% CO_2, 37°C. Medium was changed twice a week. Cumulative population doubling at each subculture was calculated by using the formula $2^X = N_H/N_I$, where N_I = the inoculum cell number, N_H = cell harvest number at confluence (>80%), and X = population doublings [19]. The population doubling increase that was calculated was then added to the previous population doubling level to yield the cumulative population doubling level.

Flow Cytometric Analysis

After culture, 1×10^6 cells were transferred to DMEM-LG containing 25% FBS and 10% dimethyl sulfoxide and frozen at −80°C overnight followed by storing at −196°C in a liquid nitrogen tank. Frozen cells were thawed at 37°C in a water bath and then cultured in DMEM-LG supplemented with 10% FBS. After trypsinization, detached cells were resuspended and then incubated with primary antibodies for 1 hour at 4°C. For stage-specific embryonic antigen (SSEA)-4 and TRA 2-54 staining, fluorescein isothiocyanate (FITC)-conjugated secondary antibody was used for labeling. After washing, cells were analyzed on FACS Calibur flow cytometer equipped with Cell Quest software (BD, Franklin Lakes, NJ, http://www.bdbiosciences.com). Monoclonal antibodies for flow cytometric analysis were purchased from the following companies: HLA-ABC, HLA-DR, CD31, CD106, CD90, CD59, CD49d, CD45, CD34, and CD44 (BD Biosciences); SSEA-4, TRA 2-54 (Chemicon, Temecula, CA, http://www.chemicon.com); CD105 (R&D Systems Inc., Minneapolis, http://www.rndsystems.com); HLA-G (Novus Biologicals, Inc., Littleton, CO, http://www.novusbio.com); CD117 (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com).

RT-PCR

RNA was isolated using Tri-reagent (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) according to the manufacturer's instructions. The purity of RNA was assessed by determining the ratio of absorbance at 260 nm to that at 280 nm (>1.8). Reverse-transcription polymerase chain reaction (RT-PCR) was performed using a GeneAmp PCR system 2400 (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com). Subsequent PCR reactions were performed using cDNA, primer pairs (supporting information Table 1), and PCR mixture (Fermentas, St. Leon-Rot, Germany, http://www.fermentas.com) according to the manufacturer's instructions. Human adult pancreatic mRNA was purchased from Ambion (Austin, TX, http://www.ambion.com). RNAs from human embryonic stem cells (CHA-3 cell line at passage [p] 72) and neuroblastoma cells (SH-SY5Y) were kindly donated by Prof. Lee (Pochon Cha University, Korea) and Prof. Kim (Kyunghee University, Korea), respectively.

Immunocytochemistry

After culture in Lab-Tek chamber slides (Nunc, Rochester, NY, http://www.nuncbrand.com), human eyelid adipose-derived stem cells (HEACs) were washed with PBS and then permeabilized in 0.5% Triton X-100 for 10 minutes. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide for 15 minutes at room temperature (RT). The slides were incubated in a blocking solution, consisting of 2% bovine serum albumin (BSA) in PBS for 1 hour at RT. Between each step, cells were washed with 0.1% BSA in PBS. Cells were incubated with primary antibodies overnight at 4°C. The primary antibodies against human proteins are listed in supporting information Table 2. After labeling with the primary antibody, cells were then incubated with biotinylated goat anti-mouse or anti-rabbit IgG (DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com) followed by horseradish peroxidase-conjugated streptavidin (DakoCytomation) for 20 minutes, respectively, at RT. Immunoreactivity was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DakoCytomation) and counterstained with Mayer's hematoxylin. Cells were photographed under a microscope using bright-field illumination (Axioskop2+; Carl Zeiss, Jena, Germany, http://www.zeiss.com). For staining of neuronal markers, cells were incubated with goat anti-mouse FITC-conjugated secondary antibodies (1:128), goat anti-rabbit FITC-conjugated secondary antibodies (1:80), or rabbit anti-goat FITC-conjugated secondary antibodies (1:400) for 30 minutes. Immunoreactive cells were visualized by fluorescence microscopy (Axioskop2+; Carl Zeiss).

Assessment of Multidifferentiation Potential

HEACs were cultured in various differentiation media and assessed as previously described [20]. To induce adipogenic differentiation, HEAC at p4 were cultured in an adipogenic medium consisting of DMEM-LG, 10% FBS, 1 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, 0.05 mg/l human insulin, and 200 μM indomethacin. After culture for 2 weeks, presence of intracellular lipid droplets, indicative of adipocytes, was assessed by oil red O staining. To induce osteogenic differentiation, cells were cultured in an osteogenic medium consisting of DMEM-LG, 10% FBS, 0.1 μM dexamethasone, 100 mM β-glycerol phosphate, and 50 μM ascorbic acid-2-phosphate. After culture for 2 weeks, mineralization of accumulated calcium, indicating osteogenic differentiation, was assessed by von Kossa staining. To induce chondrogenic differentiation, cells were cultured in a chondrogenic medium consisting of DMEM high glucose (DMEM-HG), 0.1 μM dexamethasone, 50 μg/ml ascorbic acid-2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline, 10 ng/ml transforming growth factor β1 (R&D Systems), and 50 mg/ml ITS premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 1.25 mg/ml BSA, and 5.35 mg/ml linoleic acid; BD Biosciences). After culture for 3 weeks, chondrogenic differentiation was assessed by Alcian blue staining.

Differentiation of HEACs into Insulin-Secreting Cells In Vitro

HEACs at p3-p5 were plated on collagen-coated 48-well dishes at 5×10^3 cells per well. They were then cultured in various combinations consisting of NA group containing 10 mM nicotinamide (N) and 4 nM activin A (A; Peprotech, Rocky Hill, NJ, http://www.peprotech.com), NG group containing nicotinamide and 10 nM GLP-1 (G), AG group containing A and G, and NAG group containing N, A, and G. During the first week of culture, the cells were grown in DMEM-HG containing 10% FBS and during the following 2 weeks, they were cultured in DMEM-LG containing 10% FBS.

Measurement of Insulin and c-Peptide

To measure the amount of insulin and c-peptide released in vitro, cells were treated with DMEM-LG containing 0.5% BSA for 12 hours, washed with PBS, and then stimulated by DMEM-HG for 2 hours at 37°C. The amount of insulin and c-peptide released into the media was measured using the human insulin and c-peptide enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Winston Salem, NC, http://www.mercodia.com) according to the manufacturer's instructions. For the measurement of insulin and c-peptide levels in blood, mice that fasted overnight were i.p. injected with glucose (1.5 g/kg body weight [bw]). After 30 minutes, mouse sera were taken by cardiac puncture and the level of human insulin and c-peptide was measured. Cells of MIN6N8, a mouse pancreatic cell line, were similarly cultured as above except that 1×10^5 cells per well were seeded. Mouse insulin and c-peptide were released into the media, and levels were determined using a mouse insulin ELISA kit (Mercodia) and c-peptide ELISA kit (Yanaihara Inc., Shizuoka, Japan, http://www.yanaihara.co.jp).

Dithizone Staining

Cultured cells were incubated in 100 μg/ml dithizone solution at 37°C for 15 minutes.

Transplantation of HEACs into STZ-Induced Diabetic Mice

Female C57BL/6 mice (16-18 g, 6-8 weeks) were purchased from the Samtako Bio (Osan, Korea, http://www.samtako.com). Mice were maintained in accordance with the policies of the Institutional Animal Care and Use Committee of Seoul Women's and Yonsei Universities. Experimental diabetes was induced by a single i.p. injection of STZ (180 mg/kg bw) in 100 mM citrate buffer (pH 4.5). After 7 days, the blood glucose level was measured using an ACCU-CHEK Active (Roche Diagnostics, Basel, Switzerland, http://www. roche-applied-science.com) following a 10-hour fast. Diabetic mice with glucose level $>$ 350 mg/dl were randomly allocated to differentiated cell transplant group (DC, $n = 20$), undifferentiated cell transplant group (UDC, $n = 20$), and a sham-operated group ($n = 20$). Approximately, 1.5×10^6 cells were transplanted under the left kidney capsule by direct insertion using a 30-gauge needle at 15 days after STZ injection. Trypan blue test showed that 96.9% $\pm$ 1.1% of cells were viable after release from this needle. Differentiated and undifferentiated cells were obtained by culture of HEACs for 3 weeks in the NAG condition or without additives, respectively. Blood glucose level was measured after a 10-hour fast. Nephrectomy of the graft-bearing kidney was performed 2 months later and blood glucose was monitored until death ($n = 3$). Survival curves were prepared using the Kaplan-Meier method and analyzed for a significant difference by the log-rank Mantel-Cox test using SPSS 12.0 statistical software (SPSS Inc., Chicago, http://www.spss.com).

Glucose Tolerance Test

Thirty-six days after transplantation, mice fasted overnight. Blood was sampled at 0, 30, 60, 90, 120, and 150 minutes after i.p. injection of glucose solution (1.5 g/kg bw).

Immunohistochemistry

Graft-bearing kidneys and pancreata were removed from normal and DC mice at 2 months or 1 year after transplantation. Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections, followed by permeabilization with 0.5% Triton X-100 in PBS (PBST) for 10 minutes. For antigen retrieval, tissue sections immersed in 10 mM citrate buffer solution (pH 6.0) were placed in a microwave for 20 minutes. For pancreata tissues, the sections were incubated in 0.1% horse serum at RT for 20 minutes. Then the sections were incubated overnight at 4°C with anti-insulin monoclonal (1:250; Abcam) antibody, biotin-labeled anti-mouse goat antibody, and then horseradish peroxidase-conjugated streptavidin (Vectastatin ABC Kit; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Counterstaining was done with Mayer's hematoxylin. For graft-bearing kidney tissue sections, the sections were incubated in 1% BSA in PBS at RT for 20 minutes. Then the sections were incubated overnight at 4°C with the following mixture of two primary antibodies in PBST containing 1% BSA: anti-human nuclear antigen monoclonal (1:100; Chemicon) and anti-insulin polyclonal (1:100; Chemicon) antibodies. After washing, the sections were incubated with the following mixture of two secondary antibodies for 1 hour at RT: Texas red-conjugated anti-rabbit sheep antibody (1:200; Abcam) and FITC-conjugated anti-mouse goat antibody (1:200). Cell nuclei were visualized by incubating for 5 minutes with 1 μg/ml 4′-6′ diamidino-2-phenylindole in 0.1 M PBS. Imaging was visualized by fluorescence microscope (Axioskop2+; Carl Zeiss).

Statistical Analysis

Data were expressed as mean $\pm$ SEM. Statistical significance was analyzed by one-way analysis of variance test and t test using SPSS 12.0. A P value $<$ .05 was considered statistically significant.

RESULTS

Stem Cell Properties of Human Eyelid Adipose-Derived Cells

After 3-week culture of cells isolated from human eyelid adipose tissues, plastic-adherent cells with unique morphology were obtained and are referred to as HEACs. They exhibited neuron-like bipolar morphology until the senescence at p14 or p15 (Fig. 1A). Throughout the culture, they underwent an

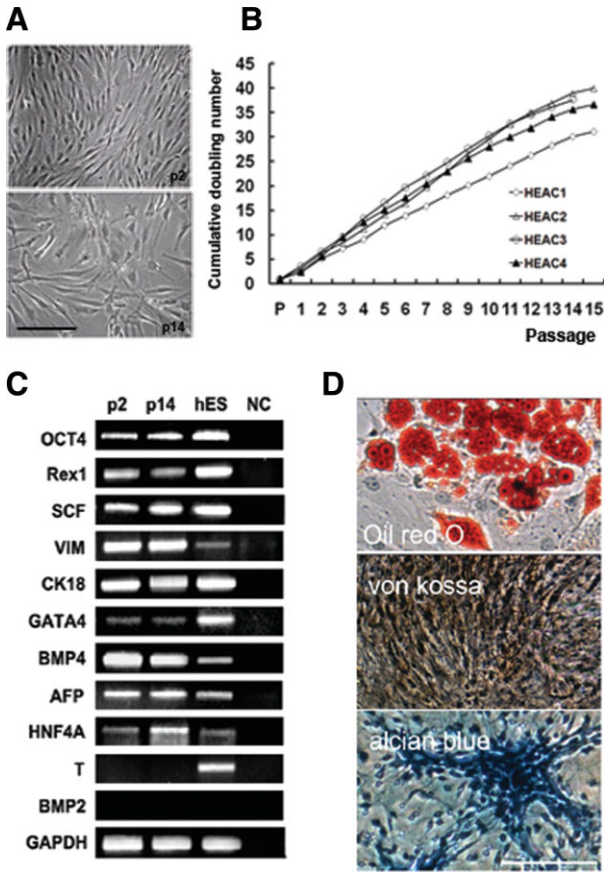

Figure 1. Stem cell properties of HEACs. **(A):** Morphology of HEACs at p2 and p14. **(B):** Cumulative doubling number of HEACs throughout ex vivo expansion. **(C):** Expression of stem cell-specific genes by HEACs and hESs. **(D):** Differentiation potential of HEACs at p4 into adipocytes, osteoblasts, and chondroblasts. Scale bar = 500 μm. Abbreviations: HEAC, human eyelid adipose-derived stem cell; hES, human embryonic stem cell; NC, negative control; p, passage.

average of 36.3 doublings and produced 6.9×10^{16} cells (Fig. 1B). HEACs both at p2 and p14 expressed stem cell-specific genes of *OCT4*, *REX1*, and *SCF*, endoderm-specific genes of *BMP4*, *AFP*, *GATA4*, and *HNF4A*, and mesoderm/endoderm-specific genes of *vimentin (VIM)* and *CK18*. They did not express mesoderm-specific *brachyury* (*T*) and endoderm-specific *BMP2* genes (Fig. 1C). Immunocytochemical analyses showed that HEACs at p4 expressed stem cell-specific proteins of TRA 1-60, SSEA-3, SSEA-4, and CD90, and other proteins, but not proteins of CD31 and HLA-DR (supporting information Fig. 1). HEACs cultured in appropriate differentiation media showed adipogenic, osteogenic, or chondrogenic differentiation potential (Fig. 1D). Flow cytometric analyses revealed that HEACs were positive for the antigens of SSEA-4, HLA-ABC, CD44, CD49d, CD59, CD90, and CD105, weakly positive for TRA 2-54 and CD34, and negative for HLA-G, HLA-DR, CD31, CD45, CD106, and CD117 antigens (Fig. 2 and supporting information Table 2).

HEACs from p2 until p14 expressed ectoderm- or neural progenitor-specific genes of *NCAM*, *FGF5*, and *NES*, neuron-specific genes of *TUBB3* and *GAP43*, neural crest-specific genes of *MSX1* and *SNAI2*, and glial cell-specific genes of *GALC* and *CNPase* (Fig. 3A). They expressed a glial cell-specific *GFAP* gene only at p2. HEACs also expressed an astrocyte-specific *S100* gene, and neuron-specific genes of *SOX9*, *MAP2*, *SYP*, *MBP*, *NEFM*, and *TFAP2A* at p8 or later. However, HEACs at all passages did not express *CD133* gene, a neural progenitor and hematopoietic stem cell marker (Fig. 3A). Human neuroblastoma cell line showed distinct expression of all of these genes. In comparison, human abdominal adipose-derived MSCs at p3 did not significantly express any of the above genes except *FGF5* and *NES*. HEACs spontaneously expressed a variety of neuronal proteins including β-tubulin III, galactocerebroside (GalC), serotonin, glial fibrillary acidic protein (GFAP), and γ-aminobutyric acid (GABA) (Fig. 3B). However, their immunoreactivity with anti-GFAP and anti-GABA antibodies reduced as the culture continued. HEACs at all passages did not express proteins of tyrosine hydrogenase and cholinergic acetyltransferase (Fig. 3B). These results suggest that HEACs are neural crest-derived stem cells, which retain many neural properties even after long-term culture.

Differentiation of HEACs into Insulin-Secreting Cells In Vitro

Until 2 weeks of culture, no colony was observed in HEACs of all groups. At three weeks, cells of only AG and NAG groups formed islet-like aggregates (supporting information Fig. 2), but cells of all groups including control group expressed *GLP1R*, *NEUROD1*, *PAX4*, *ISL1*, *GCK*, *CK19*, *PPY*, and *HNF4A* genes (Fig. 4A). In addition to these genes, HEACs of all groups except the control expressed genes of *GLUT1*, *PC1/3*, and *SST*. Those of NG, AG, and NAG groups expressed *INS* and *NEUROG3* genes. HEACs of AG and NAG groups expressed genes of *PDX1*, *NKX6.1*, and *GCG*. However, HEACs of NAG group showed higher expression levels of *PDX1*, *NEUROG3*, *INS*, and *NEUROD1* genes than those of AG group. Gene expression profiles of HEACs in NAG group were comparable to those of the human pancreas (Fig. 4A).

ELISA analyses showed that HEACs of the control group (C) followed by stimulating with high (25 mM, HG) or low (5.5 mM, LG) glucose concentration secreted 13.7 $\pm$ 9.2 pg/ml and 14.2 $\pm$ 0.6 pg/ml of insulin into the media, respectively (Fig. 4B). In contrast, HEACs of the NAG group secreted 128.5 $\pm$ 11.1 pg/ml of insulin with HG and 8.0 $\pm$ 2.5 pg/ml with LG media—values greater than those of the control (a and b, $p < .05$). Similarly, HEACs of the control group released 28.3 $\pm$ 18.0 pg/ml of c-peptide with HG and 15.8 $\pm$ 7.9 pg/ml with LG media (Fig. 4C). Those of the NAG group released 342.7 $\pm$ 70.5 pg/ml of c-peptide with HG and 21.4 $\pm$ 9.9 pg/ml with LG media—values greater than those of the control (a and b, $p < .05$). When cells of MIN6N8, a mouse pancreatic cell line, were challenged with LG and HG conditions, they showed about fourfold increase of insulin secretion as glucose concentration increased (supporting information Fig. 3). These results demonstrate that NAG combination is the best condition for the differentiation of HEACs into insulin-secreting cells and that insulin secretion by HEACs is dependent on the glucose concentration.

Immunocytochemical studies showed that HEACs of NAG group were strongly positive for the human proinsulin, insulin, and c-peptide (Fig. 4D). Cells in colony-forming areas of NAG group also gave an intense staining with dithizone (Fig. 4E).

Normalization of Diabetes in Mice by Transplantation with HEACs

STZ-treated C57BL/6 mice received saline or cell transplant under the kidney capsules. Of 20 mice that received HEACs of NAG group (DC mice) from the above experiments, 10 mice showed normalized blood glucose level and survived for longer than 60 days (Fig. 5A). Among them, eight mice were sacrificed at 61 days. At the time of sacrifice, all mice

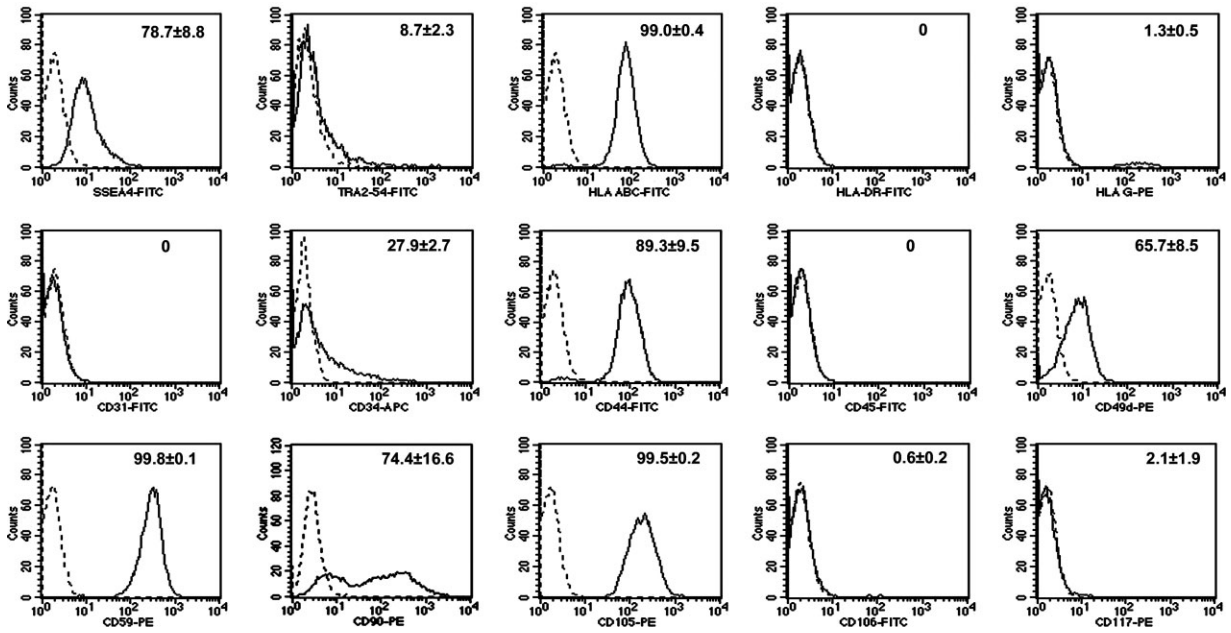

Figure 2. Flow cytometric analyses of human eyelid adipose-derived stem cells (HEACs). HEACs at passage 3 were strongly positive for SSEA-4, HLA-ABC, CD44, CD49d, CD59, CD90, and CD105; weakly positive for TRA 2-54 and CD34; and negative for HLA-DR, CD31, CD45, CD106, and CD117 antigens. Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

showed blood glucose level ranging between 118 and 134 mg/dl, slightly higher than the normal levels ranging between 108 and 123 mg/dl. Two mice were allowed to live up to one and half years. The other 10 DC mice died within 53 days after transplantation. Mice that received a transplant of saline (sham) or undifferentiated HEACs (UDC) began to die by 10 days after transplantation and all 40 mice died within 43 days. Body weight of the sham and UDC group mice gradually decreased after transplantation (Fig. 5B). In contrast, body weight of DC mice began to increase after 18 days. Body weight of normal mice gradually increased. Following transplantation, blood glucose levels of the sham and UDC group mice gradually increased during 36 days (Fig. 5C). In contrast, blood glucose level of DC mice decreased during 61 days, reaching the level of normal mice. Following removal of the left kidneys transplanted with HEACs from 3 DC mice at 61 days after transplantation, blood glucose level of these mice markedly increased during the next 7 days. Glucose tolerance tests were done for 10 DC mice showing normoglycemia at 36 days after transplantation, and showed that blood glucose levels of both the normal and DC mice dropped to initial levels within 150 minutes, although DC mice showed higher glucose level than the normal mice (Fig. 5D). The results demonstrate that insulin-secreting cells differentiated from HEACs could normalize type I diabetes in mice.

Regulation of Mouse Blood Glucose Level by Human Insulin from Transplanted HEACs

ELISA analyses showed that the blood of five randomly chosen DC mice after 61 days of transplantation contained near background values of mouse insulin and c-peptide levels, whereas that of normal mice exhibited 3.0 ± 0.7 ng/ml of mouse insulin and 4.1 ± 0.9 ng/ml of mouse c-peptide (Fig. 6A). However, DC mice showed 0.4 ± 0.1 ng/ml of human insulin and 0.8 ± 0.2 ng/ml of human c-peptide (Fig. 6B). Normal mice exhibited a small amount of human insulin and human c-peptide in their blood. These results clearly show that only human, but not mouse, insulin and c-peptide were present in the blood of DC mice.

Kidneys of DC mice at 2 months (DcK1) or 1 year (DcK2) after transplantation showed expression of human genes of *PDX1*, *NEUROG3*, *NKX6.1*, *GCK*, *PC1/3*, *GLUT1*, *INS*, *GCG*, *GAPDH*, and *hAlu*, most of which were also expressed by HEACs cultured in NAG condition before transplantation (Figs. 4A and 6C). In contrast, normal mouse kidneys never showed expression of these human genes. Before and after differentiation into insulin-secreting cells in vitro, HEACs consistently expressed transcripts of the immune-related genes of *HLA-ABC*, *β2M*, *CD40*, *CD59*, *TAP1*, and *TAP2*, but they did not express *HLA-DR*, *HLA-DM*, *CD80*, and *CD86*—genes that are involved in cell-mediated immune responses (Fig. 6D). DcK1 and DcK2 showed a similar expression pattern, except that *β2M* and *CD40* genes were no longer expressed. In addition, DcK2 did not express *HLA-ABC* gene.

Many cells of kidneys of DC mice reacted in common with both antibodies against insulin and human nuclear antigen (Fig. 7). Pancreas of the same mice showed only a few insulin-positive pancreatic islets, the size of which was greatly diminished compared with the normal pancreas. These observations show that transplanted HEACs survived and actively synthesized human insulin in the kidneys of immunocompetent mice for 2 months after transplantation and that little regeneration of mice β cells occurred in pancreas of DC mice.

DISCUSSION

The highlights of this study are the isolation of adult stem cells from the human eyelid adipose tissue, successful differentiation into insulin-producing cells in vitro, and normalization of hyperglycemia in STZ-induced diabetic mice without immunosuppressive agents following transplantation of these insulin-producing cells.

Human tissue cells that were manipulated to differentiate into insulin-secreting cells in vitro have been shown to lower

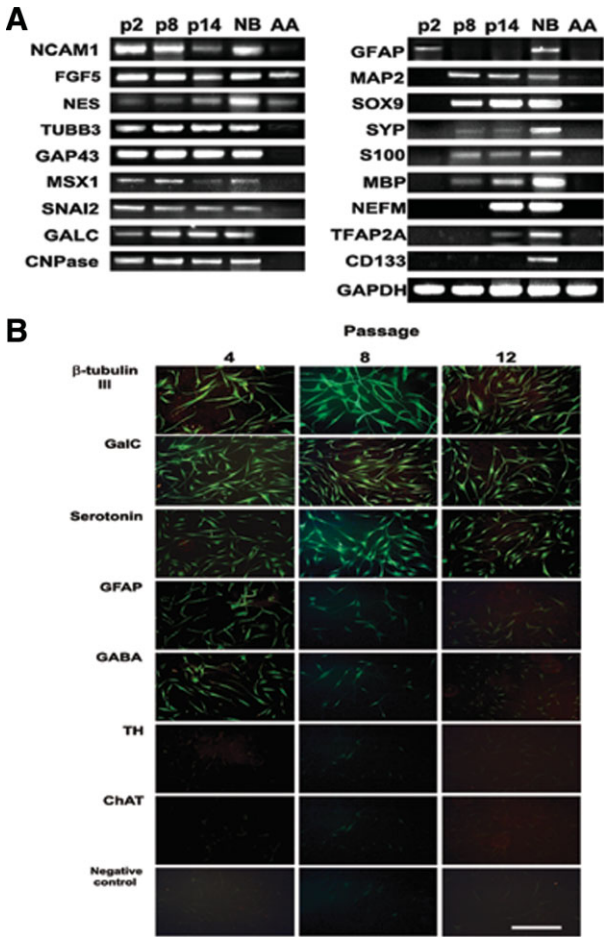

Figure 3. Neural properties of human eyelid adipose-derived stem cells (HEACs). **(A):** Expression of neuron-related genes by HEACs at p2, p8, and p14. **(B):** Immunocytochemistry of HEACs at p4, p8, and p12. Scale bar = 500 μm. Abbreviations: AA, human abdominal adipose-derived stem cells at p3; NB, neuroblastoma cells; p, passage.

the glucose level when transplanted into diabetic animals [7, 8, 21–23]. Some of these studies demonstrated the presence of human insulin and/or c-peptide in blood of recipient animals, suggesting that donor cell might have participated in lowering the blood glucose level [8, 23]. However, it is obscure in these studies whether the recipients themselves produced their own insulin responsible for the normoglycemia. In the present study, we observed that only human, but not mouse, insulin and c-peptide were detected in the blood of DC mice recovered from hyperglycemia. Removal of the HEAC-bearing kidneys from these mice resulted in the hyperglycemia. Expression of human β cell-specific genes, and human insulin- and nuclear protein-positive cells, was found in these kidneys. Regeneration of islets in the pancreata of the same mice was not seen. Our observations clearly demonstrate that insulin from human donor cells is solely responsible for the normalization of hyperglycemia.

HEACs exhibit some of the characteristics typical of MSCs in terms of the mesodermal differentiation potential, expression of CD90 and CD105, and null expression of CD34, CD45, CD106, and HLA-DR in addition to a variety of proteins [24, 25]. However, they are markedly different from MSCs isolated from other adipose tissues. Undifferentiated HEACs spontaneously express many neural cell-related mRNAs and a variety of neural proteins such as GABA, GalC, serotonin, β-tubulin III, and GFAP, most of which are also observed in human neural crest cells [26] and in neuroblastoma cells. In contrast, MSCs from the trunk adipose tissues do not show most of these neural cell-like characters unless they are cultured in the neurogenic conditions consisting of particular chemicals, growth factors, or neural tissues [24, 27–30]. Undifferentiated MSCs from abdominal adipose in the present study also did not express most of neural cell-related mRNAs seen in neuroblastoma cells. HEACs are of the bipolar neuronal shape, whereas other MSCs are spindle shaped. The origin of cell sources may explain the difference between HEACs and other adipose-derived MSCs. In mice, it has been observed that the cephalic neural crest cells produce a subset of facial adipocytes but do not contribute to adipocytes at the truncal level during normal development [31]. Although embryonic truncal adipocytes may arise from MSCs derived from truncal neural crest, these MSCs appear to be replaced by as-yet-unidentified cells during later development [32]. Wnt-1 lineage tracing analysis has also demonstrated that mouse truncal adipose-derived MSCs do not represent a neural crest-derived population residing in adult adipose [33]. Based upon these findings, it is concluded that HEACs are neural crest-derived multipotent stem cells that are distinguished from trunk adipose-derived MSCs. Given that mesoderm-like structures including muscles, cartilages, bones, and arterial endothelial cells in the head and neck are derived from cranial neural crest cells [13], mesodermal differentiation potency of HEACs further supports their origin from neural crest.

In vitro differentiated HEACs successfully engrafted in the kidneys of half of the immunocompetent mice for 2 months or longer. Similar engraftment of xenogeneic transplants overcoming immune attack has been observed in a variety of human cells and animal models. Although the tolerance across the immune barrier is not absolute [34], the engraftment has been explained by the low level of HLA class I, and the absence of HLA class II [18], CD80 and CD86 molecules [35], all of which play key roles in T-cell-mediated immune response. We also observed that differentiated HEACs as well as undifferentiated ones did not express *HLA-DR*, *HLA-DM*, *CD80*, and *CD86* genes. Moreover, mouse kidneys transplanted with differentiated HEACs showed little expression of human *β2M* gene, which is a component of *HLA-ABC* and *CD40* gene, a costimulatory factor on antigen-presenting cells. These characteristics might enable HEACs to overcome the T-cell-mediated graft rejection following xenotransplantation. MSCs are known to modulate the intensity of an immune response by inhibiting antigen-specific T-cell proliferation and cytotoxicity, and have immunosuppressive activities via diverse soluble factors [36]. Whether HEACs could exhibit similar immunosuppressive activities remains in question. In the present study, however, half of DC mice failed to recover from hyperglycemia. HEACs in these mice might have been rejected by immune attack or could not secrete enough amount of insulin. The mechanism of the immune privilege of HEACs warrants further investigation.

We successfully differentiated HEACs into insulin-secreting cells in vitro. Since we did not use any insulin that might be absorbed by cells during in vitro culture [37, 38], the insulin that appeared in the media was de novo synthesized and secreted by HEACs. The amount of c-peptide released into the media was comparable with that released by pancreatic islet cells [39]. Unique properties of HEACs expressing many genes that play important roles in pancreatic differentiation and/or insulin secretion most likely play a role in the successful differentiation of HEACs into insulin-secreting cells. Many of pancreatic genes such as NEUROD1, ISL1, PAX4, NES [40], HNF4A [41], GLP1R [42], and GCK [43] are also

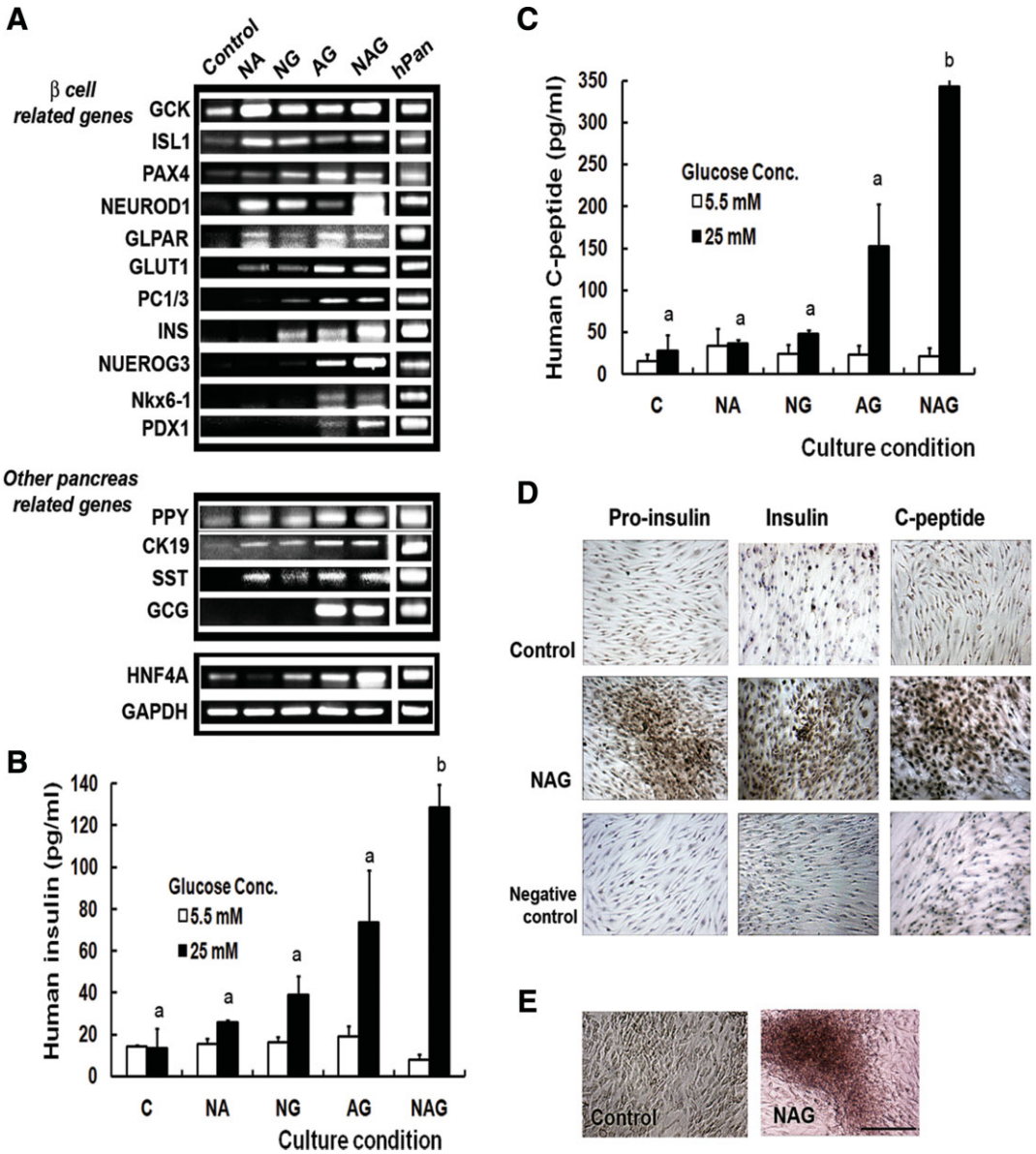

Figure 4. Differentiation of human eyelid adipose-derived stem cells at passage 3 into insulin-secreting cells in vitro. After culture in various differentiation conditions, cells were analyzed by dithizone staining **(A)**, expression of genes **(B)**, enzyme-linked immunosorbent assay for the secretion of insulin **(C)** and c-peptide **(D)**, and immunocytochemistry **(E,** ×100). Scale bar = 500 μm. a and b, $p < .05$. Abbreviations: AG, activin A and GLP-1; C, control; Conc, concentration; hPan, human pancreas total RNA; NA, nicotinamide and activin A; NAG, nicotinamide, activin A, and GLP-1; NG, nicotinamide and GLP-1.

expressed in cells of the nervous system. Previously, brain-derived neural progenitor cells were shown to differentiate into β-cell-like insulin-positive cells in vitro [44]. Conversely, mouse adult pancreas could generate multiple cell types with phenotypic characteristics of neurons and glial cells as well as insulin-producing β-like cells in vitro [45, 46]. Similarities of regulatory genes involved in both neural and β-cell differentiation pathways may enable ectoderm- and pancreas-derived stem cells to transdifferentiate.

Cytokine mixture also seems to contribute to efficient differentiation of the HEACs. Nicotinamide enhances in vitro differentiation of human fetal pancreatic cells, favoring the expression of insulin gene [47]. Activin A [48] and GLP-1 [49] facilitate β-cell differentiation and potentiate glucose-dependent insulin secretion of fetal and adult pancreatic cells. We found that a mixture of all three compounds was optimal to enable the differentiation of HEACs into insulin-secreting cells. Lowering the glucose concentration during the last differentiation stage following high concentration treatment was shown to increase insulin secretion from embryonic stem cells [50] and to increase the expression of β-cell-related genes in neural progenitor cells [43]. Collectively, unique properties of HEACs, combination of selected cytokines, and two-step culture method might cooperatively work, resulting in the efficient differentiation of HEACs into insulin-secreting cells in vitro.

Conclusion

In conclusion, our results demonstrate that differentiated HEACs could directly regulate blood glucose levels in diabetic mice by releasing human insulin, rather than supporting

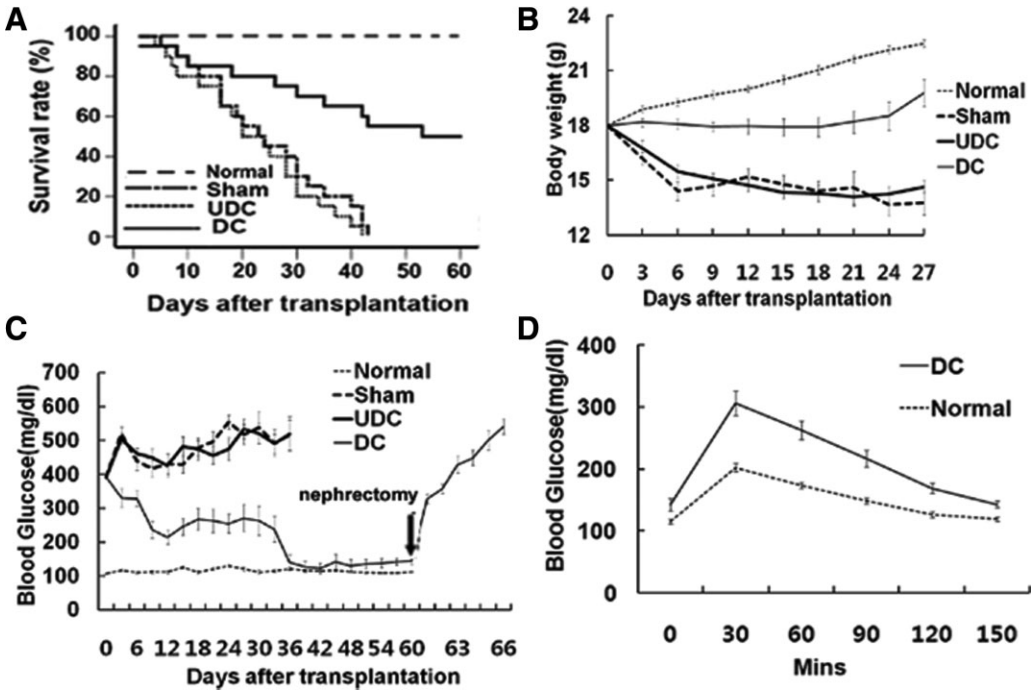

Figure 5. Physiological responses of diabetic mice to human eyelid adipose-derived stem cell transplantation. Following injection with saline (Sham), UDC, or DC, **(A)** survival rate, **(B)** body weight, **(C)** blood glucose levels, and **(D)** glucose tolerance test were determined in 10 DC mice that showed normoglycemia after 36 days. Data are shown as mean ± SEM. Abbreviations: DC, differentiated cell transplant group; Mins, minutes; UDC, undifferentiated cell transplant group.

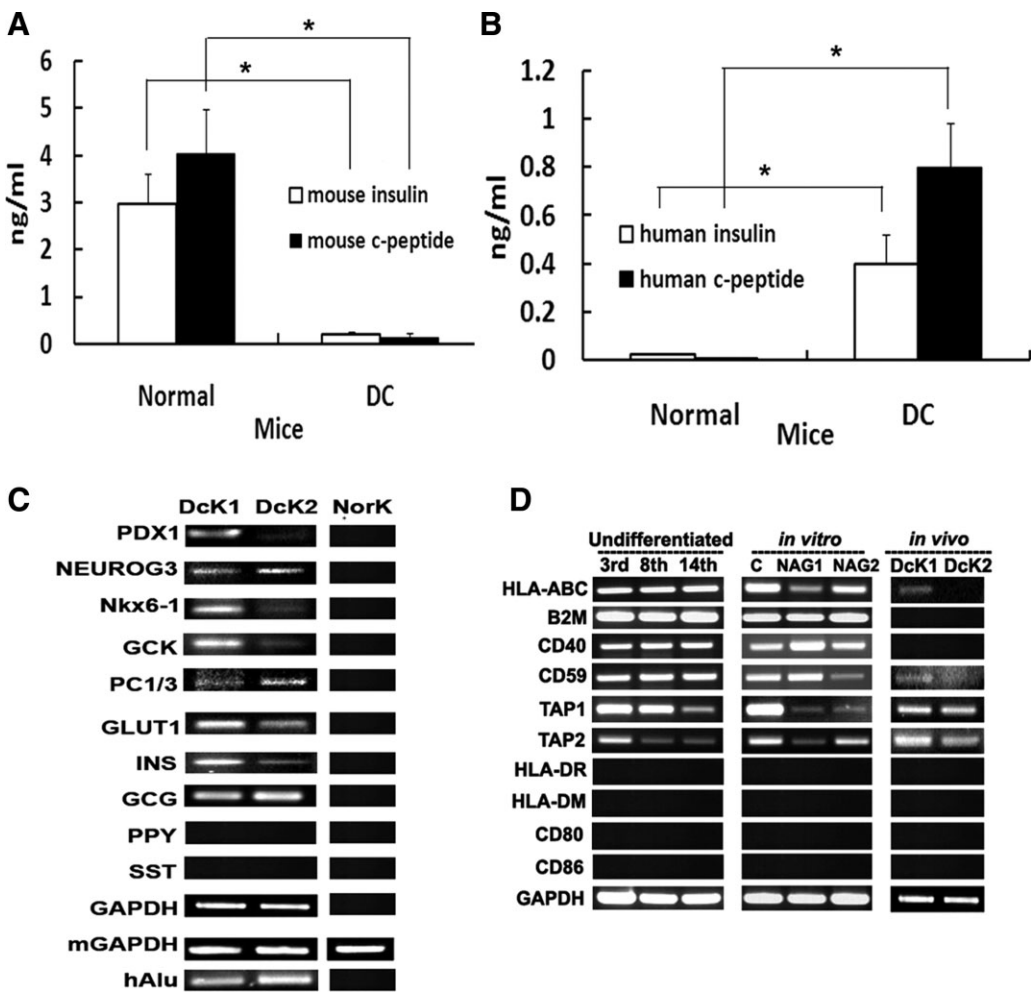

Figure 6. Roles of human eyelid adipose-derived stem cells (HEACs) transplanted into immunocompetent mice. **(A):** Mouse and **(B)** human insulin and c-peptide in the sera of normal and DC mice ($n = 5$). *, $p < .05$. **(C):** Human genes expressed in kidneys of normal and DC mice. **(D):** Expression of human immune genes by HEACs in vitro and in vivo. Abbreviations: C, control; DC, differentiated cell transplant group.

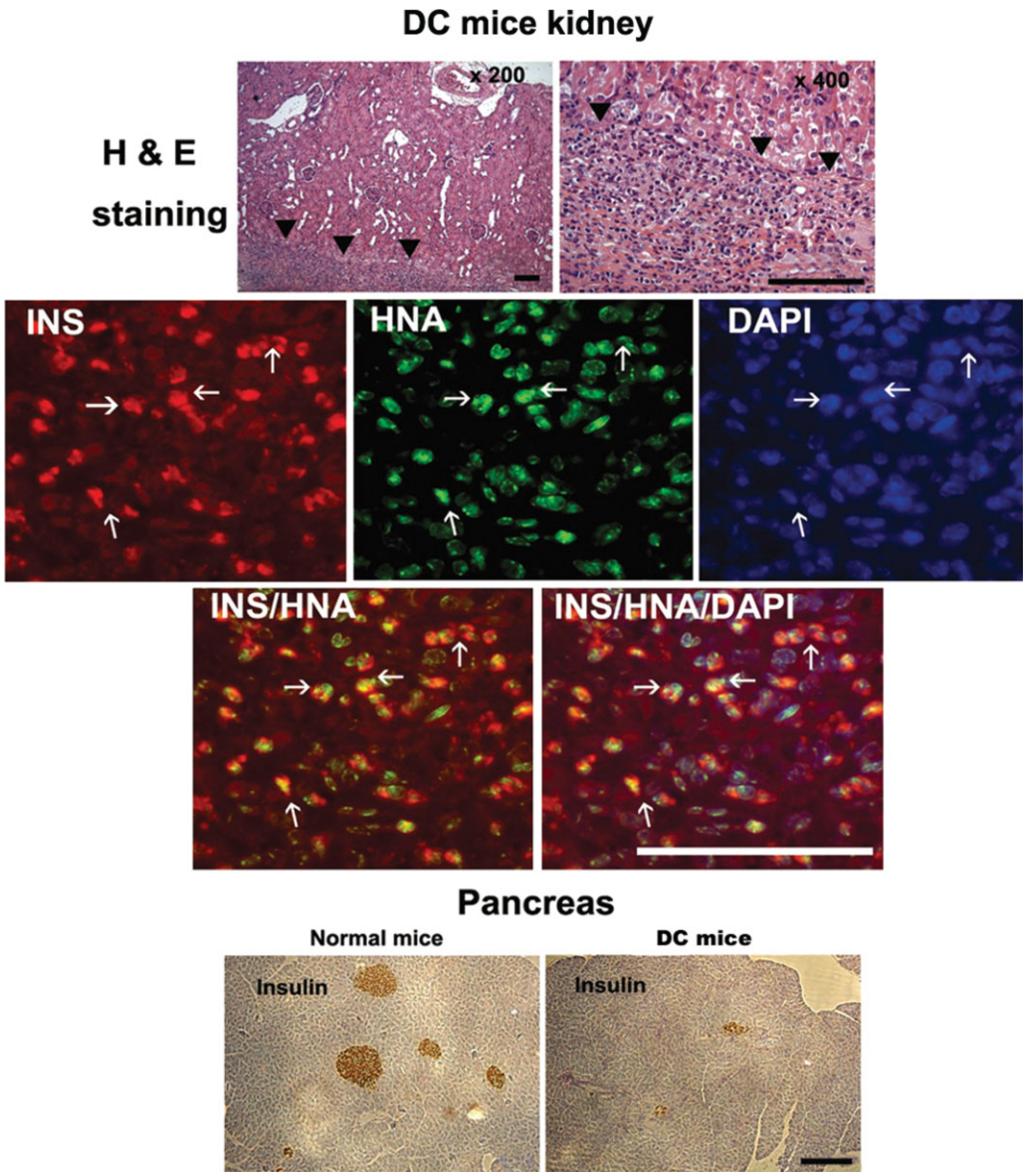

Figure 7. Immunohistochemistry of kidneys and pancreata from normal and DC mice. First row: H&E-stained kidneys of DC mice. Arrowheads indicate boundary between human and mouse cells; second and third rows: kidneys of the same mice triple-labeled with INS, HNA, and DAPI; fourth row: pancreata of normal and DC mice stained with INS. Scale bar = 100 μm. Abbreviations: DAPI, 4′-6′ diamidino-2-phenylindole; DC, differentiated cell transplant group; HNA, human nuclear antigen.

the regeneration or repopulation of mouse β cells. These findings may be useful when considering the clinical application of HEACs to diabetes mellitus and neural diseases.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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