

## Fetal Liver-Derived Stem Cells Ameliorate Adipocyte Functions in Obese Mice

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### Abstract

Fetal liver (FL) contains hepatic stem cells, hematopoietic stem cells, and mesenchymal stem cells, as well as pluripotent stem cells and very small embryonic-like stem cells. FL has thus been available as a source of stem cells for regenerative medicine. Our previous report suggested that combining FL cell transplantation with the transplantation of fetal thymus prevented tumor growth in tumor-bearing mice. Moreover, in animal models, intra bone marrow-bone marrow transplantation (IBM-BMT) has proven to be the best approach for allogenic BMT. We here propose that transplanting FL cells by IBM-BMT can improve adipocyte functions in obese mice. FL cells were collected from FLs of C57BL/6 mice at 16 days post coitum, and then transplanted by IBM-BMT to db/db mice, an animal model of type 2 diabetes with obesity. Our results showed that the body weight was significantly lower in the treated db/db mice than in the sham-treated db/db mice. The plasma IL-6 level significantly decreased and adiponectin level significantly increased after the transplantation of FL cells. Blood glucose levels also significantly decreased although not to within the normal range. This is the first report that the transplantation of FL cells may improve adipocyte functions, resulting in decreased body weight in obese mice.

**Keywords:** Fetal liver-derived stem cells; Transplantation; Adipocyte; Obesity

### Introduction

Fetal liver (FL) is the primary fetal hematopoietic organ. Hematopoietic stem cells (HSCs) have been shown to migrate into the FL and subsequently into bone marrow and spleen during the embryonic period. The first HSCs appear in the FL of mice at 11.5 days post coitum (dpc) and undergo expansion after 12.5 dpc, accompanied by rapid differentiation into various hematopoietic progenitors. Myeloid and B cell precursors sequentially increase in the FL while T cell precursor cells peak at 13 dpc. After birth, HSCs migrate into the bone marrow and become quiescent [1]. The number of HSCs that express Scal-1+Lin-CD45+ has been reported to be highest in the FL of mice at 15.5 dpc, and FL was thus regarded as a source of HSCs [2]. In addition to HSCs, FL contains hepatic stem cells and mesenchyma-derived cells such as endothelial cells and mesenchymal stem cells (MSCs), and these stem cells have also been used in regenerative therapy for the liver and pancreas [3].

The frequency of HSCs in FL cells is comparable to that in bone marrow, and HSCs from FL have a greater proliferative capacity than adult bone marrow stem cells [4,5]. Furthermore, one report noted that HSCs expand by self-renewal of these cells during FL development [6]. FL-derived MSCs facilitate the expansion of hepatic progenitor cells, and significantly improve expansion compared with bone marrow-derived MSCs, suggesting that FL-derived MSCs benefit hepatocyte expansion [7]. One report showed that the differentiation potential and stromal capacity of FL-derived MSCs are similar to those of bone marrow-derived MSCs, whereas the osteoblast differentiation potential was different due to differences in microenvironment between FL and bone marrow [8]. Furthermore, hepatocytes and pancreatic cells have the common endodermal progenitor cells. Insulin-positive cells have been shown to be induced from FL progenitor cells, and these cells are responsive to the high glucose levels in alloxan-induced diabetic mice [9]. Human FL-derived MSCs may enhance islet-like cell cluster differentiation when cocultured with pancreatic progenitor cells.

Human FL progenitor cells were induced to differentiate into insulin-positive cells, and these cells can reverse hyperglycemia in mice, suggesting that human FL cells may be a source for cell therapy for diabetes patients [10].

Fetal stem cell transplantation has been used in the treatment of various diseases [11]. Our previous report suggested that combining FL cell transplantation with the transplantation of fetal thymus prevented tumor growth, resulting from increased CD62L- CD44+ effector memory T cells and decreased myeloid suppressor cells in tumor-bearing mice [12]. Moreover, intra bone marrow-bone marrow transplantation (IBM-BMT) appears to offer the best approach for allogenic BMT, as manifested by a reduced incidence of GVHD and the restoration of donor-derived T cell functions [13]. However, it is still not known whether FL cell transplantation improves obesity and hyperglycemia in type 2 diabetes mellitus (T2DM). db/db mice are an animal model of T2DM, and show severe hereditary obesity, display hormonal imbalances and hematolymphoid defects, and hyperglycemia resulting from leptin receptor-deficiency [14]. Our results showed that, when FL cells of C57BL/6 mice were allogeneically transplanted to db/db mice by IBM-BMT, the body weight of the treated db/db mice was significantly lower than that of sham-treated db/db mice. Blood glucose levels also significantly decreased, although not to within the normal range. However, this is the first report that FL cell transplantation improved adipocyte functions in db/db mice.

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## Method

### Animals

Five-week-old male db/db mice were purchased from Charles River Laboratories (Yokohama, Japan), and pregnant C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan). All mice were maintained under specific pathogen-free conditions. The Committee on the Ethics of Animal Experiments of Kansai Medical University approved our experiments. All protocols (Permit Number: 10-108) for these animal experiments were performed in accordance with the Guidelines for Animal Experimentation, Kansai Medical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Each experiment was repeated three times (n=6 in each group).

### FL cell isolation and transplantation

FLs were dissected, and tissue pieces were then digested in 0.05% collagenase type 1 (Wako Pure Chemical Industries, Osaka, Japan) for 10 min at 37°C. The digested product was stained with anti-Ter119 antibody (BioLegend, San Diego, CA), then depleted of Ter119-positive cells using Dynabeads sheep anti-rat IgG (Invitrogen Dynal AS, Oslo, Norway). The cells were used for FACS analysis.

The db/db mice were irradiated by a Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada) with two 137Cs sources. FLs were isolated from the B6 mice embryos at 16 dpc under a dissecting microscope. They were then digested in collagenase, and the cell suspension then passed through mesh with a pore size of 70µm (BD Falcon). These cells were injected into the 6wk-old db/db mice ( $1 \times 10^7$ /mouse) by IBM-BMT [15]. The same volume of saline was injected by IBM-BMT into age-matched db/db mice as a sham-treated group. These experimental animals were followed up for 6 weeks after treatment.

### Flow cytometric analyses

FITC-Scal 1, FITC or PE-CD45, FITC-H2Kb, PE-c-kit, FITC-CD4, FITC-CD8 and FITC-CD31 antibodies (BD Bioscience Pharmingen, San Diego, CA) were used for FACS analysis. Ter119-depleted FL cells were double stained with anti-FITC-CD45 and PE-Scal1 antibodies, anti-FITC-CD45 and PE-c-kit antibodies, and anti-FITC-H2Kb and PE-CD45 antibodies. PE-anti-DIK antibody (Medical & Biological Laboratories Co., LTD., Japan) was used to detect hepatic stem cells. These stained cells were analyzed by FACScan (BD Bioscience Pharmingen) after washing. Isotype-matched immunoglobulins (BD Bioscience Pharmingen) were used as controls.

### Cytokine, low-density lipoprotein (LDL) and insulin measurements and morphology analysis

Plasma adiponectin, IL-6 and leptin levels were determined using ELISA assay kits (R&D Systems, Inc., MN). LDL levels were measured according to the manufacturer's protocol using HDL and LDL/VLDL cholesterol assay kit (Abcam®, UK). Insulin was measured using an ELISA kit (Morinaga, Yokohama, Japan). All assays were performed according to the manufacturer's protocol.

The adipose and pancreas tissue of the db/db mice was removed 6 weeks after transplantation and FLs were removed from the embryos at 16 dpc. The sections of adipose tissue and fetal livers were stained with hematoxylin and eosin. The sections of adipose tissue were stained with F4/80 antibody (AbD Serotec, Oxford, UK). The sections of fetal livers were stained with SSEA-1 (Cell Signalling Technology, MA) and Oct-4 (Santa Cruz Biotechnology, Inc, CA) antibodies. The pancreata were

stained with polyclonal guinea pig anti-swine insulin antibody (N1542, Dako Cytomation, CA) and monoclonal rabbit anti-human glucagon (8233s, Cell Signaling Technology, Massachusetts). These stained sections were examined on a microscope (Olympus, Japan).

### Statistical analysis

The results are represented as means  $\pm$  SD. The Student's *t* test was used to determine any statistical significance. A *p*-value of  $<0.05$  was considered to be a significant difference.

## Results

### Analysis of FL cells at 16 dpc

FL was analyzed by HE staining (Figure 1A) and FACS analysis (Figure 1B-E). FACS results showed that the cell percentages were as follows: single Scal-1+, 0.4%; single CD45+, 16.33%; Scal-1+CD45+ double-positive, 40.12% (Figure 1C); single c-kit+, 3.24%; and c-kit+CD45+ double-positive, 46.66% (Figure 1D); single H2Kb+, 1.82%; and H2Kb+ CD45+ double-positive, 27.86% (Figure 1E). In contrast, the percentages of isotype controls were 0.75% (FITC) and 0.79% (PE) (Figure 1B).

### Analysis of body weight and adipocyte size in sham-and FL-treated db/db mice

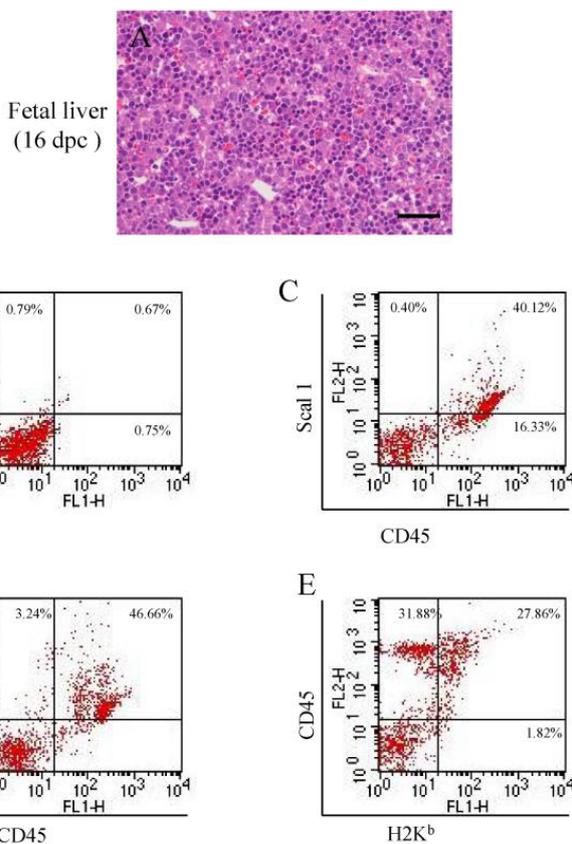
The body weight significantly decreased (red line) from 2 wks ( $34.3 \pm 1.2$  vs  $42.5 \pm 0.9$  g,  $*p < 0.01$ ) to 4 wks ( $33.5 \pm 2.9$  vs  $51.9 \pm 2.3$  g,  $*p < 0.01$ ) after FL transplantation (Figure 2A), although there was a little increase in FL-treated db/db mice at 6 wks ( $39.9 \pm 3.7$  vs  $53.2 \pm 2.9$  g,  $*p < 0.05$ ) after treatment, though the levels were still significantly lower than in the sham-treated db/db mice (black line). The fasting blood glucose levels were significantly lower ( $357.6 \pm 54.3$  vs  $544.3 \pm 29.9$  mg/dl,  $*p < 0.05$ ) although the blood glucose levels were unable to recover to within the normal range (Figure 2B) during the 6 wks after transplantation. Figure 2C and D show the HE staining of visceral fat. The qualitative analysis of a single adipocyte showed that the mean area of the adipocyte was significantly smaller in the FL-treated db/db mice than in the sham-treated db/db mice (Figure 2E,  $0.40 \pm 0.1$  vs  $1.0 \pm 0.2$ ,  $*p < 0.001$ ).

### Analysis of morphology and plasma adiponectin and IL-6 levels

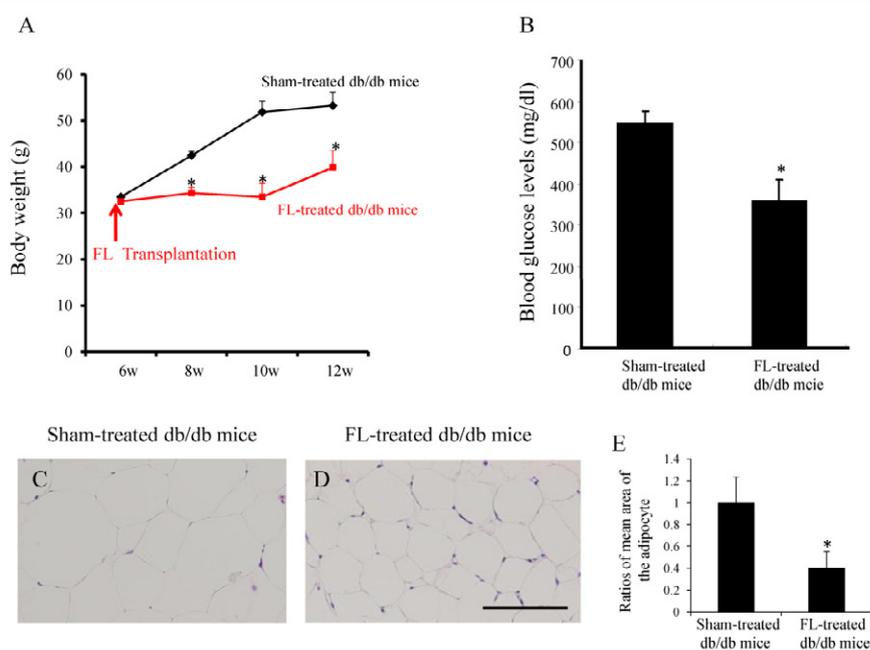
F4/80 is a marker of mature macrophages, and F4/80-positive cells were observed in the visceral fat of the sham-treated db/db mice (arrows in Figure 3A). The number of F4/80-positive cells decreased after FL transplantation (Figure 3B). Plasma adiponectin (Figure 3C) levels were significantly higher in the FL-treated db/db mice than the sham-treated db/db mice ( $6981 \pm 656.7$  vs  $5113 \pm 350.2$  ng/ml,  $*p < 0.05$ ). In contrast, plasma IL-6 (Figure 3D) levels were significantly lower in the FL-treated db/db mice than in the sham-treated db/db mice ( $17.3 \pm 3.7$  vs  $44.6 \pm 7.3$  ng/ml,  $*p < 0.05$ ). Moreover, plasma leptin (Figure 3E) levels were significantly lower in the FL-treated db/db mice than in the sham-treated db/db mice ( $33573.1 \pm 3385.0$  vs  $48394.8 \pm 5332.4$  pg/ml,  $*p < 0.01$ ). Plasma LDL (Figure 3F) levels were also significantly lower in the FL-treated db/db mice than the sham-treated db/db mice ( $0.48 \pm 0.11$  vs  $0.76 \pm 0.12$  µg/ml,  $*p < 0.05$ ), suggesting that adipocyte functions may have been improved by the FL cell transplantation in these experiments.

### Ratios of CD4/CD8 in peripheral blood and morphology of pancreata

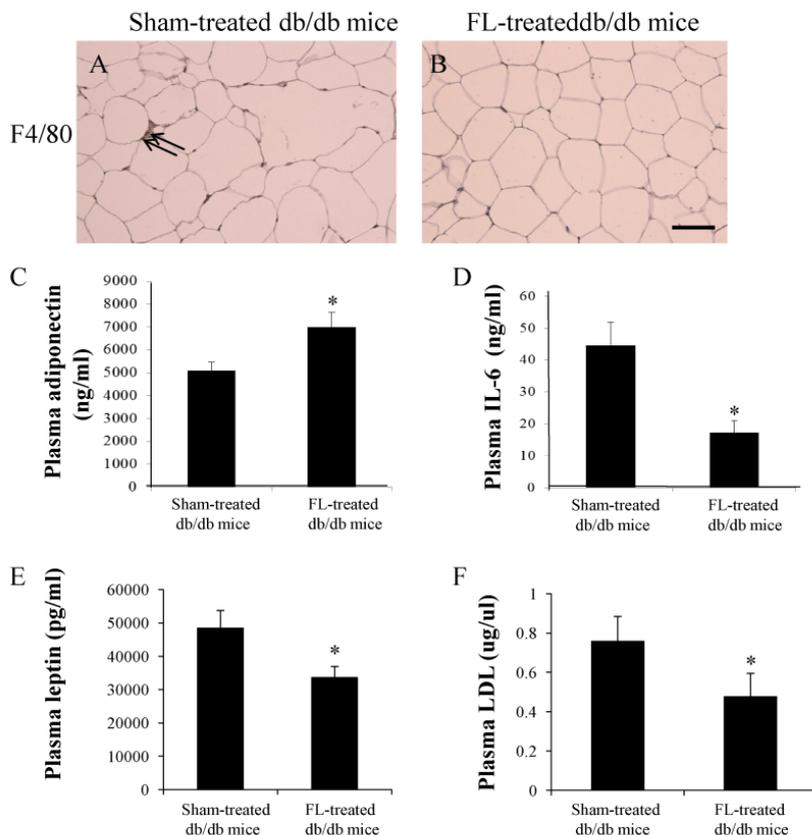
Figure 4A shows the ratios of CD4/CD8-positive cells in the



**Figure 1: Analysis of FL at 16 dpc**  
(A) HE staining. Scale bar =25  $\mu$ m (B-E) Percentages of Scal-1, CD45, c-kit and H2Kb positive cells in the FL were measured by FACS analysis

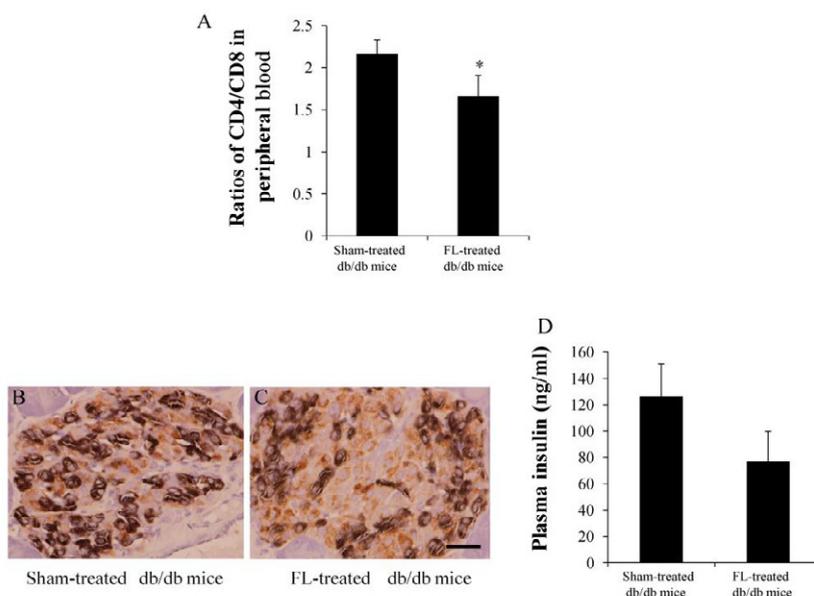


**Figure 2: Body Weight and Blood Glucose Levels**  
(A) Body weights (\*p<0.05). (B) Fasting blood glucose levels (\*p<0.05). (C and D) HE staining in the Visceral fat. (E) Analysis of adipocyte size in the FL-treated and sham-treated db/db mice. The mean area of a single adipocyte cell was significantly lower in the FL-treated db/db mice than in the sham-treated db/db mice. The results are mean  $\pm$ SD, n=6 in each group



**Figure 3: Imaging Analysis of Adipose Tissue and Cytokines**

(A and B) F4/80 staining in the visceral fat. (C) Plasma adiponectin levels (\* $p < 0.05$ ) (D) Plasma IL-6 levels (\* $p < 0.05$ ). (E) Plasma leptin levels (\* $p < 0.01$ ) (D) Plasma LDL levels (\* $p < 0.05$ ). The results are mean  $\pm$ SD,  $n = 6$  in each group. Scale bar = 25 $\mu$ m



**Figure 4: Ratios of CD4/CD8 and Imaging Analysis in the Pancreata**

(A) Ratios of CD4/CD8 in the peripheral blood. (B and C) Immunohistochemistry staining for insulin (brown) and glucagon (black). There was considerably more insulin content in residual beta cells (brown color in C) when compared to sham-treated db/db mice (B). (D) Plasma insulin levels. The results are mean  $\pm$ SD,  $n = 6$  in each group. Scale bar = 25 $\mu$ m

peripheral blood. The ratio was improved in the FL-treated db/db mice when compared with that in sham-treated db/db mice ( $1.66 \pm 0.25$  vs  $2.16 \pm 0.17$ ,  $*p < 0.05$ ).

Insulin and glucagon double-staining was used to estimate islets. There were more insulin-positive cells (brown color) in the FL-treated db/db mice (Figure 4C) than in those of sham-treated db/db mice (Figure 4B). Furthermore, plasma insulin (Figure 4D) levels were lower in the FL-treated db/db mice than the sham-treated db/db mice ( $77.2 \pm 22.6$  vs  $126.0 \pm 24.9$  ng/ml,  $p = 0.625$ ), suggesting that the beta cells were functioning better in the FL-treated db/db mice.

## Discussion

Our results showed that the body weight decreased and the adipocytes became smaller in the db/db mice after treatment. First, the abnormal HSCs (which are leptin receptor-deficient) in the db/db mice were replaced by FL-derived normal HSCs (we will carry out additional experiments to confirm whether this is associated with an improvement in the immunofunctions). Second, adipocyte secretes leptin and adiponectin to regulate metabolism. Inflammatory cytokines such as IL-6 have been shown to be related to insulin resistance in obese subjects [16]. The FL cell transplantation normalized the CD4:CD8 ratio, resulting in decreased the inflammatory cytokine, LDL and leptin levels, and increased plasma adiponectin levels in the peripheral blood, thus improving adipocyte functions as a result of the adipocytes being smaller. Further studies will focus on the mechanisms of the adipocyte functions that were improved, including molecular signalling pathways, and we will also observe body weight and blood glucose levels for a longer term than 6 wks.

Liver and pancreas both develop from the endoderm during embryonic development. Insulin-producing cells were induced from human fetal liver progenitor cells by the overexpression of PDX-1, a key regulator for pancreas development [10]. One report has shown that insulin-positive cells can be transdifferentiated from human liver cells when treated with PDX-1 *in vitro* [17]. PDX-1 and Ngn-3 induced insulin biosynthesis in the liver in response to glucose in the streptozotocin-induced diabetic mice [18]. Some transcription factors such as PDX-1, Isl-1, Pax4, and NKX6.1 are associated with pancreatic development, and these factors were also detected in the trans-differentiated human hepatic progenitors [19]. We will study whether FL cells benefit liver and pancreas regeneration in db/db mice, as one review claims that FL cells have been shown clinically to regenerate liver and pancreas [20]. Third, blood glucose levels did not recover to within the normal range, although they decreased in the FL-treated db/db mice more than in the sham-treated db/db mice. Hyperinsulinemia was detected in both groups, and the plasma insulin levels showed that there was no significant difference between the two groups, although the plasma insulin levels were lower in the FL-treated db/db mice (data not shown), suggesting that their insulin sensitivity may have been improved. Further studies are required to confirm whether FL cells could migrate into the pancreas and improve beta cell functions.

SSEA-1 and Oct 4 are embryonic stem cell markers, but no SSEA-1 or Oct 4-positive cells were observed in the FLs at 16 dpc (data not shown), suggesting that there are no embryonic stem cells in the FLs at 16 dpc. FL is a major hematopoietic organ during embryonic development, but some stem cells such as pluripotent stem cells (PSCs) and very small embryonic like stem cells (VSELs) have been found in FL [2,21]. The FL cells isolated from mouse embryos at 13.5 dpc were analyzed, and c-Kit-CD49f+CD29+CD45-TER119- FL cells were found to be the best candidate hepatic stem cells. In contrast, c-Met+

CD49f+low c-Kit-CD45-TER119- FL cells present functions of PSCs in the developing mouse liver [21]. VSELs that express Scal-1+Lin-CD45- have been observed in pregnant mice at 12.5, 15.5 and 17.5 dpc. Moreover, the percentage of Oct4+ VSELs was highest in FL at 12.5 dpc, and decreased with maturation [2]. From these experiments, our results suggest that FL cell transplantation that mainly consists of HSCs does not normalize blood glucose levels in db/db mice. As previously reported, db/db mice treated with IBM-BMT+ thymus transplantation (TT), which results in increased plasma adiponectin levels and insulin sensitivity, normalized hyperglycemia and kidney functions [22,23]. Thus, we will combine FL cell transplantation with TT to treat db/db mice. Moreover, FL cells at 12-13 dpc will be used instead of FL cells at 16 dpc, since there have been reports suggesting that there are VSELs and PSCs in the FL, and that these stem cells have the capacity to differentiate into insulin-positive cells and may normalize hyperglycemia [2].

Our FACS results showed that 30% of CD45+ FL cells were H2Kb+, which is an antigen for major histocompatibility complex I (MHC I) of C57BL/6 mice. MHC I is an important surface antigen in allogenic transplantation, because different mice strains have different MHC I antigens. Recipient-derived T cells are reactive with the MHC I of donor cells, inducing the rejection of the donor cells. In contrast, donor-derived T cells induce graft versus host disease (GVHD) by responding to the MHC I of the recipients when carrying out allogenic transplantation. Thus, the risk of rejection is reduced because of the lower expression of MHC I when transplanting FL cells. Meantime, no CD4+ or CD8+ T cells were detected in the FLs, also reducing the risk of GVHD. In conclusion, FL cell transplantation improves adipocyte functions in obese mice without GVHD.

## Conclusions

FL can be obtained from fetuses after spontaneous abortion, and can be considered a source of stem cells because it contains VSELs, PSCs and HSCs. Although there remain ethical controversies regarding the clinical use of fetal tissue-derived stem cells, FL cell transplantation plays an important role in not only liver and pancreas regeneration, but also in HSC transplantation. Moreover, FL cell transplantation may reduce the risk of rejection or GVHD when carrying out allogenic transplantation. This may open up a new avenue in regeneration medicine.

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