Transplantation of insulin secreting cells differentiated from human adipose tissue-derived stem cells into type 2 diabetes mice

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Transplantation of insulin secreting cells differentiated from human adipose tissue-derived stem cells into type 2 diabetes mice

Directed by Professor Chul Woo Ahn

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December, 2011

Ji Sun Nam

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ABSTRACT

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(Directed by Professor Chul Woo Ahn)

There has been a shift in paradigm about the pathogenesis of type 2 diabetes, from the emphasis on insulin resistance to a depletion of insulin secretion. Although there are various drugs and ways to ameliorate insulin resistance, there are very limited ways to preserve or recover insulin secretory capacity.

The purpose of this study was to evaluate the efficacy of cell therapy using insulin secreting cells differentiated from human eyelid adipose tissue-derived stem cells (hEA) into type 2 diabetes mice in terms of glucose lowering effect, survival benefit, and its effect on other metabolic parameters. After differentiating hEA using ISI media, these insulin secreting cells (hEA-ISC) were transplanted into renal capsules of type 2 diabetes mouse model established by a low dose streptozotocin injection followed by a high fat diet. Mice that received differentiated cells (DC group) showed a significant lowering of blood glucose level up to 60 days and improved glucose tolerance

compared to those that received undifferentiated cells (UDC group) and sham-operated mice (sham group). There was a survival benefit, with 10 out of 15 mice surviving in DC group while 6 out of 15 in UDC group and none of 8 sham mice survived at 60 days after transplantation. Significantly increased levels of human insulin and human c-peptide were detected in sera of DC group (both $p < 0.001$), but the combined human and mouse insulin and c-peptide levels were not different among the groups. Human genes were expressed in kidneys of transplanted mice, including insulin, GLUT1, GLUT2, NueroD1, Pdx1, and glucokinase. Immunohistochemical analysis showed co-existence of human and mouse cells in transplanted kidneys, and triple-staining with human insulin (hINS), human nuclear antigen (hNUCLEI), and DAPI (4',6-diamidino-2-phenylindole) showed co-localization of human insulin and human nuclear antigen. Also, DC and UDC group showed a significantly lower serum IL-6 level compared to sham group (both $P < 0.05$), and DC group showed a tendency toward lower triglyceride and free fatty levels compared to sham group ($P = 0.093$ and 0.065, respectively).

Cell therapy using differentiated hEAs is effective in lowering blood glucose level in type 2 diabetes mice by increasing circulating insulin level, and it also has a favorable effect on metabolic parameters and IL-6. The cause and effect relationship remains to be determined.

Key words : adipose tissue-derived stem cell, insulin secreting cells, type 2 diabetes mellitus, cell therapy

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I. INTRODUCTION

Recently, there has been a shift in paradigm about the pathogenesis of type 2 diabetes. Earlier, insulin resistance was deemed the main underlying cause of type 2 diabetes, but throughout the second half of the 20th century, beta-cell dysfunction became another main pathophysiology[1]. Many studies support evidence that beta-cell dysfunction is the primary defect or at least plays a non-inferior role in the pathogenesis of type 2 diabetes[1,2]. For example, beta cell function continues to decline, whereas there is a minimal if any change in insulin sensitivity[3,4], and beta cell dysfunction is already present in normal glucose-tolerant individuals genetically predisposed to develop type 2 diabetes[5]. Thus, currently, the treatment of type 2 diabetes targets to restore beta cell mass and improve insulin sensitivity.

Various drugs are available for ameliorating insulin resistance, but there are limited ways to preserve or restore beta cell function. Currently, insulin therapy is the only practical method for replacing depleted beta cells in type 1 and 2 diabetes patients, but it costs weight gain and hypoglycemia, and many patients, especially type 2 diabetes patients, are reluctant to begin a self-injection. Pancreas or islet cell transplantation is an alternate therapy, but it remains an experimental procedure[6]. Due to a limited supply of donor organs, procedure-related morbidities, a low success rate, and adverse effects from permanent immunosuppressive therapy, it is indicated in selected type 1 diabetes patients with life-threatening hypoglycemia, and type 2 diabetes patients are excluded[7].

Thus, various efforts have been made in search of an alternative source of beta cells, and stem cells gained a spotlight. Stem cell therapy is an attractive treatment option for diabetes for its abundant source and glucose-dependent insulin secretory function[6,8]. Currently, attempts are being made to find the appropriate cell source and design optimal ways to differentiate these cells into insulin secreting cells[8]. Earlier studies used embryonic or induced-pluripotent stem cells as beginning materials for insulin secreting cells for their ability to differentiate into diverse cells, but their clinical application is hampered by the teratogenicity and ethnic concerns[9,10].

Meanwhile, adult stem cells are relatively safe in these aspects, and depending on the source of the cells, some are easily acquired without an

invasive procedure, and they allow autologous transplantation, thereby circumventing the adverse effects of immunosuppression[10]. Adult stem cells derived from various sources including hepatocytes[11,12], peripheral blood-derived blood monocytes[13], and umbilical cord blood[14] have been successfully differentiated into insulin secreting cells and showed a glucose-lowering effect in diabetes murine models. Neural-crest derived cells are another good candidate source of stem cells for producing insulin secreting cells. Stem cells from neural crest-derived cells had been shown to differentiate into mesodermal cells like muscle, cartilage, and ectodermal neurons[15] as well as endothelial cells like hepatocytes[16] and insulin-secreting cells[17]. These adipose tissue-derived stem cells can be easily obtained from human eyelid adipose tissue without an invasive procedure, and they contain good proliferation and multi-differentiation potential with high levels of stem cell-related antigens[18]. Also, a lack of HLA-DR expression and immunosuppressive properties of these cells have been reported[19], and a successful engraftment was shown in immunocompetent mice without immunosuppressive agents[17]. Currently, there are continuous efforts, such as testing various combinations of growth factors and cytokines in culture media and genetic manipulation, to establish an efficient way to differentiate these cells and obtain a high-yield insulin[20-23].

However, most of the studies were targeted toward treating type 1 diabetes. There are very few works done with cell therapy in type 2 diabetes. In 2004,

Ende et al. transplanted human umbilical cord blood mononuclear cells into types 2 diabetes mice, and demonstrated improved blood glucose levels and survival[24]. Recently, Alipio et al. showed a reversal of hyperglycemia in type 1 as well as type 2 diabetes mouse models using induced-pluripotent stem cell-derived pancreatic beta-like cells[25]. Lastly, there are few human clinical trials, including one that showed the efficacy and safety of autologous bone marrow derived stem cell transplantation in patients with type 2 diabetes[26] and another one demonstrating a reduced insulin requirement using placenta-derived mesenchymal stem cells[27]. However, all these animal and human studies on type 2 diabetes were conducted with undifferentiated cells or differentiated iPS cells, and to our knowledge, there is no prior study that transplanted differentiated adult stem cells.

In this study, we transplanted insulin secreting cells differentiated from human eyelid-derived adipose tissue stem cells into immunocompetent type 2 diabetes mouse model, and assessed the effects on glucose level, metabolic profiles as well as surrogate markers of insulin resistance, and survival.

II. MATERIALS AND METHODS

1. Human eyelid adipose-derived stem cell (hEA) isolation and culture

The human eyelid adipose tissue was obtained from nine subjects 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, USA) in phosphate-buffered saline (PBS) for 30 minutes at 37℃ 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, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 3.7 mg/ ml sodium bicarbonate at 5% CO_2, 37℃. Medium was changed twice a week.

2. Differentiation of hEA into insulin-secreting cells (hEA-ISC) in vitro

Cells were induced into differentiated insulin-secreting cells (hEA-ISC) using insulinogenic medium (IS1 kit, Bcellbio, Seoul, Korea). Briefly, hEA at passage 3 and passage 4 were plated on 48 well dishes (Nunc, Rochester, MN) at 5 x 10^3 cell//well and then cultured in control or insulinogenic differentiation medium for 21 days. For first three days, cells were cultured in RPMI1640 containing 25 mM glucose or 1X IS1-01 supplement, and then cells were cultured in

RPMI1640 containing 25 mM glucose, 10% FBS or 1X IS1-02 supplement for 4 days. After 1 week, the medium was changed RPMI1640 supplement with 5.5 mM glucose, 10% FBS or 1X IS1-03 supplement for 3 days, and during the subsequent 10 days, cells were cultured in RPMI 1640 containing 5.5 mM glucose, 10% FBS or 1X IS1-04 supplement.

3. Immunocytochemistry

hEA-ISC cultivated in Lab-Tek chamber slides (Nunc) were washed with PBS and then permeabilized in 0.5% Triton X-100 for 10 min. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide (DakoCytomation, Carpinteria, CA, USA), for 15 min at room temperature (RT). The slides were incubated in a blocking solution, PBS containing 2% BSA, for 1 h at RT. After washing with PBS containing 0.1% BSA, cells were treated with mouse anti-human insulin monoclonal antibody (1:100; Abcam, Cambridge, UK) or mouse anti-human c-peptide monoclonal antibody (1:100; Monosan, AM Uden, Netherland) for 1 h at RT. Cells were then incubated with biotinylated goat anti-mouse or anti-rabbit IgG (Vectastatin ABC kit; Vector Laboratories, Burlingame, CA, USA followed by horseradish peroxidase-conjugated streptavidin (Vector Laboratories) for 20 min, respectively, at RT. Immunoreactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DakoCytomation) and counterstained with Mayer's haematoxylin (Sigma). Cells were photographed

under a microscope using a bright-field illumination (Axioskop2+, Carl Zeiss, Oberkochen, Germany).

4. Measurement of insulin and c-peptide

To measure the amount of human insulin and c-peptide secreted in vitro, differentiated cells were treated with DMEM-HG for 2 h at 37℃. The amount of insulin and c-peptide, a peptide fragment released from the proinsulin molecule during processing into mature insulin, in media was measured using the ultrasensitive human insulin and c-peptide ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions**.** For the measurement of human insulin and c-peptide levels in serum, blood were taken by cardiac puncture from mice which were fasted overnight and then intraperitoneally injected with glucose (1.5g/kg body weight), and serum levels were measured. Mouse insulin and c-peptide in mouse serum were determined using mouse-specific insulin ELISA kit (Mercodia) and c-peptide ELISA kit (Yanaihara, Shizuoka, Japan).

5. Reverse-transcription polymerase chain reaction (RT-PCR)

RNA was isolated using Tri-reagent (Invitrogen, Carlsbad, CA, USA) 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). RT-PCR was performed using a Primus 96 Universal (Peqlab Biotechnologie,

Erlangen, Germany). Subsequent PCR reactions were performed using cDNA, primer pairs (Table 1) and PCR mixture (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. RNAs from human embryonic stem cells were kindly donated from Prof. Kim (Korea University, Korea), and human total RNAs from pancreas (hPAN) was purchased from Ambion (Austin, TX, USA).

Table 1. Primers used in the present study

Gene	*Primer sequence*	*Accession number*	*Size (bp)*	*Anneal -ing temp .(°C)*
GAPDH	5`-ACA ACT TTG GTA TCG TGG AA-3` 5`-AAA TTC GTT GTC ATA CCA GG-3`	NM_002046	456	53
Oct-5	5`-CGA CCA TCT GCC GCT TTG AG-3` 5`-CCC CCT GTC CCC CAT TCC TA-3`	NM203289	573	61
Rex-1	5`-ATG GCT ATG TGT GCT ATG AGC-3` 5`-CCT CAA CTT CTA GTG CAT CC-3`	NM_174900	449	57
SCF	5`-CCA TTG ATG CCT TCA AGG AC-3` 5`-CTT CCA GTA TAA GGC TCC AA-3`	NM000899	275	55
BMP-4	5`-AGC CAT GCT AGT TTG ATA CC-3` 5`-TCA GGG ATG CTG CTG AGG TT-3`	NM130851	384	55
Brachyury (T)	5`-GAG CTC ACC AAT GAG ATG AT-3` 5`-GGC TCA TAC TTA TGC AAG GA-3`	NM003181	335	57
AFP	5`-TTT TGG GAC CCG AAC TTT CC-3` 5`-CTC CTG GTA TCC TTT AGC AAC TCT-3`	NM001134	451	57
GATA-4	5`-TCC CTC TTC CCT CCT CAA AT-3` 5`-TCA GCG TGT AAA GGC ATC TG-3`	NM002052	194	57
HNF-4a	5`-GAG CAG GAA TGG GAA GGA TG-3` 5`-GGC TGT CCT TTG GGA TGA AG-3`	NM178849	205	57
VIM	5`-CCT TCG TGA ATA CCA CGA CCT GC-3` 5`-TAA TAT ATC GCC TGC CAC TGA G-3`	NM003380	321	56
BMP-2	5`-TTG CGG CTG CTC AGC ATG TT-3` 5`-TTC CGA GAA CAG ATG CAA GAT G-3`	NM001200	316	55

CK18	5`-GAG ATC GAG GCT CTC AAG GA-3` 5`-CAA GCT GGC CTT CAG ATT TC-3`	NM000224	357	57
GCK	5'- GAA TAC CCC CCA GAG ACC TTT TC-3’ 5'- GGT TTC TTC CTG AGC CAG CG-3'	NM_0333508.1	176	61
ISL1	5'-AGC ATC AAT GTC CTC TCA ACT TCC-3’ 5'-TGT TTG GCA AGG CAA TGA CC-3’	NM_002202.2	496	57
HNF4A	5`-GAG CAG GAA TGG GAA GGA TG-3` 5`-GGC TGT CCT TTG GGA TGA AG-3`	NM178849	205	57
NEUROD1	5’-CAG AAC CAG GAC ATG CCC-3’ 5’-ATC AAA GGA AGG GCT GGT G-3’	NM_002500.2	218	59
NEUROG3	5'-CGT GAA CTC CTT GAA CTG AGC AG-3‘ 5'-TGG CAC TCC TGG GAC AGA TTT C-3'	AF234829	211	61
GLUT1	5’-CTC ACT GCT CAA GAA GAC ATG G-3’ 5’-CTG GGT AAC AGG GAT CAA ACA G-3’	NM 006516.2	366	60
Nkx6-1	5'-ACA CGA GAC CCA CTT TTT CCG-3’ 5’-TGC TGG ACT TGT GCT TCT TCA AC-3’	NM_006168.2	336	59
PC1/3	5’-TTG GCT GAA AGA GAA CGG GAT ACA TCT-3’ 5’-ACT TCT TTG GTG ATT GCT TTG GCG GTG-3’	NM_000439.4	457	60
SST	5’-GGC TGC GCT GTC CAT CGT CCT G-3’ 5’-TTG CGT TCT CGG GGT GCC ATA GC-3’	NM_001048.3	276	63
PDX1	5'-CCC ATG GAT GAA GTC TAC C-3’ 5'-GTC CTC CTC CTT TTT CCA C-3'	NM_000209.3	262	54
INS	5'- AAC CAA CAC CTG TGC GGC TC-3‘ 5’-AAG GGC TTT ATT CCA TCT CTC TCG-3’	NM_000207.2	322	59
hGAPDH	5’-ACA ACT TTG GTA TCG TGG AA-3’ 5’-AAA TTC GTT GTC ATA CCA GG-3’	NM_002046.3	458	55
mGAPDH	5'-AAT CCC ATC ACC ATC TTC CA-3’ 5'-GGC AGT GAT GGC ATG GAC TG-3'	NM_008084.2	333	55
hAlu	5’-CGA GGC GGG TGG ATC ATG AGG T-3’ 5’-TCT GTC GCC CAG GCC GGA CT-3’	NT.007592.15	230	66
Nkx2-2	5’-TTC TAC GAC AGC AGC GAC AAC C-3’ 5’-CGT CAC CTC CAT ACC TTT CTC G-3’	NM_002509.2	393	61
GLPAR	5`-GTT CCC CTG CTG TTT GTT GT-3` 5`-CTT GGC AAG TCT GCA TTT GA-3`	NM002062	228	55
PPY	5`-CTC TGT TACT AC AGC CAC TG-3` 5`-AGT CGT AGG AGA CAG AAG GT-3`	NM002722	261	55

6. Establishment of type 2 diabetes mouse model

Female C57BL/6 mice (16-18g, 6-week old) were purchased from the Samtako Bio Korea (Osan, Korea). All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use of Committee of Seoul Women's University. Non-genetic, experimental type 2 diabetes was induced by a single intraperitoneal low dose streptozotocin (STZ, 120 mg/kg body weight, Sigma) in 100 mM citrate buffer (pH 4.5) at 8 weeks of age followed by high fat diet[28]. Three weeks after STZ injection, mice were placed on 60% high fat diet (D12451, 45% of energy as fat; Research Diets, New Brunswick, NJ, USA) or normal diet for 5 weeks. Body weight and blood glucose level were measured weekly from tail bleeds using ACCU-CHECK Active (Roche Diagnostics, Basel, Switzerland). Then, blood samples were obtained by cardiac puncture from selected mice from normal control, STZ only, and STZ followed by high fat diet (STZ/HFD) groups and assessed for serum insulin, free fatty acid, triglyceride, and glucose levels.

7. Intraperitoneal insulin tolerance test (IP-ITT)

After 5 weeks of high fat diet, intra-peritoneal insulin tolerance test was performed to assess the insulin sensitivity. Animals were injected with 0.5U/kg of rapid-acting insulin (Humalog®, Lilly, Indianapolis, IN, USA) after 4 hours of fasting. Blood glucose levels were measured from tail bleeds using glucometer before insulin injection and at 15, 30, 60, and 90 minutes

postinjection. Kitt was calculated from following formula.

KITT (%/min) = 0.693/ t½ , where t½ was calculated from the slope of the plasma glucose concentration during the period from 15 to 90 minutes after insulin injection, using the least square analysis.

Animals with Kitt value of less than 5% calculated from insulin tolerance test were deemed to have insulin resistance.

8. Transplantation of hEA-ISC into type 2 diabetes mice

Diabetes mice with glucose level of higher than 300 mg/dl and Kitt value of greater than 5% were selected, and those with similar degrees of hyperglycemia and body weight were randomly allocated to saline-injected sham group (sham group, n = 8), hEA-ISC transplant group (differentiated cell (DC) group, n = 15) and a hEA transplant group (undifferentiated cell (UDC) group, n = 15). Normal mice were used as a control group (control group, n = 8). Total 1.5 x 10^6 cells were loaded using a PE50 polyethylene tubing (Becton Dickinson, Sparks, MD, USA) were transplanted underneath the kidney capsule using a Hamilton syringe (Fisher, Pittsburg, PA, USA) at 9 weeks after STZ injection. No immunosuppressive agent was given to mice throughout the experiment. Differentiated and undifferentiated cells were obtained by culture of hEAs for 3 weeks using IS1 kit or without additives, respectively. Blood glucose level was measured feeding condition weekly thereafter for 60 days, and 1 mouse that received hEA-ISC was observed for 210 days.

9. Intraperitoneal glucose tolerance test (IP-GTT)

Intraperitoneal Glucose Tolerance Test (IP-GTT) was performed in fasting anaesthetized mice at 4 weeks after transplantation. After baseline blood glucose measurements, mice received an i.p. injection of 1.5 g of glucose per kilogram body weight. Blood glucose levels were measured from tail bleeds with a glucometer (ACCU-CHECK Active) at 0, 15, 30, 60, 90, 120, and 150 minutes.

10. Metabolic parameters and interleukin-6 (IL-6)

Blood samples were obtained from cardiac puncture at the time of sacrifice. Plasma free fatty acids, triglyceride, total cholesterol, adiponectin, and IL-6 levels were measured using quantification kits (Triglyceride, free fatty acids and cholesterol: BioVision, Mountain View, CA, USA; adiponectin and IL-6: AbFrontier, Seoul, Korea) according to the manufacturer's instructions. Blood serum sample taken from transplanted mice were plated into 98 well culture dishes (Nunc), and then enzyme or reaction mix solution were added and mixed. Samples were incubated for 60 min and measured at OD 570nm for colorimetric assay in a microtiter plate reader.

11. Immunohistochemistry

Sixty days after transplantation, graft-bearing kidneys and pancreata were

removed from control and DC group. Tissues were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 4-um section, and permeated with 1% Triton X-100 in PBS (PBST) for 10 minutes. Then, tissue sections were immersed in 10 mM citrate buffer solution (pH 6.0) and placed in microwave for 20 minutes for antigen retrieval. For graft-bearing kidneys, sections were incubated in 1% BSA in PBS at room temperature for 2 minutes. Then, the sections were incubated overnight at 4°C with mixture of mouse anti-human nuclei monoclonal (1:100; Chemicon, Temecula, CA, USA) and rabbit anti-insulin polyclonal antibodies (1:100; Abcam) in PBST containing 1% BSA. After washing, they were incubated with mixture of two secondary antibodies, Texas red-conjugated anti-rabbit sheep IgG (1:200; Abcam) and FITC-conjugated anti-mouse goat IgG (1:200; Abcam) for 1 h at RT. Cell nuclei were visualized by incubating for 1 min with 1 μg/ml 4'-6' diamidino-2-phenylindole (DAPI) in PBS. Fluorescence imaging was visualized by confocal microscope (Nikon, Tokyo, Japan). Sections from pancreata were incubated in 0.1% horse serum at room temperature for 20 minutes. Then the sections were incubated with anti-mouse insulin monoclonal antibody (1:100; Abcam) for 1 h at RT, biotin-labeled anti-mouse/rabbit house antibody and then horseradish peroxidase-conjugated streptavidin (Vectastatin ABC Kit). Counterstaining was done with Mayer's hematoxylin. Imaging was visualized by microscope (Axioskop2+).

12. Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was analyzed by non parametric analysis because most of the variables were not normally distributed. The Kruskal-Wallis multiple comparison nonparametric test was performed and post hoc was applied determine individual differences between means. A p value < 0.05 was considered to be statistically significant. Survival curves were prepared using the Kaplan-Meier method and analyzed for a significant difference by the log-rank Mantel-Cox test using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA).

III. RESULTS

1. Isolation and differentiation of hEA into insulin-secreting cells in vitro

After 3 weeks of culture, plastic-adherent cells with unique morphology were isolated from human eyelid adipose tissues, and hEA at passage 2 and passage 14 expressed stem cell-specific genes including OCT4, Rex1, and stem cell factor (SCF), endoderm-specific genes of bone morphogenetic protein 4 (BMP4), alpha-fetoprotein (AFP), GATA4, and hepatocyte nuclear factor 4 alpha (HNF4A) , and mesoderm/endoderm-specific genes like vimentin (VIM) and cytokeratin 18 (CK18) (Figure 1). However, they did not express mesoderm-specific genes of brachyury (T) or endoderm-specific gene of bone morphogenetic protein 2 (BMP-2) (Figure 1).

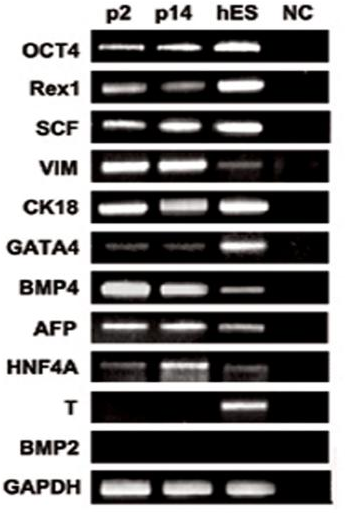


Figure 1. mRNA expression of stem cell-specific genes by hEA and hES. p2,

hEA at passage 2; p4, hEA at passage 4; hES, human embryonic stem cell; NC, negative control; SCF, stem cell factor; VIM, vimentin; CK18, cytokeratin 18; BMP4, bone morphogenetic protein-4; AFP, alpha-fetoprotein; HNF4A, human nuclear factor 4 alpha; T, brachyury.

After 3 weeks of culture in IS1 differentiation medium, differentiated hEA formed islet-like aggregates, and genetic analysis by RT-PCR showed various beta cell related genes, including glucokinase (GCK), insulin gene enhancer protein (ISL1), human nuclear factor 4 alpha (HNF4A), paired box gene 4 (PAX4), neurogenic differentiation 1 (NEUROD1), neurogenin-3 (NEUROG3), glucose transporter 1 and 2 (GLUT1, GLUT2), proprotein convertase 1 (PC1/3), PC2, homeobox protein Nkx-6.1, pancreatic and duodenal homeobox 1 (PDX1), and insulin (INS) (Figure 2-A). Immunocytochemistry showed marked expressions of human insulin and c-peptide in hEA-ISC compared to hEA and negative control, which was treated with secondary antibodies without primary antibodies of anti-human insulin or anti-human c-peptide (Figure 2-B). ELISA analysis showed increased insulin and c-peptide levels secreted by hEA-ISC compared to hEA in high glucose concentration (25 mM) media (Figures 2-C).

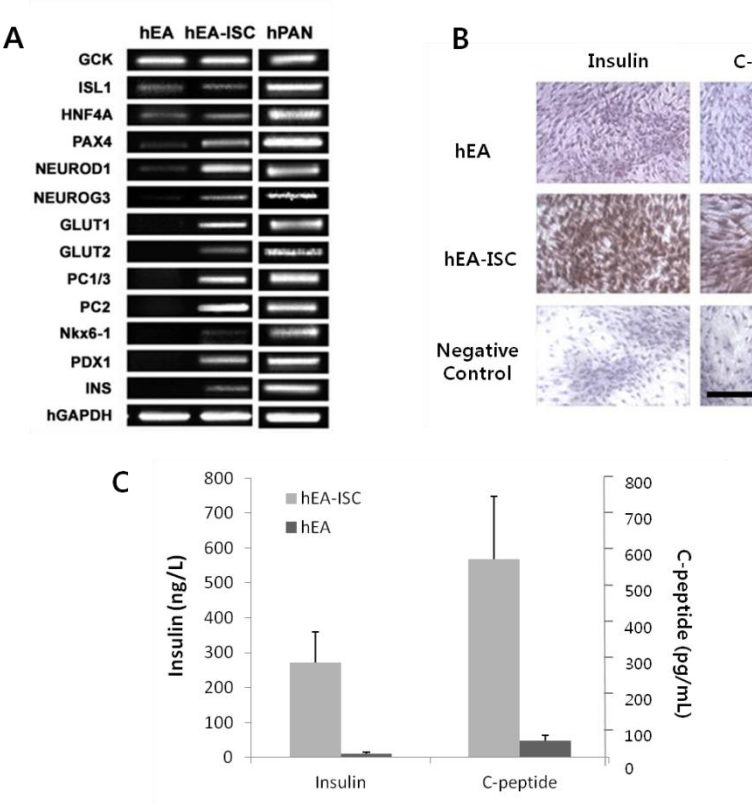


Figure 2. Differentiation of hEA at passage 3 into insulin-secreting cells in vitro. After differentiation, cells were analyzed for expression of beta cell-related genes (A) and immunocytochemistry (B, x100, Scale bar = 200 μm). ELISA for insulin and c-peptide secretion in high glucose concentration (25 mM) by hEA and hEA-ISC (C). hPAN, human pancreas total RNA; GCK. glucokinase; ISL1, insulin gene enhancer protein; HNF, hepatic nuclear factor; PAX4, paired box gene 4, NEUROD1, neurogenic differentiation 1; NEUROG3, neurogenin-3; GLUT, glucose transporter; PC1/3, proprotein convertase 1; PC2, proprotein convertase 2; PDX1, pancreatic and duodenal homeobox 1; INS, insulin. Data are shown as mean ±SEM.

2. Establishment of type 2 diabetes mouse model with insulin resistance

Type 2 diabetes mouse model, characterized by insulin resistance and relative insulin deficiency was induced by a low dose streptozotocin followed by a high fat diet. There was a gradual increase in blood glucose level and body weight compared to normal control group and group that received low-dose streptozotocin only (Figure 3). Also, after 5 weeks of HFD, serum insulin levels of STZ/HFD were higher than those of STZ only mice and lower than control mice, indicating insulin resistance (Figure 4). Free fatty acid and triglycerides levels were significantly higher in the STZ/HFD mice compared to control mice (both $P < 0.05$, Figure 4). Insulin tolerance test was performed on all STZ/HFD mice to ensure the insulin resistance component of type 2 diabetes, and these mice were categorized into two groups according to Kitt value calculated from insulin tolerance test. Mice with Kitt value of less than 5% were arbitrarily defined to have insulin resistance, and thus these mice were selected for transplantation. They showed a blunted response to short acting insulin (Figure 5).

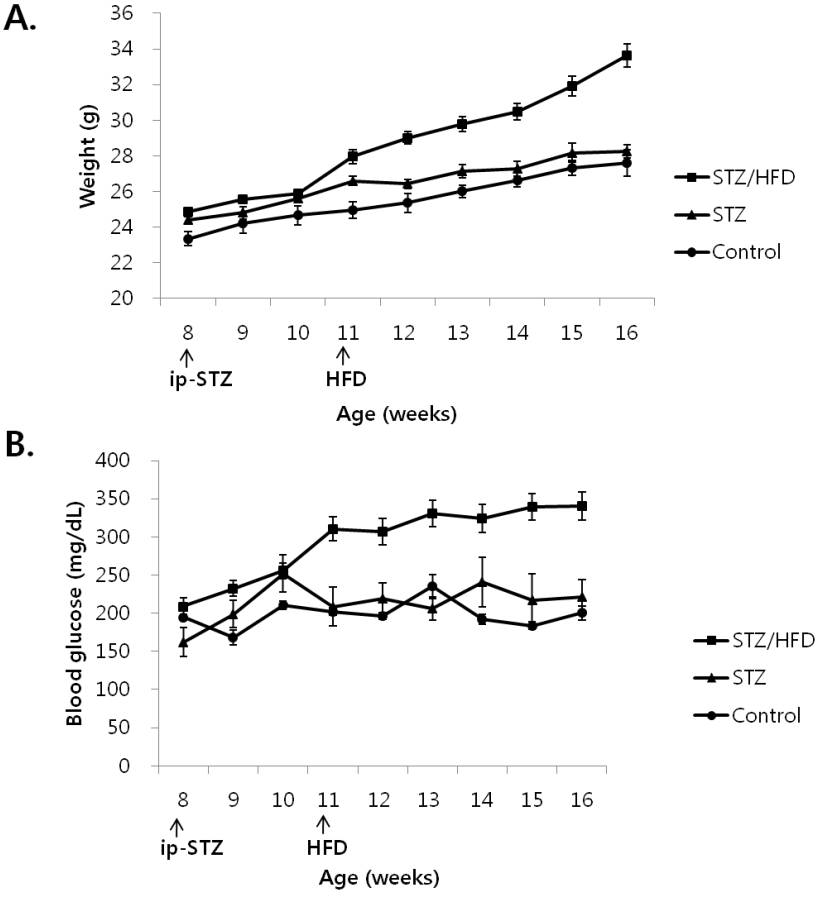


Figure 3. Body weight and blood glucose levels of mice treated with low dose streptozotocin and high fat diet. STZ/HFD mice were more obese (A) and hyperglycemic (B) compared to STZ only and control mice. ip-STZ, intraperitoneal streptozotocin; HFD, high fat diet; STZ/HFD, mice that received both low dose STZ and high fat diet; STZ, mice that received low dose STZ only; Control, control mice. Data are shown as mean ± SEM.

A.

	mouse insulin (ng/mL)	FFA(mM)	TG(nM)	BG(mg/dL)	wt(g)
Control	3.1 ± 0.5	1.2 ± 0.1	0.3 ± 0.1	210 ± 6.5	28.1 ± 1.3
STZ	1.9 ± 9.6	1.3 ± 0.1	0.4 ± 0.1	221 ± 23	28.3 ± 0.4
STZ/HFD	2.2 ± 0.8	1.8 ± 0.1	1 ± 0.2	407 ± 55	31.4 ± 1.6

B.

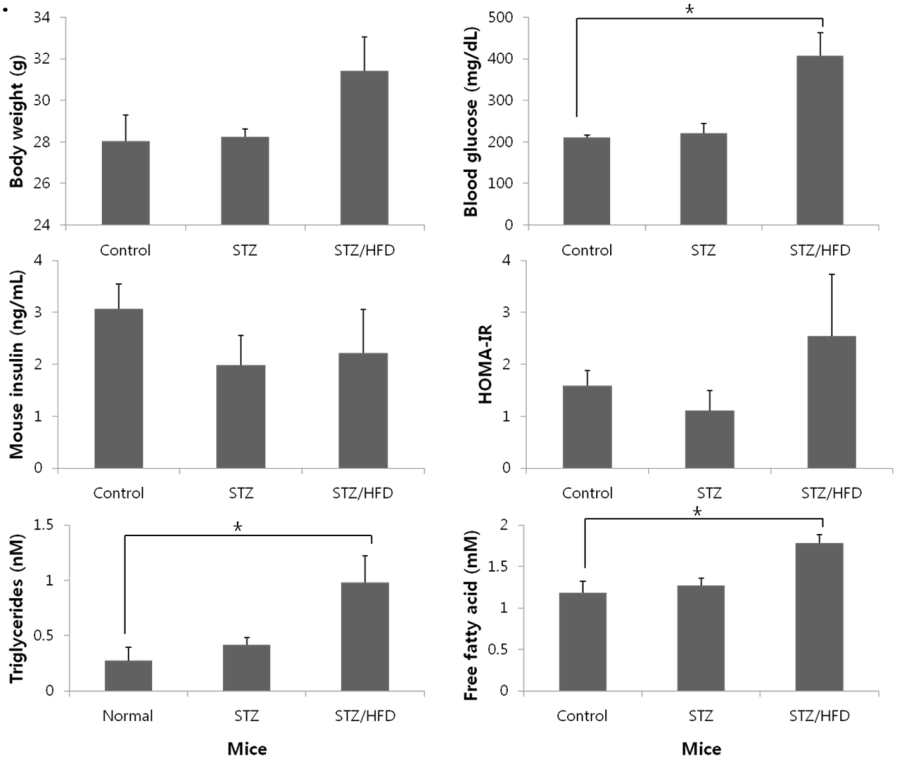


Figure 4. Biochemical profiles of mice that received low dose STZ only (STZ), mice that received both low dose STZ and high fat diet (STZ/HFD), and normal control. FFA, free fatty acid; TG, triglycerides; BG. Blood glucose, wt; body weight. Data are shown as mean ± SEM, *$P < 0.05$

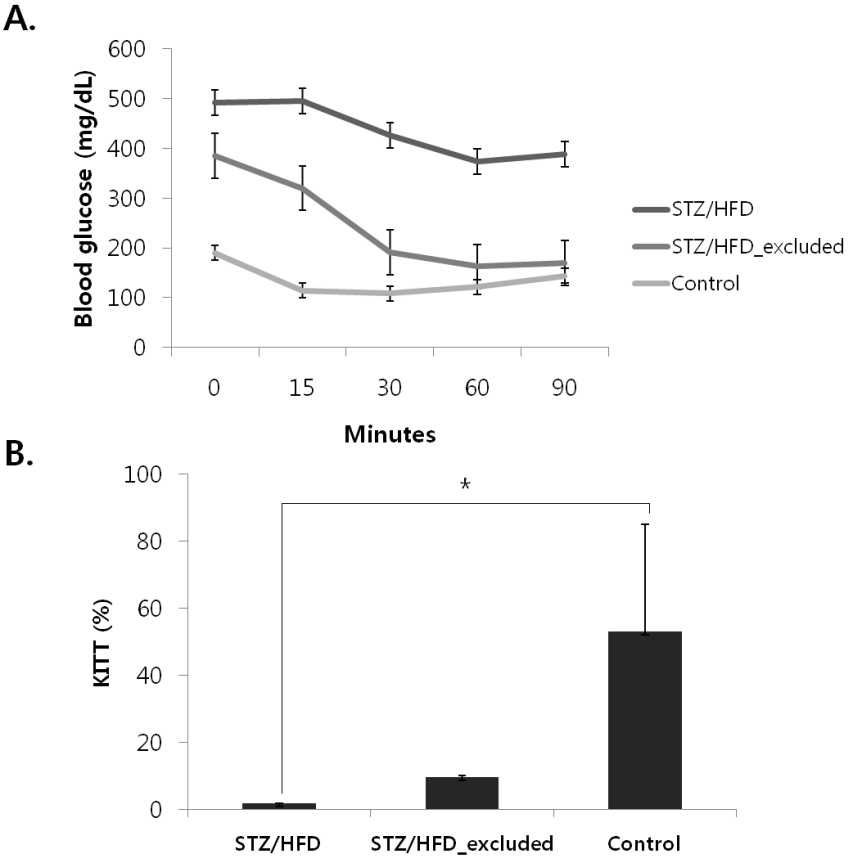


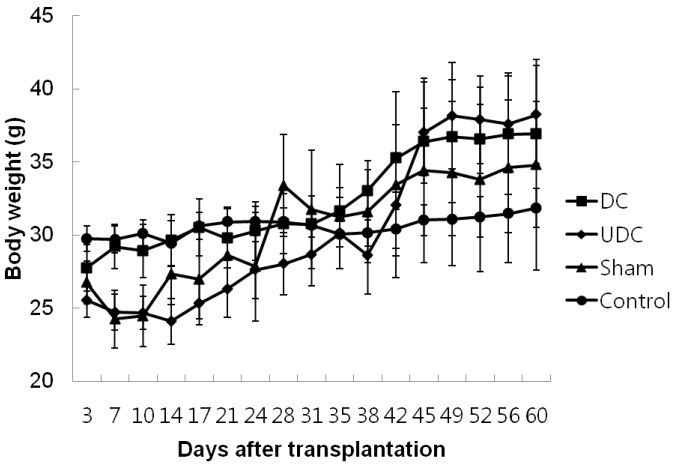
Figure 5. Intraperitoneal insulin tolerance test of mice that received streptozotocin and high fat diet. STZ/HFD are mice with a relative insulin resistance, defined by Kitt of less than 5% and STZ/HFD_excluded are mice with a relatively preserved insulin sensitivity with Kitt of greater than or equal to 5% and thus excluded from transplantation. STZ/HFD group shows a blunted response to short acting insulin (A) and there was a significant difference in Kitt value between STZ/HFD and control mice (B). Data are shown as mean ± SEM, *$P < 0.05$.

3. The Effect of transplantation of hEA-ISC into type 2 diabetes mice

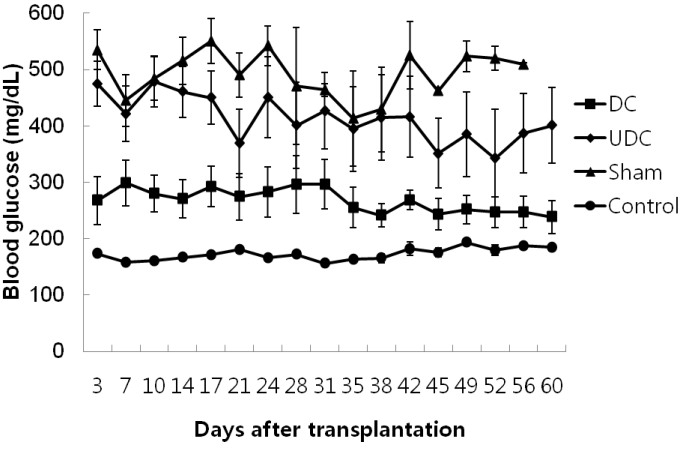
A. Body weight, blood glucose level, glucose tolerance, and survival

After transplantation, there were no significant differences in body weight of mice that received hEA-ISC (DC group), hEA (UDC group) and sham-operation (sham group) (Figure 6-A). Meanwhile, a significant lowering of blood glucose level was observed in DC group compared to UDC and sham group at 3 days following transplantation and it was maintained until 60 days when the mice were sacrificed (Figure 6-B). Intra-peritoneal glucose tolerance test showed an improved glucose tolerance in DC group compared to UDC and sham group. It was more prominent when subgroup analysis was done after dividing the DC group into responders whose mean glucose was maintained below 250 mg/dL throughout the follow-up period, and non-responders whose mean glucose level was above 250 mg/dL (Figure 6-C). Among 15 mice in DC group, 3 mice died within 30 days following transplantation and 2 within 60 days. From 15 mice in UDC group, 5 died within 30 days and 3 within 60 days. From sham group (n = 8), 6 died within 30 days, and the remaining 2 died before 60 days, and all 8 control mice survived until the end of the study (Figure 6-D).

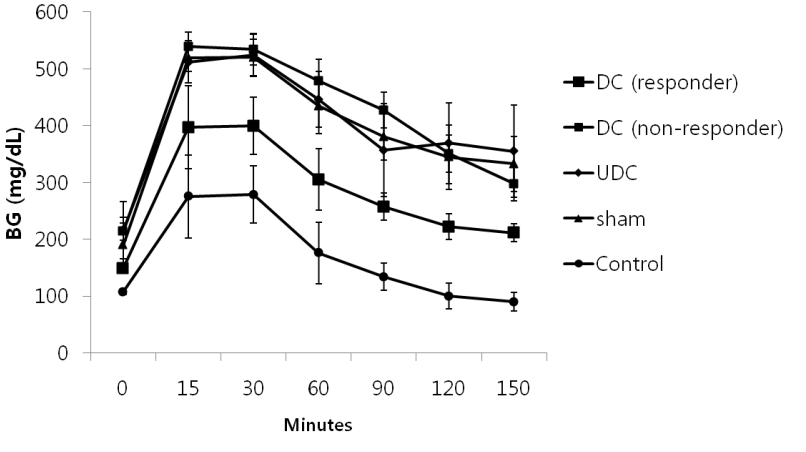
A.


Body weight (g)
45
40
35
30
25
20
3 7 10 14 17 21 24 28 31 35 38 42 45 49 52 56 60
Days after transplantation
DC
UDC
Sham
Control

B.


Blood glucose (mg/dL)
600
500
400
300
200
100
0
3 7 10 14 17 21 24 28 31 35 38 42 45 49 52 56 60
Days after transplantation
DC
UDC
Sham
Control

C.


BG (mg/dL)
600
500
400
300
200
100
0
0 15 30 60 90 120 150
Minutes
DC (responder)
DC (non-responder)
UDC
sham
Control

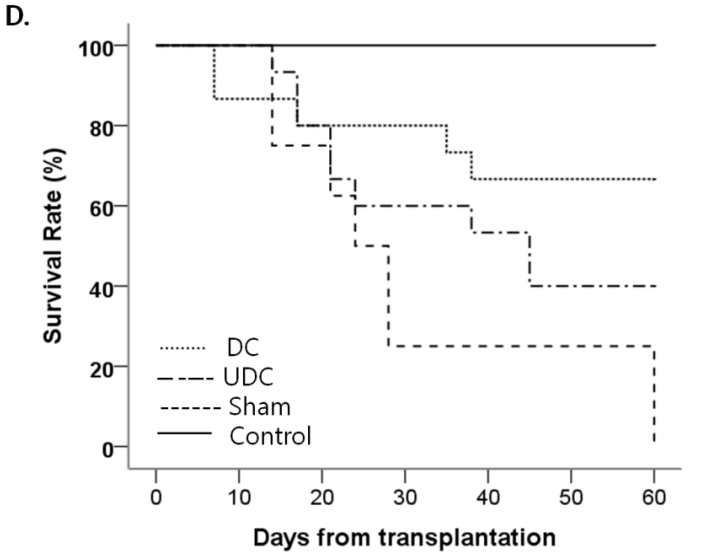


Figure 6. The effect of transplantation of hEA-ISC and hEA and sham operation on type 2 diabetes mice. hEA-ISC transplantation had no effect on body weight (A), but showed favorable results on blood glucose levels (B), glucose tolerance (C), and survival (D). DC, differentiated cell group; DC (responder), responders of DC group; DC (high), non-responders of DC group ; UDC, undifferentiated cell group; sham, sham-operated group.

B. Plasma human and mouse insulin and c-peptide levels

At the time of sacrifice at 60 days after transplantation, DC group showed significantly increased levels of human insulin ($P < 0.001$) and human c-peptide ($P < 0.001$) compared to those of UDC, sham, and control group (Figure 7-A). Meanwhile, no significant differences were observed in mouse insulin and mouse c-peptide levels (Figure 7-B). When total insulin, combining human and

mouse insulin levels, and total c-peptide, combining human and mouse c-peptide levels were compared, there were no differences among the groups (Figure 7-C).

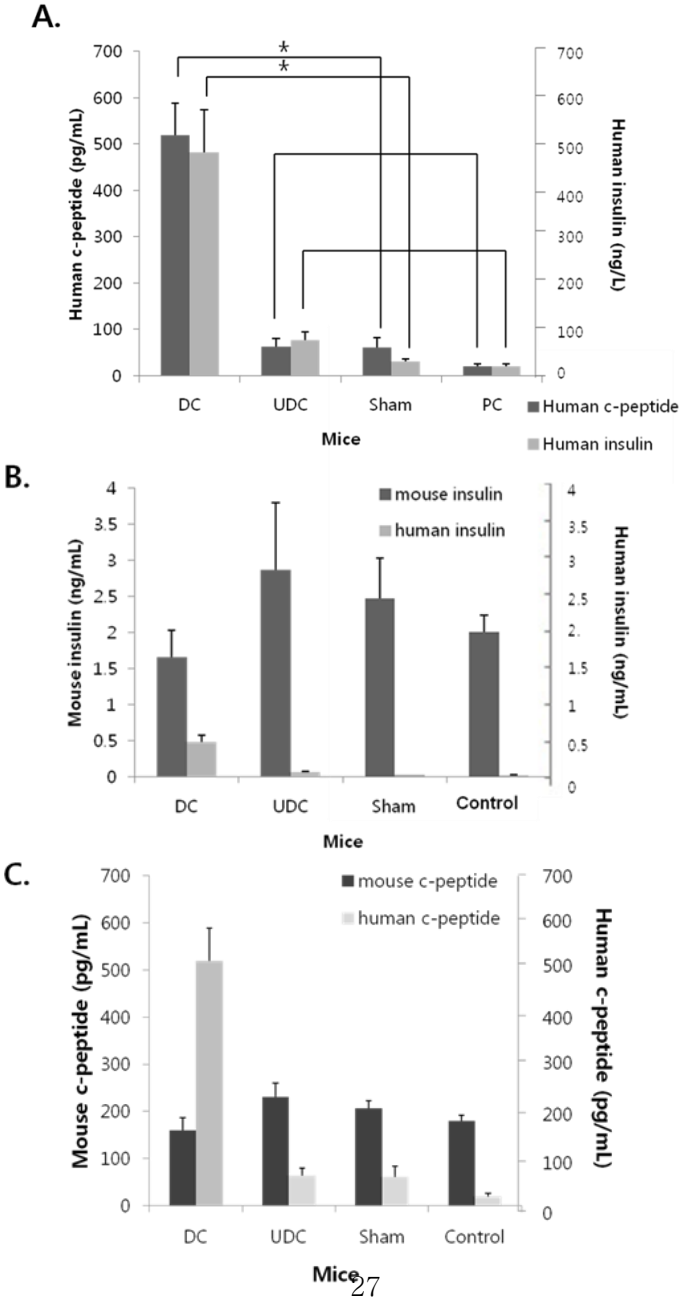


Figure 7. The effect of transplantation of hEA and hEA-ISC on human and mouse insulin and c-peptide levels. Transplantation of hEA-ISC led to increased human insulin and c-peptide levels (A), mouse insulin levels (B), and mouse and c-peptide levels (C) in type 2 diabetes mice. *$P < 0.001$.

C. Human gene expression in kidneys of transplanted mice

Kidneys of DC group at 60 days (DCK1) and 210 days (DCK2) post transplantation showed the expression of human genes including insulin gene enhancer protein (ISL1), glucokinase, pancreatic and duodenal homeobox 1 (Pdx1), homeobox protein Nkx-2.2 (Nkx2.2), neurogenin-3 (NeuroG3), neurogenic differentiation 1 (NeuroD1), proprotein convertase 1 (PC1/3), proprotein convertase 2 (PC2), glucose transporter 1 (GLUT1), glucose transporter 2 (GLUT2), insulin, and glucagon. In contrast, none of these human genes were expressed in normal mouse kidney (NorK) (Figure 8).

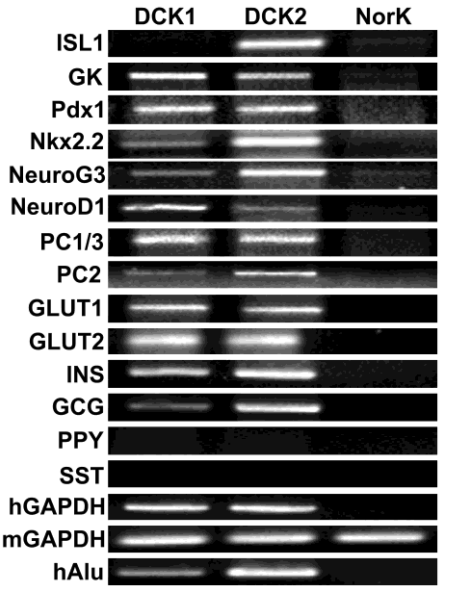


Figure 8. mRNA expressions of human genes in kidneys of normal and DC mice. DCK1, kidney of mouse with hEA-ISC transplantation at 60 days after transplantation; DCK2, kidney of mouse with hEA-ISC transplantation at 210 day after transplantation; Nork, normal mouse kidney; ISL1, insulin gene enhancer protein; GK, glucokinase; Pdx1, pancreatic and duodenal homeobox 1; Nkx2.2, homeobox protein Nkx-2.2; NeuroG3, neurogenin-3; NeuroD1, neurogenic differentiation 1; PC1/3, proprotein convertase 1; PC2, proprotein convertase 2;, GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; INS, insulin; GCG, glucagon; PPY, pancreatic polypeptide; SST, somatostatin; hGAPDH, human glyceraldehydes-3-phosphate dehydrogenase; mGAPDH, mouse glyceraldehydes-3-phosphate dehydrogenase; hAlu, human Alu element.

D. Immunohistochemistry of kidneys and pancreas

H&E staining demonstrated human cells in kidneys of mice in DC group, and clear boundary was observed by the renal capsule (Figure 9-A). Many cells of kidneys showed co-localization when triple-labled with DAPI, human nuclear antigen (hNUCLEI), and human insulin (hINS) (Figure 9-B). In pancreas of mice in DC group, insulin staining islets were diminished in size as well as in number compared to pancreas of a normal mouse (Figure 9-C).

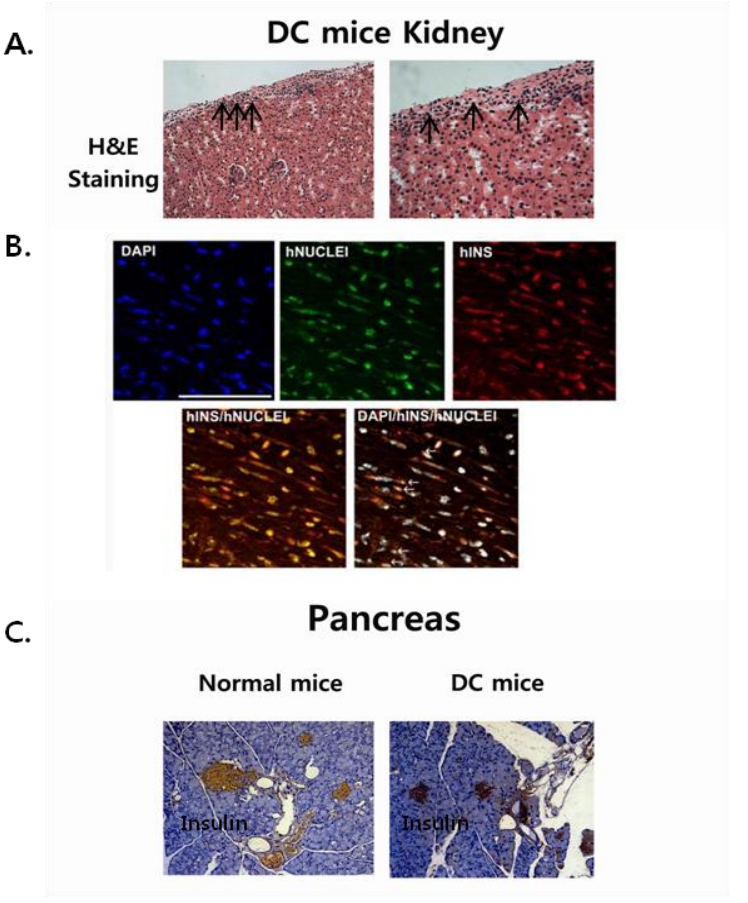


Figure 9. Immunohistochemistry of kidneys and pancreata of mice from control and DC group. H&E stained kidneys of DC mice, arrow indicating the boundary between human and mouse cells (A, x400); kidneys of the same mice triple-labled with DAPI, hNUCLEI and hINS (B, x600, scale bar = 100um); Pancreata of normal and DC mice stained with INS (C, x400). H&E, hematoxylin and eosin statin; DAPI, 4',6-diamidino-2-phenylindole; hNUCLEI, human nuclear antigen; hINS, human insulin.

E. Metabolic parameters and interleukin-6 (IL-6)

Metabolic and anti-inflammatory effects of transplantation were assessed 60 days after transplantation. DC and UDC groups showed a significant decrease in IL-6 compared to sham group (both $P < 0.05$), and DC group showed a tendency toward lower triglyceride and free fatty levels compared to sham group ($P = 0.093$ and 0.065, respectively) and these values were comparable to those of control group (Figures 10). There was no difference in serum adiponectin level in DC group compared to sham group.

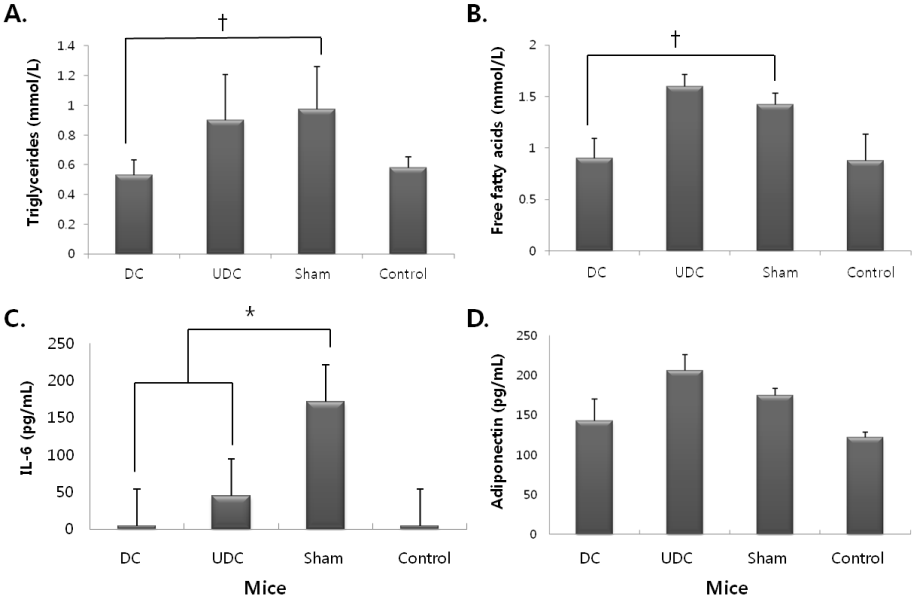


Figure 10. Metabolic parameters and IL-6 after transplantation. Triglycerides (A), free fatty acids (B), IL-6 (C), adiponectin levels (D) at 60 days after transplantation. DC, differentiated cell group; UDC, undifferentiated cell group; Sham, sham-operated group. *$P < 0.05$, †$P < 0.1$.

IV. Discussion

Ultimately, majority of type 2 diabetes patients, who account for more than 90-95% of all diabetes, experience beta cell exhaustion, and many require insulin therapy[29], which has been an only therapeutic option. However, these days, pancreas transplantation for type 2 diabetes has become an emerging concept and likewise, cell therapy for type 2 diabetes is gaining highlights[1,30]. In this study, we assessed the efficacy of transplantation of insulin secreting cells differentiated from human adipose tissue-derived stem cells into type 2 diabetes mouse model, which are readily applicable to humans for its low teratogenic potential and ethnic concerns.

In our previous work done with type 1 diabetes mice, transplantation of hEA-ISC was effective in normalizing blood glucose level and prolonging the survival[17]. In type 2 diabetes model, it did not completely normalize the blood glucose level, but significantly lowered the glucose level compared to UDC and sham-operated groups. Considering the insulin resistance part of the pathogenesis of type 2 diabetes, it was somewhat expected. Among 10 mice that survived until 60 days after transplantation, only 1 mouse had the average glucose level of 179 mg/dL, which was comparable to that of the normal control group. Meanwhile, 8 mice maintained a mean glucose level between 200 to 250 mg/dL, which was slightly higher than the mean glucose level of control group (214.4 ± 20.8 vs. 172.7 ± 10.6, $P < 0.01$). Two mice that died within 7 days after

transplantation seem to have died from procedure-related complication or possibly from graft-versus-host reaction. The other 3 that died between 20 and 60 days after transplantation had mean glucose levels of above 500 mg/dL, and we consider them to be a graft-failure group. Compared to 50% success rate of type 1 diabetes[17], 9 out 15 type 2 diabetes mice showed an alleviation of glucose level following transplantation.

In previous studies conducted on type 2 diabetes with undifferentiated cells, transplantation of undifferentiated cells lowered blood glucose level by increasing plasma insulin level[26,27]. Therefore, we thought that providing insulin secreting cells would be more effective in restoring the depleted circulating insulin, and our results demonstrated superior glucose lowering effect of differentiated cells compared to that of undifferentiated cells, which had a minimal or no glucose lowering effect nor survival benefit. As expected, human insulin and human c-peptide levels were much higher in the DC group, but when insulin and c-peptide levels of mouse and human were combined, total levels were not different among the groups.

While type 1 diabetes is almost completely dependent on exogenous insulin to survive, type 2 diabetes is more complex with various metabolic derangements related to insulin resistance and a relative insulin deficiency[2,3]. A significant improvement in glucose level in DC group, without a complimentary increase in total plasma insulin level, implicates that increased serum insulin level may not be the only factor in lowering glucose level in DC mouse.

These days, there are clear evidences that recognize the critical role of chronic inflammation in the development of type 2 diabetes[31,32]. For example, obese adipose tissue was shown to activate CD8T cells, which results in the activation of macrophages in adipose tissue[33], which then infiltrates into adipose tissue[32]. Adipocytes regulate and mediate inflammatory cytokines including tumor necrosis factor-alpha, IL-6, and free fatty acids, which interact with each other to affect insulin resistance[34]. Therefore, inflammatory signals interfaced with metabolic disorders are considered new therapeutic targets for metabolic diseases[32]. Meanwhile, a recent study showed a reduced blood glucose level accompanied by a reduction in IL-6 and IL-1ß and increased plasma adiponectin and insulin sensitivity in db/db mouse after intra-bone marrow-bone marrow transplantation using bone marrow cells of normal mice[35]. The improved adiponectin level and insulin sensitivity were associated with increases in pAKT and pAMPK expression on liver and pancreas[35].

Therefore, in addition to an increment in circulating insulin level, we hypothesized that hEA-ISC transplantation provide metabolic or anti-inflammatory benefits that would alleviate insulin resistance, and assessed several metabolic and inflammatory mediators. As a result, IL-6 was significantly reduced in DC group compared to that of sham group, and triglyceride and free fatty acid levels were also decreased in DC group compared to sham group with a marginal significance. Not only DC, but UDC group also showed a significantly lower IL-6 compared to sham group.

Considering the fact that in hEA-ISC, only about 10% are purely differentiated cells, and the rest are undifferentiated cells, our results support the previous studies which suggested that the transplantation of undifferentiated, mesenchymal stem cells have favorable effect on metabolic, inflammatory parameters via paracrine effects on adipocytes and macrophages[34,35]. It can be speculated that the increased insulin levels combined with reduced IL-6 level and metabolic mediator associated with insulin resistance led to the lowering of blood glucose level in DC group.

However, our results do not imply that stem cell transplantation directly had favorable effects on these parameters, and consequently lowered the blood glucose level. There is a matter of the chicken or egg question. These changes may be the consequence of the improved glucose level, or maybe the other way around. Moreover, adipose-derived stem cell transplantation was shown to decrease the expression of caspase-12, which mediates endoplasmic reticulum (ER) stress-induced apoptosis in neuronal cells[36]. It would be interesting to assess the ER stress markers after transplantation in the future.

Immunocompetent mice were used in this study without any immunosuppressive agents. Immunomodulatory properties of mesenchymal cells have been studied extensively. Initially, bone marrow mesenchymal cells have been shown to have immunosuppressive effects on MHC-mismatched lymphocytes proliferation and can inhibit various immune cells T cell[37]. Recently, adipose tissue-derived stem cells were shown to have similar

immunomodulatory effects[19], and successfully inhibited graft versus host disease in mice transplanted with haploidentical hematopoietic grafts[38]. Likewise, hEA and hEA-ISC did not express HLA-DR, HLA-DM, CD80, and CD86 genes[17], which are known to play key roles in T-cell mediated response. Since we did not assess the immune reaction, we can only assume that there was no significant immune reaction at least in the mice that maintained a good glucose control for 60 days. A further study in the immune characteristics of the hEA and hEA-ISC, and the in vivo immune reactions after transplantation is warranted.

There are several limitations to this study. First, for study animals, instead of using genetically manipulated type 2 diabetes mouse models, we established type 2 diabetes by injecting a low dose streptozotocin followed by a high fat diet based on previous studies[28,39], and therefore there remains a concern for the validity of the animal model. However, we assessed the beta cell dysfunction and insulin resistance by measuring serum insulin level and conducting insulin tolerance test, and selected mice with hyperglycemia and insulin resistance. Secondly, the duration of follow-up period was relatively short. However, at the time of sacrifice for serum and IHC analysis at 60 days following transplantation, we saved 1 DC mouse, which was maintained until 210 days. Its glucose level was maintained below 250 mg/dL at the time of sacrifice and its RT-PCR of the transplanted kidney showed expression of human genes.

Type 2 diabetes is a very complex disease caused mainly by insulin resistance

and relative insulin depletion, but also intermingled by inflammation, autoimmunity, ER stress, and other unknown factors. Our data show that adipose tissue-derived insulin secreting cell-based therapy is effective in ameliorating hyperglycemia by restoring circulating insulin level and possibly attenuating metabolic derangements.

V. Conclusion

These results demonstrate that cell therapy using differentiated hEA-ISC is effective in lowering blood glucose level in type 2 diabetes mouse model by increasing circulating insulin level and it also has a favorable effect on metabolic parameters and inflammatory markers. The cause and effect relationship remains to be determined.

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ABSTRACT (IN KOREAN)

사람 지방유래 성체줄기세포의 인슐린 분비세포로의 분화 및 이식이 제2형 당뇨병 생쥐모델에 미치는 영향

<지도교수 안철우>

연세대학교 대학원 의학과

남 지 선

최근 제2형 당뇨병의 발병기전에 있어서 패러다임의 변화가 일어나고 있다. 이전에는 말초조직에서의 인슐린 저항성이 주요 기전으로 생각되었으나 점차 췌장 베타세포 기능부전의 중요성이 부각되고 있다. 현재까지 인슐린 저항성을 호전시키는 경구용 약제들이 많이 개발되어 사용 중이나 아직까지 당뇨병 환자에서 감소된 베타세포의 부피 혹은 기능을 복구시킬 수 있는 치료법은 매우 제한되어 있다.

본 연구에서는 배아줄기세포, 태아줄기세포, 유도만능줄기세포, 성체줄기세포 중 세포치료제의 재료로 기형종 발생 위험이나 윤리적인 문제가 적어 바람직하다고 알려져 있는 성체줄기세포를 대상으로 연구하였으며, 세포를 얻기 위해 침습적인 처치가 필요 없는 방법으로 지방조직을 얻어 줄기세포(hEA)를 분리한 뒤 이를 인슐린분비세포 (hEA-ISC)로 분화시킨 후 이를 제2형 당뇨병 생쥐모델에 이식하였다. 이식이 혈당, 생존율, 및 여러 대사 지표 및 염증관련 지표에 미치는 영향에 대해 알아보았다.

지방조직유래 줄기세포를 사람의 눈밑지방조직으로부터 분리하여 ISI 분화액에서 3주 간 분화배양하여 hEA-ISC를 얻었으며 1 x 10^6 세포들을, 저용량 streptozotocin 주사 후 5주일 간의 고지방식이를 하여 성립한 제2형 당뇨병 생쥐의 신피막에

이식하였다. 실험군으로 hEA-ISC 이식군 (분화세포군, DC 군)과 hEA 이식군 (미분화세포군, UDC 군)으로 나누고 대조군으로 sham 수술군 (sham 군) 과 정상대조군 (control 군)을 사용하였다.

연구 결과, DC 군에서 이식 후 3일째부터 혈당이 정상화되지는 않았으나, UDC와 sham 군에 비하여 의미 있게 감소하였으며, 이는 관찰기간인 60일 간 지속되었다. 당부하검사 결과 비슷한 양상을 나타내었다. DC 군에서 생존율의 증가를 보였으며, 60일까지 관찰한 결과 15마리 중 10마리가 생존하였으며, UDC 군에서는 15마리 중 6마리가 생존하였고, sham 군에서는 8마리 모두 그 전에 사망하였다. DC 군의 혈청 내 사람 인슐린 및 c-peptide 수치가 나머지 군에 비해 의미 있게 증가되어 있었고, DC 군의 이식신에서 INS, GLUT1, GLUT2, NeuroD1, Pdx1 및 GK와 같은 사람 유전자가 발현되었다. 또한, 면역조직화학검사 결과 이식신에서 사람과 생쥐 세포가 공존하고 있었으며, 사람 insulin (hINS), human nuclear antigen (hNUCLEI) 및 DAPI로 염색한 결과 같은 세포 내에 공존하는 것이 관찰되었다. 이식 후 대사지표들을 측정하였으며, DC와 UDC 군에서 sham 군에 비해 혈청 IL-6 수치가 통계적으로 유의하게 감소되어 있었으며 ($P < 0.05$), 중성지방 및 free fatty acid 수치가 DC 군에서 sham 군에 비해 감소하는 경향을 보였다 (각각 $P = 0.093$ 과 0.065, 순서대로).

hEA-ISC를 이용한 세포치료는 제2형 당뇨병 생쥐모델에서 의미있는 혈당강하효과를 나타내며, 생존율을 향상시킨다. 그 기전으로 혈중 인슐린 수치의 증가와 더불어 대사 및 염증관련 지표에도 긍정적인 역할을 하는 것으로 생각되며, 정확한 인과관계 및 기전에 대해서는 추후 연구 되어야 할 것이다.

핵심되는 말 : 지방조직유래 줄기세포, 인슐린 분비세포, 제2형 당뇨병, 세포치료