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

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ABSTRACT

Currently, there are limited ways to preserve or recover insulin secretory capacity in human pancreas. We evaluated the efficacy of cell therapy using insulin-secreting cells differentiated from human eyelid adipose tissue-derived stem cells (hEAs) into type 2 diabetes mice. After differentiating hEAs into insulin-secreting cells (hEA-ISCs) in vitro, cells were transplanted into a type 2 diabetes mouse model. Serum levels of glucose, insulin and c-peptide were measured, and changes of metabolism and inflammation were assessed in mice that received undifferentiated hEAs (UDC group), differentiated hEA-ISCs (DC group), or sham operation (sham group). Human gene expression and immunohistochemical analysis were done. DC group mice showed improved glucose level, and survival up to 60 days compared to those of UDC and sham group. Significantly increased levels of human insulin and c-peptide were detected in sera of DC mice. RT-PCR and immunohistochemical analysis showed human gene expression and the presence of human cells in kidneys of DC mice. When compared to sham mice, DC mice exhibited lower levels of IL-6, triglyceride and free fatty acids as the control mice. Transplantation of hEA-ISCs lowered blood glucose level in type 2 diabetes mice by increasing circulating insulin level, and ameliorating metabolic parameters including IL-6.

1. Introduction

Recently, there has been a shift in paradigm about the pathogenesis of type 2 diabetes in which beta cell dysfunction is considered to be a primary defect or at least non-inferior to insulin resistance in the pathogenesis of type 2 diabetes [1–3]. Various drugs have been shown to ameliorate insulin resistance, yet there are limited ways to preserve or restore beta cell function. Stem cell therapy is an attractive treatment option for its abundant source and its potential to acquire glucose-dependent insulin secretory function [4]. Adult stem cell, in particular, is good candidate for its safety in terms of tumorigenicity and ethical concerns compared to embryonic or induced pluripotent stem cells [5,6], and depending on the source of the cells, some are easily acquired without an invasive procedure. Moreover, they allow autologous transplantation, thereby circumventing the adverse effects of immunosuppression [6].

Human adult stem cells derived from various sources including bone marrow mesenchymal stem cells [7], peripheral blood monocytes [8] umbilical cord mesenchymal stem cells [9] 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 in preparing insulin-secreting cells [10]. These adipose tissue-derived stem cells can be easily obtained from human facial adipose tissue without an invasive procedure, and they exhibit good proliferation and multi-differentiation potential with high levels of stem cell-related antigens [11]. Also, a lack of HLA-DR expression and immunosuppressive properties of these cells have been reported [12],

Abbreviations: DC, differentiated cell; hEAs, human eyelid adipose-derived stem cells; hEA-ISCs, insulin secreting cells differentiated from human eyelid adipose-derived stem cells; UDC, undifferentiated cell.

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and a successful engraftment was shown in immunocompetent mice without immunosuppressive agents [10].

The efficacy of stem cell therapy in type 2 diabetes has been suggested by several studies. Mononuclear cells from human umbilical cord blood were shown to improve blood glucose levels and survival when transplanted into type 2 diabetic mice [13]. Human pancreatic beta-like cells derived from induced-pluripotent stem cell (iPS) also exhibited a reversal of hyperglycemia in mouse diabetic models of type 2 as well as type 1 [14]. Moreover, clinical trials demonstrated the efficacy and safety of stem cell transplantation using autologous bone marrow cells [15] and placenta-derived mesenchymal stem cells [16] in patients with type 2 diabetes. However, all these studies on type 2 diabetes were conducted with undifferentiated cells or differentiated iPS cells, and there is no prior study that transplanted differentiated adult stem cells.

Mounting evidence points to the involvement of chronic inflammation and immune dysfunction in the pathogenesis of type 2 diabetes [17], and adult stem cells were shown to have anti-inflammatory, immune modulatory effects [18,19]. In the present study, we transplanted insulin-secreting cells derived from human eyelid-derived adipose stem cells (hEA-ISCs) into immunocompetent type 2 diabetes mouse model, and assessed their effects on glucose level, metabolic profiles as well as surrogate markers of insulin resistance, inflammatory marker, and survival.

2. Materials and methods

2.1. Isolation and differentiation into hEA-ISCs

The human eyelid adipose tissue was obtained from nine subjects undergoing cosmetic surgery with informed consent. All experiments were approved by Institutional Review Boards of Seoul Women's University and Yonsei University. Human eyelid adipose-derived stem cells (hEAs) were obtained as described elsewhere [10]. Briefly, minced adipose tissue was reacted with 0.075% type I collagenase for 30 min at 37 °C with gentle stirring. Cell suspensions were cultured in DMEM-LG supplemented with 10% FBS at 5% CO_2, 37 °C. Medium was changed twice a week. Then, hEAs at passage 3–4 were induced into insulin-secreting cells using differentiation medium (IS1 kit, Bcellbio, Korea), for 21 days according to the manufacturer's instructions.

2.2. Immunocytochemistry

The cells cultivated in chamber slides were incubated with antigen specific antibodies followed by biotinylated 2nd antibodies, and horseradish peroxidase-conjugated streptavidin. Immunoreactivity was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with haematoxylin. Negative control staining was performed by omitting primary antibody.

2.3. Flow cytometric analysis

Cells were fixed with 10% formaldehyde for 10 min, then permeabilized with Phosphate-buffered Saline with Tween (PBST) for 15 min. After washing, cells were incubated with rabbit anti-human insulin antibody for 20 min at 4 °C, then labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody. Flow cytometry analyzes were performed using a FACS Caliber apparatus (Beckman Coulter).

2.4. Measurement of insulin and C-peptide

Cells were incubated with DMEM-LG containing 0.5% BSA for 12 h, washed with PBS, and then stimulated by DMEM-HG for 2 h at 37 °C. The amount of insulin and c-peptide in media was measured using the ultrasensitive human insulin and c-peptide ELISA kit (Mercodia). For the measurement of insulin and c-peptide levels in blood, mice fasted overnight were intraperitoneally injected with glucose (1.5 g/kg body weight), then blood sample collected by cardiac puncture at 30 min. Mouse insulin and c-peptide in serum were determined using mouse insulin ELISA kit (Mercodia) and c-peptide ELISA kit (Yanaihara).

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

RNA was isolated using Tri-reagent (Invitrogen) according to the manufacturer's instructions. Subsequent PCR reactions were performed using cDNA, primer pairs (Supplementary Table 1) and PCR mixture (Fermentas) 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.

2.6. Establishment of type 2 diabetes mouse model

Eight-week-old female C57BL/6 mice (Samtako Bio, Korea) were induced by a single intraperitoneal low dose streptozotocin (STZ, 120 mg/kg) followed by high fat diet (HFD) [20]. Three weeks after STZ injection, mice were fed on high-fat diet consisting (as a percentage of total kcal) of 45% fat, 35% carbohydrate and 20% protein (D12451; Research Diets). After 5 weeks of HFD feeding, mice were intraperitoneally injected with 0.5 units/kg of rapid-acting insulin (Humalog®; Lilly) under fasted condition for 4 h. Blood glucose levels were measured at 0, 15, 30, 60, and 90 min post-injection [21]. The rate constant for plasma glucose disappearance (K_{itt}) was calculated from following formula [22].

2.7. Transplantation into type 2 diabetes mice

Diabetes mice with glucose level of higher than 300 mg/dl and K_{itt} value of less than 5% were selected and randomly allocated to sham, hEAs, and hEA-ISCs group. Total 1.5×10^6 cells loaded into a PE50 tube were transplanted underneath the kidney capsule using a Hamilton syringe. Blood glucose level was measured under feeding condition weekly thereafter until 60 days, and 1 mouse that received hEA-ISCs was observed for 210 days.

2.8. Immunohistochemistry

The methods have been described previously [10]. Briefly, graft-bearing kidneys were embedded in paraffin and cut into 4-μm section. The sections were incubated with antigen specific antibodies followed by fluorescence conjugated 2nd antibodies and visualized under confocal microscopy (Nikon). Cell nuclei were visualized by DAPI. Pancreata sections were incubated with anti-mouse insulin monoclonal antibody. Then, cells were incubated with biotinylated 2nd antibody followed by horseradish peroxidase-conjugated streptavidin. Immunoreactivity was visualized using DAB and counterstained with haematoxylin.

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

Plasma free fatty acids, triglyceride, total cholesterol, adiponectin, and IL-6 levels were measured using quantification kits (Triglyceride, free fatty acids and cholesterol: BioVision; adiponectin and IL-6: AbFrontier) according to the manufacturer's instructions.

2.10. Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was analyzed by nonparametric 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).

3. Results

3.1. Isolation and differentiation of hEAs into insulin-secreting cells in vitro

Differentiated hEAs formed islet-like aggregates, and genetic analysis by RT-PCR showed various β-cell related genes (Fig. 1A). Expressions of GCK, ISL1, and HNF4A were expressed by both hEAs and hEA-ISCs to a similar degree while there were stronger expressions of PAX4 and NEUROD1 in hEA-ISCs compared to hEAs. GLUT2, PC1/3, PC2, Nkx6-1, PDX1, and INS genes were observed only in hEA-ISCs. Gene expressions of hEA-ISCs were comparable to those of human pancreas. Immunocytochemistry of hEA-ISCs showed marked expressions of human insulin and c-peptide in comparison with undifferentiated hEAs and negative control (Fig. 1B). Flow cytometry analysis revealed the insulinogenic differentiation efficiency of 13.1% (Fig. 1C). ELISA analysis showed that hEA-ISCs released 272.6 ± 86.3 pg/ml/5 × 10^4 cells of human insulin and 568.8 ± 180.0 pg/ml/5 × 10^4 cells of human c-peptide in response to 25 mmol glucose concentration whereas undetectable amounts were secreted from hEAs.

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

Beginning on the day of HFD feeding, STZ/HFD mice gained a greater body weight with a marginal significance and higher blood glucose level compared to control mice (Supplementary Fig. 1). After 5 weeks of HFD feeding, serum insulin level of STZ/HFD mice were lower than that of control mice, free fatty acid and triglyceride levels of STZ/HFD mice were significantly higher compared to those of control mice (both $p < 0.05$, Supplementary Fig. 2A). Mice with K_{itt} value of less than 5% showed a blunted response to short acting insulin (Supplementary Fig. 2B and C). These mice were arbitrarily defined to have insulin resistance, and used for transplantation experiment.

3.3. The effect of transplantation of hEA-ISCs into type 2 diabetes mice

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

Before transplantation, there was no significant difference in baseline body weight among mice that received hEA-ISCs

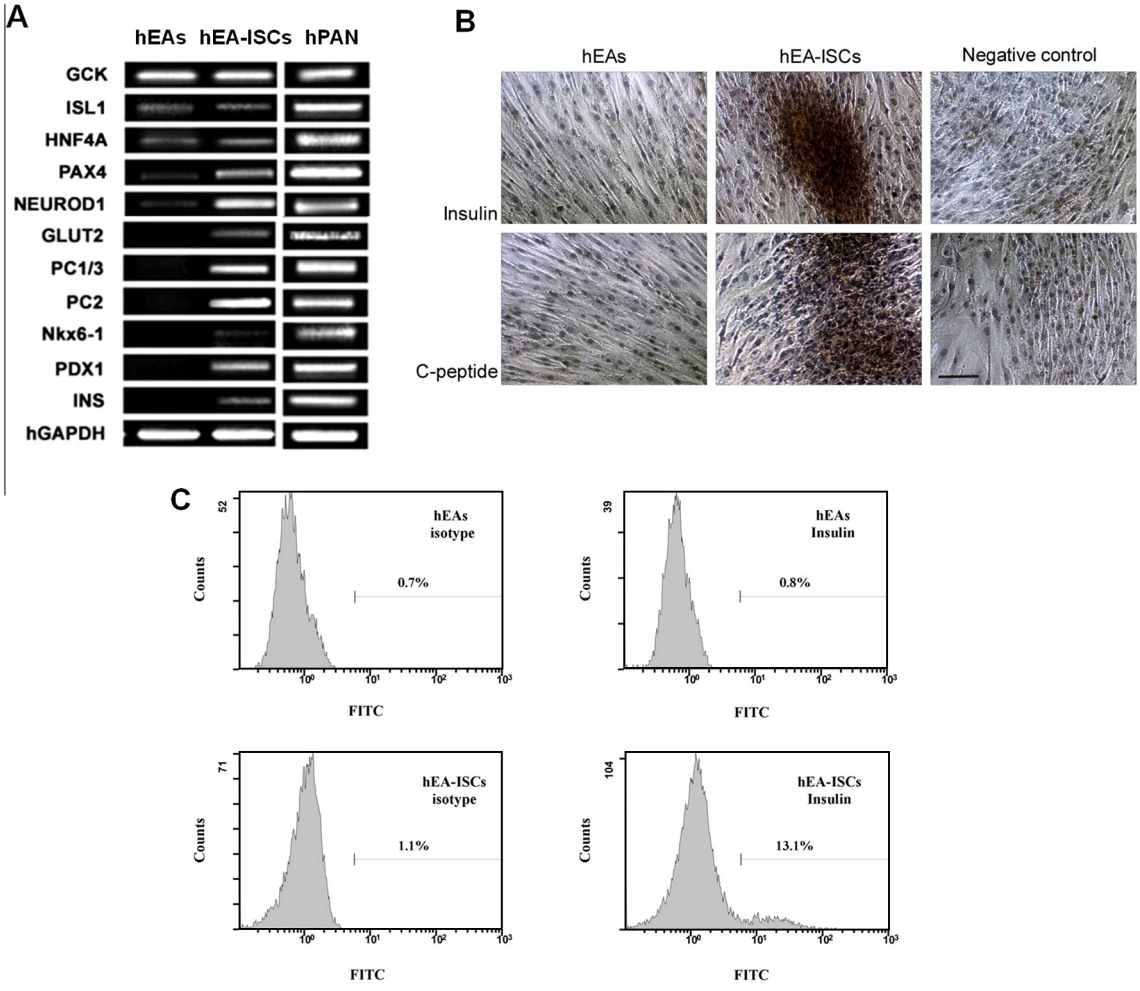

Fig. 1. Differentiation of hEAs at passage 3 into insulin-secreting cells in vitro. After differentiation, cells were analyzed for expression of β-cell-related genes (A), immunocytochemistry (B), (×100, scale bar = 200 μm), and differentiation efficiency of hEAs into hEA-ISCs by flow cytometric analysis (C).

(DC group), hEAs (UDC group), and sham-operation (sham group) (31.7 ± 2.2 vs. 28.9 ± 4.3 vs. 27.9 ± 3.7, respectively) and it was maintained throughout the follow-up period (Fig. 2A). Baseline blood glucose levels were also not significantly different among the DC, UDC, and sham groups (22.9 ± 6.9 vs. 25.6 ± 6.2 vs. 29.7 ± 3.8, respectively), and significant lowering of blood glucose level was observed in DC group compared to UDC and sham groups at 3 days following transplantation (Fig. 2B). Glucose levels were not normalized to the level of control group, but the lowered glucose level was maintained until 60 days (Fig. 2B). Of 15 mice in DC group, 3 mice died within 30 days following transplantation and 2 within 60 days. Of 15 mice in UDC group, 5 mice died within 30 days and 3 within 60 days. Of 8 mice in sham group, 6 died within 30 days, and the remaining 2 died within 60 days. All of 8 control mice survived until the end of the study (Fig. 2C).

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

After 60 days of transplantation, DC group mice showed the presence of significant amount of human insulin ($p < 0.001$) and human c-peptide ($p < 0.001$), 482.9 ± 91.5 and 519.3 ± 69.5 pg/ml, respectively, in their blood while those of UDC group mice showed little amount of both human proteins (Fig. 3A). In contrast, no significant difference was observed in mouse insulin and c-peptide levels among the experimental groups (Fig. 3B). Results showed that the summation of human and mouse c-peptide levels is significantly higher in DC group compared to other groups.

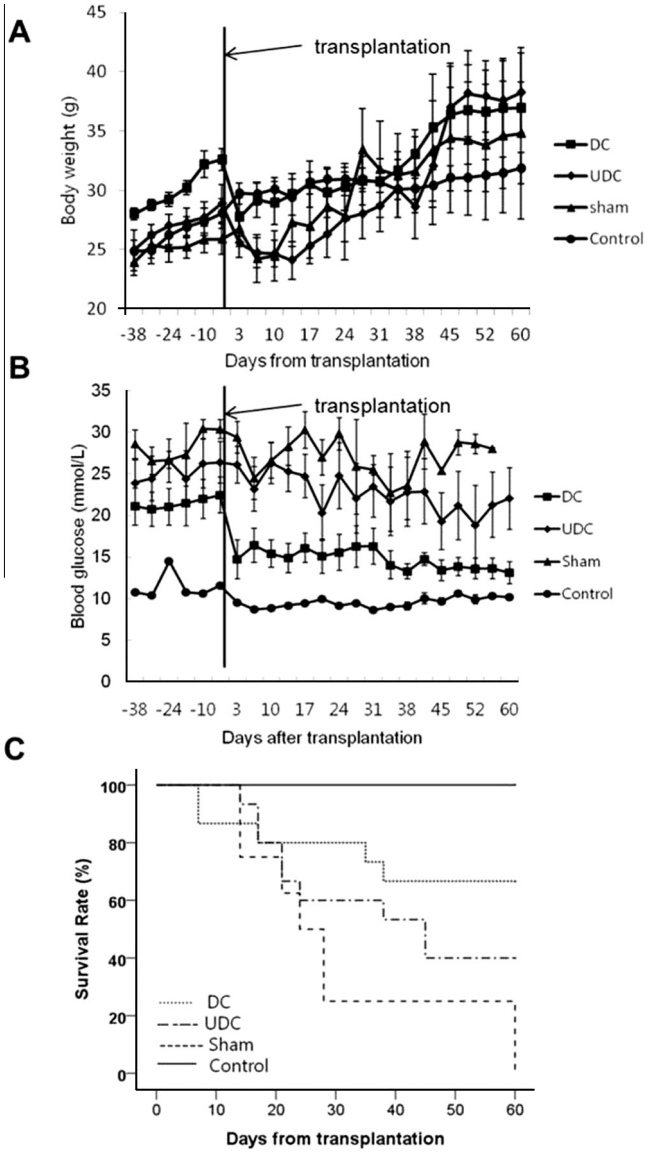

Fig. 2. The effect of transplantation of hEA-ISCs and hEAs and sham operation on body weight (A), blood glucose levels (B), and survival (C) of type 2 diabetes mice. DC, differentiated cell group; UDC, undifferentiated cell group; sham, sham-operated group.

3.3.3. Human gene expression and immunohistochemistry of kidneys and pancreas of transplanted mice

Kidneys of DC group at 60 days (DCK1) and 210 days (DCK2) post transplantation showed the expression of human genes while none of these human genes were expressed in normal mouse kidney (NorK) (Fig. 3C). Immunohistochemical studies showed that engrafted cells co-express both human insulin (hINS) and antigen (hNUCLEI), demonstrating the presence of human cells in kidneys of mice in DC group (Fig. 3D and E). In pancreas of mice of DC group, insulin-stained islets were diminished in their size as well as the number compared to pancreas of normal mice (Supplementary Fig. 3).

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

After 60 days of transplantation, DC group showed significantly lower triglycerides than sham group, but not UDC group (Fig. 4A). However, compared with sham groups, free fatty acids of DC group exhibited similar level with the control group, whereas UDC group showed no improvement (Fig. 4B). Furthermore, IL-6 level showed significant improvement in DC group (4.3 ± 2.0 pg/ml) compared with UDC group (44.3 ± 25.4 pg/ml), which are comparable to the level of control group (3.8 ± 0.9 pg/ml) (Fig. 4C). There was no significant difference in adiponectin level among the groups (Fig. 4D).

4. Discussion

Majority of type 2 diabetes patients experience beta cell exhaustion, and many require insulin therapy [23]. In an effort to overcome problems with insulin, cell therapy for type 2 diabetes is gaining traction [1]. Previously we observed that transplantation of hEA-ISCs was very effective in normalizing blood glucose level and prolonging the survival of a type 1 diabetes mouse model [10]. In this study, we assessed effects of hEA-ISCs transplantation on a type 2 diabetes mouse model.

Following transplantation of hEA-ISCs, type 2 diabetes mouse showed significantly lowered glucose level compared to UDC and sham-operated group. Although the transplantation did not completely normalize the glucose level, the observed lowering is interesting since insulin resistance in addition to relative insulin deficiency is an important factor in the pathogenesis of type 2 diabetes. Among 10 mice that survived until 60 days after transplantation, only 1 mouse exhibited the average glucose level of 179 mg/dl, which was comparable to that of the normal control group. Other 8 mice maintained a mean glucose level between 200 and 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 appeared to result from procedure-related complication. The other 3 mice that died between 20 and 60 days after transplantation showed mean glucose level of above 500 mg/dl. Thus, a sustained high glucose level is likely to be a cause of their death, and we can assume the initial engraftment failure to be a possible reason. However, future studies are warranted to clarify the reason for persistent hyperglycemia and characterize responders and non-responders. Although we cannot confer significance, it was interesting to see one mouse survive until 210 days, and future study with a longer follow-up period is warranted.

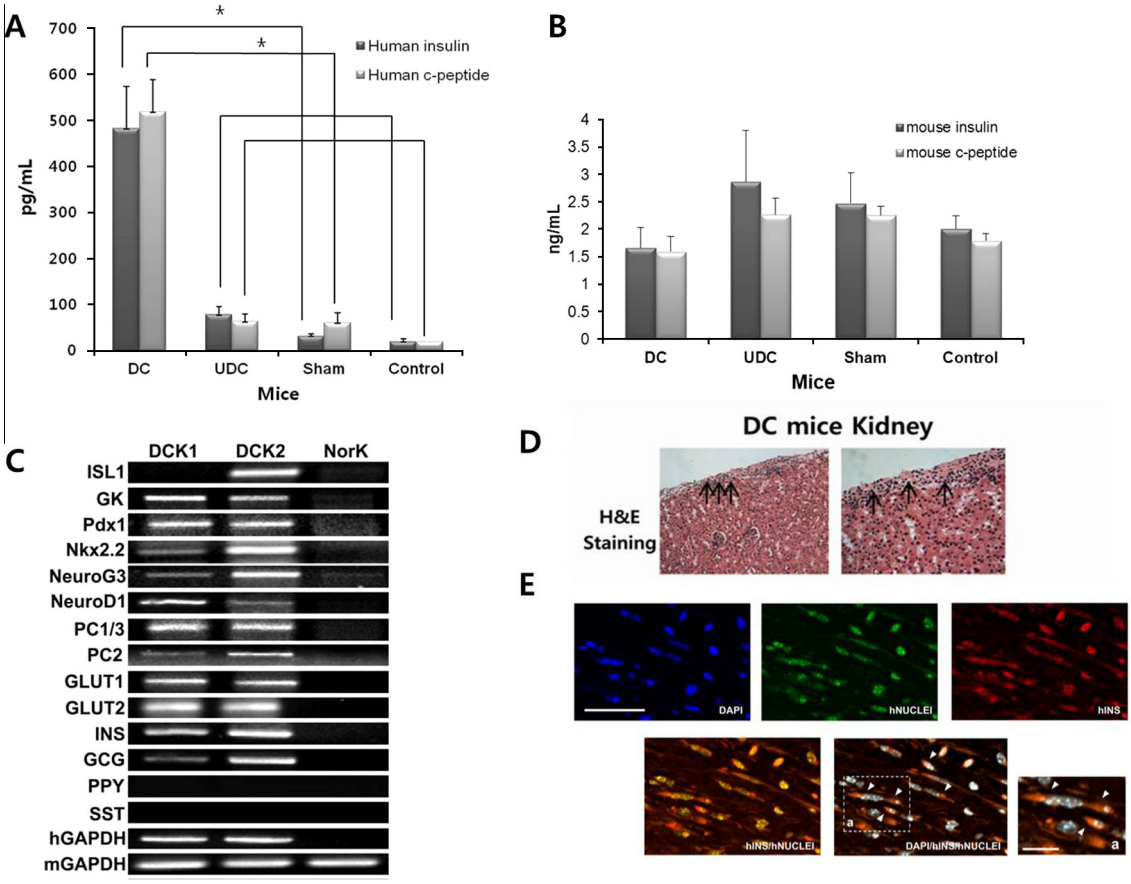

Fig. 3. The effect of transplantation of hEAs and hEA-ISCs on type 2 diabetes mice. Human insulin and c-peptide levels (A) and mouse insulin and c-peptide levels (B) in type 2 diabetes mice. $^{*}p < 0.001$. Gene Expressions of human genes in kidneys of normal (NorK) and DC mouse (C). DCK1, kidney of mouse with hEA-ISCs transplantation at 60 days after transplantation; DCK2, kidney of mouse with hEA-ISCs transplantation at 210 days. Immunohistochemistry of kidneys of mice in normal control group (normal mice) and DC group (DC mice) (D) and (E). H&E stained kidneys of DC mice, arrow indicating the boundary between human and mouse cells (D, ×400); kidneys of the same mice triple-labled with DAPI, hNUCLEI and hINS (E, ×600, scale bar = 100 μm) H&E, hematoxylin and eosin statin; DAPI, 4′,6-diamidino-2-phenylindole; hNUCLEI, human nuclear antigen; hINS, human insulin.

Previous studies suggested that undifferentiated human cells could lower blood glucose level in patients or mice with type 2 diabetes accompanied by increased plasma insulin level, although the source of increased insulin has not been addressed [15,16]. In contrast, the present study demonstrates that differentiated hEA-ISCs were more effective in restoring the depleted circulating insulin, and in survival of diabetic mice. Furthermore, high concentration of human insulin and c-peptide and the summed amount of human and mouse c-peptide were detected in the blood of mice transplanted with hEA-ISCs. Undifferentiated hEAs showed neither glucose-lowering effect nor survival benefits, and little amount of human insulin was present in the blood of recipient mice. Nevertheless their own insulin level was close to normal in these recipient mice.

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]. This seems to be the reason for the suboptimal normalization of blood glucose level in this study. To overcome the insulin resistance, more number of cells might have been needed compared to those needed to normalize the blood glucose level in type 1 diabetes mice, and oral administration of insulin sensitizers after transplantation may have further lowered the blood glucose level. Evidences suggest the critical role of chronic inflammation in the development of type 2 diabetes [24]. First, the relationship between metabolic inflammation and insulin resistance has been widely studied. 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 [25]. Therefore, inflammatory signals interfaced with metabolic disorders are considered as new therapeutic targets for metabolic diseases [24]. Meanwhile, a recent study showed a reduced blood glucose level accompanied by a reduction in IL-6 and IL-1 and increased plasma adiponectin and insulin sensitivity in db/db mouse after intra-bone marrow–bone marrow transplantation [25].

We have observed that IL-6 was significantly reduced in hEA-ISCs transplantation group compared to the level of sham group, and triglyceride and free fatty acid levels also decreased with a marginal significance. Moreover, hEAs transplantation showed a tendency toward lowering of IL-6 compared to sham group although without a statistical significance. Considering that hEA-ISCs consist of about 10% differentiated cells and the rest undifferentiated cells, our results may support the previous observation that the transplantation of undifferentiated stem cells could have favorable effects on metabolic, inflammatory parameters via paracrine effects on adipocytes and macrophages as well as antioxidant effects [18,25]. Ikehara et al. have demonstrated that bone marrow stem cell transplantation with concurrent thymus transplantation lowers blood glucose level and increase insulin sensitivity and adiponectin level in type 2 diabetes mice by normalizing T-subsets and cytokine imbalance [25]. Our study also showed a highest

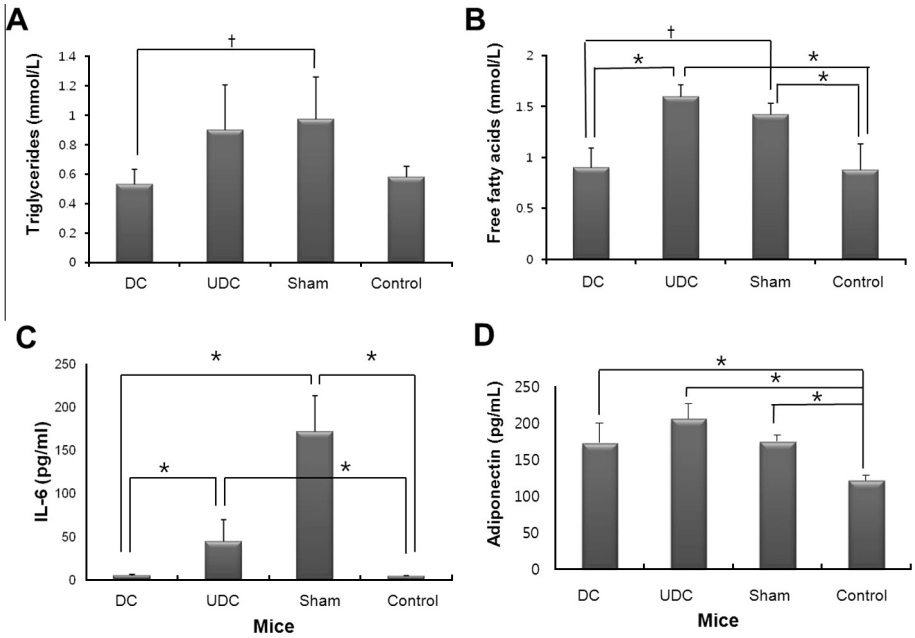

Fig. 4. Metabolic parameters and IL-6 after transplantation. Triglycerides (A), free fatty acids (B), IL-6 levels (C), adiponectin (D) at 60 days after transplantation. DC, differentiated cell group; UDC, undifferentiated cell group; sham, sham-operated group. $^{*}p < 0.05$, $^{\dagger}p < 0.01$.

serum adiponectin level in UDC group, but it was not significantly different compared to sham-operated group. Unlike previous studies done with other undifferentiated cells, hEAs transplantation alone was not enough to lower blood glucose level, but our results suggest that hEA-ISCs transplantation could not only raise circulating insulin level but also have anti-inflammatory effects by lowering inflammatory cytokines which eventually may alleviate insulin resistance.

In this study, immunocompetent mice were used without any immunosuppressive agents. Previously we have shown that hEAs and hEA-ISCs do not express HLA-DR, HLA-DM, CD80, and CD86 genes [10], which are known to play key roles in T-cell mediated response. This is in accordance with previous studies that showed immunomodulatory capacities of mesenchymal stem cells [26,27]. Although immune reaction was not assessed in the present study, we could assume that there was no significant graft vs. host immune reaction at least in the mice that maintained a lowered glucose control for 60 days. A further study in the immune characteristics of the hEAs and hEA-ISCs, and the in vivo immune reactions after transplantation is warranted. Also, there was a high rate of animal attrition during the course of the study, especially in sham operated group, with the death in 6 out of 8 mice by 30 days after transplantation. We presume hyperglycemia to be the main cause of their deaths, but a future study with a greater number of animals is needed to clarify the reason for death as well as to gain a more statistical significance.

Type 2 diabetes is a very complex disease caused mainly by insulin resistance and relative insulin depletion, but also is intermingled by inflammation, autoimmunity, and other unknown factors. From our study, we cannot state that hEA-ISC transplantation alone is a competent treatment option for type 2 diabetes, but when combined with oral hypoglycemic agents, especially insulin sensitizing drugs, they may have a synergistic effect and liberate patients with beta cell exhaustion from insulin injection. Future studies with oral hypoglycemic agents are warranted. Moreover, with advantages of human tissue-derived differentiated cells in terms of safety and ethical issues, hEA-ISCs is a good candidate to apply in patients with type 2 diabetes. Despite of the complicated causes of type 2 diabetes, our data suggest that cell therapy using adipose stem cells-derived insulin-secreting cells could be effective in ameliorating hyperglycemia by restoring circulating insulin level and possibly attenuating metabolic derangements through anti-inflammatory effect.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.059.

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