Title:
Niche-dependent regulations of metabolic balance in high-fat diet induced diabetic mice by mesenchymal stromal cells

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One Sentence Summary: Niche-dependent metabolic regulation by MSCs
**Abstract:**

Mesenchymal stromal cells (MSCs) have great potentials to maintain glucose homeostasis and metabolic balance. Here, we demonstrate that, in mice continuously feed with high-fat diet (HFD) and developed non-insulin dependent diabetes, two episodes of systemic MSC transplantations effectively improve the glucose tolerance, blood sugar homeostasis and reduce the body weight through targeting pancreas and insulin-sensitive tissues and organs via site-specific mechanisms. MSCs support pancreatic islet growth by direct differentiation into insulin-producing cells and by mitigating the cytotoxicity of interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α) in the pancreas. Localization of MSCs in the liver and skeletal muscles in diabetic animals is also enhanced and therefore improves glucose tolerance, although long-term engraftment is not observed. MSCs prevent HFD-induced fatty liver development and restore glycogen storage in hepatocytes. Increased expression of IL-1 receptor antagonist and Glut4 in skeletal muscles after MSC transplantation result in better blood sugar homeostasis. Intriguingly, systemic MSC transplantation does not alter adipocyte number, but it decreases HFD-induced cell infiltration in adipose tissues and reduces serum levels of adipokines, including leptin and TNF-α. Taken together, systemic MSC transplantation ameliorates HFD-induced obesity and restores metabolic balance through multi-systemic regulations which are niche-dependent. Such findings have supported systemic transplantation of MSCs to correct metabolic imbalance.

**Key Words:** diabetes, fatty liver, inflammation, insulin-producing cell differentiation, mesenchymal stromal cells, obesity
Introduction

Impairment of glucose tolerance and insulin resistance initiates type 2 diabetes; however, exhaustion of the insulin supply due to beta (β)-cell apoptosis is the ultimate pathomechanism of both type 1 and 2 diabetes (1,2). The liver, adipose tissues, skeletal muscles, and vascular tissues are insulin-sensitive tissues responsible for blood sugar homeostasis (1,3). Inflammation adversely affects insulin sensitivity and worsens diabetic retinopathies, neuropathies, and vasculopathies (4-6). Proinflammatory cytokines produced by macrophages, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1, create a vicious cycle of reduced insulin sensitivity (3,7-9).

To the present, more than 300 clinical trials of mesenchymal stem/stromal cell (MSC) transplantations have been conducted worldwide with a wide clinical application targeting more than 50 indications (www.clinicaltrial.gov). Systemic transplantation is the most favorable route for MSC delivery from a safety and systemic regulation point of view, and 56% of MSC clinical trials is allogenic transplantation (10). It is well accepted that the majority of MSCs are initially distributed in vital organs (11-16), and are also frequently found in tissues of mesenchymal origin (11,12) when systemically administered. Nevertheless, the long-term engraftment of MSCs is minimal, and the engraftment rate is not correlated with therapeutic response in human studies (17). The acutely diseased and damaged tissues show increased localization of MSCs from the circulation (13-16).

Regarding the possibility of treating diabetes with MSC transplantation, an in vitro study of directly co-culturing MSCs with islet cells has demonstrated the potential of MSCs to give rise to insulin-producing cells (18). In animal experiments of MSC transplantation for treating
hypercglycemia and diabetes-related complications, it is indicated that paracrine and anti-inflammatory effects of MSCs are the primary mechanisms of action (19-22) rather than direct engraftment and differentiation into insulin-producing cells (23, 24).

IL-1 receptor antagonist (IL-1 RA) produced by MSCs has been postulated to mediate anti-inflammatory and anti-fibrotic effects (25, 26), which is responsible for the modulation of diabetogenesis in the animal experiment (25). We have previously reported that indoleamine 2,3-dioxygenase (IDO), IL-10 (hIL-10), soluble TNF receptor II (sTNF RII), and IL-1 RA are inducible immune-modulators produced by human MSCs in response to the stimulation of macrophage-produced IL-1α, β and TNF-α (27). We have also demonstrated that, in a murine model of type 1 diabetes, systemic transplantation of MSCs regulates blood sugar homeostasis by differentiation into insulin-producing cells in liver when islet cells are totally depleted in the pancreas; and such heterotopic engraftment is not observed in non-diabetic mice (28). All the above findings indicate that the therapeutic mechanism of MSCs via systemic administration may be guided by specific tissue and organ micro-environments; and the micro-environments in type 1 diabetes are different from those in non-diabetic conditions. However, the interaction between MSCs and pancreas as well as insulin-sensitive tissues in type 2 diabetes remains elusive so far.

The purpose of this study is to investigate how MSCs contribute to the maintenance of blood sugar homeostasis in type 2 diabetes. Herein, experimental model of type 2 diabetes is established in mice under continuous high-fat diet (HFD) feeding for 6 months. Therapeutic effects of systemic MSC transplantation on the correction of body weight and the maintenance of blood sugar homeostasis, as well as the mechanism of action in the pancreas and insulin-sensitive tissues/organs including liver, skeletal muscles, and adipose tissues are to be elucidated.
in this study. We hypothesize that improvement of blood sugar homeostasis after MSC transplantation is mediated by multiple niche-specific regulatory mechanisms in pancreas and other insulin-sensitive tissues/organs.

Research Design and Methods

Animals

Seven-week-old male B6 mice were purchased from the National Laboratory Animal Center (NLAC, Taipei, Taiwan) and housed following the animal care guidelines of the NLAC. The use of animals was approved by the Institutional Animal Care and Use Committee of Wan Fang Hospital, Taipei Medical University (TMU-WFH).

Isolation and Culture of MSCs

MSCs were isolated from human orbital fat tissues following a previously reported protocol (29), An Institutional Review Board approval was obtained prior to the commencement of the study of isolating MSCs, and written informed consents to donate orbital fat tissues were obtained from patients prior to blepharoplastic surgery.

Induction of Diabetes

Diabetes was induced by continuous feeding of a HFD (D12492, Research Diets, New Brunswick, NJ, USA) (30). Thirty six mice were used in the study of systemic MSC transplantation. Mice were divided into four groups: (1) eight mice with normal diet (ND)-fed mice (D12450B, Research Diets) receiving two PBS injections (ND), (2) ten mice with ND-fed mice receiving two MSC injections (ND+MSCs), (3) eight mice with HFD-fed mice receiving two PBS injections (HFD), and (4) ten mice HFD-fed mice receiving two MSC injection (HFD+MSCs). The body weight and fasting blood glucose level were measured weekly. A glucose tolerance test (GTT) was performed every 2 weeks before the onset of diabetes. The
onset of diabetes in mice fed the HFD was defined as an elevated fasting blood glucose level on two consecutive tests plus impaired glucose tolerance on two consecutive GTTs compared to mice fed the ND. After the onset of diabetes, GTT was performed at 2 weeks before the first dose of MSC transplantation (week 10) and 2 weeks after each MSC transplantation (week 14 and week 22).

**Blood Sugar Measurement and the GTT**

Blood sugar measurement and the GTT were performed following the same protocol as in our previous study (30). Briefly, the tail capillary blood sugar level was measured using a OneTouch Ultra meter (LifeScan, Milpitas, CA, USA) after a mouse was starved for 8 h. For the GTT, 1 g/kg body weight of glucose in 0.9% NaCl was intraperitoneally injected, and tail capillary blood sugar levels were measured before (0 min) and 30, 60, 90, and 120 min after the glucose injection.

**MSC Transplantation**

For systemic MSC transplantation, $4.2 \times 10^7$ cells/kg body weight in 0.2 ml PBS for each dose, the same therapeutic dose as in our previous experiments used for type 1 diabetes (28) and acute lung inflammation (31), were slowly injected into mice via the tail vein. The same volume of PBS was injected into control mice.

**Measurement of Serum Insulin, IL-1, Leptin, and TNF-α Levels**

The serum level of mouse insulin was measured with a Mercodia Mouse Insulin Enzyme-linked Immunosorbent Assay (ELISA) Enzyme immunoassay (Mercodia AB, Uppsala, Sweden), and human insulin was detected with a Mercodia Ultrasensitive Insulin ELISA (Mercodia AB) as per the manufacturer's instructions. Mouse IL-1, leptin, and TNF-α levels were determined using reagents of the ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's
instructions. All ELISA data were analyzed with an ELISA reader (Spectra MAX 250, Spectra Devices, Sunnyvale, CA, USA). Six mice were used in each group of each experiment.

**Histopathology and Quantification of Adipocyte Numbers**

Tissues/organs were harvested, fixed in formalin, and embedded in paraffin blocks for sectioning (at a thickness of 3–4 µm). For the histopathological evaluation, tissue sections were stained with hematoxylin and eosin (Sigma-Aldrich). For adipocyte number calculation, six images of adipose tissue sections under the 200x magnification were captured in each sample, and six mice were used in each group.

**Immunohistochemical and Periodic acid–Schiff (PAS) Staining**

For immunohistochemical staining, pancreas sections were incubated with rabbit antibodies against human β2 microglobulin (hβ2M; 1:800, Abcam, Cambridge, MA, USA), rabbit antibodies against human insulin (1:100, Abcam), or rabbit antibodies against mouse insulin (1:3000, ImmunoStar, Hudson, WI, USA) at room temperature for 1 h, followed by goat antibodies against rabbit immunoglobulin (IgG; 1:200, Abcam) for another 40–60 min. For PAS staining, tissue sections were covered with 0.1% Triton X-100 (Sigma-Aldrich) for 5 minutes, followed by 1% Periodic-acid (Sigma-Aldrich) for 10 minutes, and final Schiff’s fuchsin-sulfite reagent (Sigma-Aldrich) in the dark for 30 minutes. Tissue sections were assessed by fluorescence microscopy (Leitz, Wetzlar, Germany). Image acquisition was performed with the SPOT RT Imaging system (Diagnostic Instruments, Sterling Heights, MI, USA).

**Quantitative real-time RT-PCR**

Total RNAs were reverse-transcribed into cDNAs using an Omniscript RT kit (Qiagen). A real-time RT-PCR was performed using a SYBR supermix kit (Bio-Rad). Samples from each animal were subjected to 40 cycles of 95°C for 15 s, followed by 60°C for 30 s and 72°C for 30 s. A primer for detecting both mouse and human 18s rRNA was included in every plate as an internal loading control. The mRNA level of each sample for each gene was normalized against that of
18s rRNA mRNA. The relative mRNA level was determined as $2^{[(Ct/18S rRNA − Ct/gene of interest)]}$.

Primers for the real-time RT-PCR in this study were listed in table 1.

**Quantitative Western Blot Analysis**

Protein samples were prepared according to a previously described protocol (30). Proteins (30 µg) from each sample was subjected to SDS-PAGE (10% (w/v) acrylamide) and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were blotted with rabbit antibodies against mouse carnitine palmitoyltransferase (Cpt) 1A (1:2000, Proteintech Group, Chicago, IL, USA), or rabbit antibodies against mouse Glut4 (1:2000, Abcam) followed by horseradish peroxidase-conjugated secondary antibody (1:5000, Abcam). Protein intensities were determined using an enhanced chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA, USA) and the density of protein bands from at least three animals in each group was assessed using a computing densitometer with Image-J software (LabWorks, UVP, Upland, CA, USA).

**Statistical Analysis**

Statistical analyses were performed using the Statistical Package for Social Science software (Version 16, SPSS, Chicago, IL, USA). Differences in blood glucose levels for the ND and HFD groups at the same time point were assessed using a two-tailed, non-paired t-test, and a $p<0.05$ was considered statistically significant. Differences in blood sugar levels and body weights at the same time point among ND, ND+MSCs, HFD, and HFD+MSCs groups, or differences in any test among three and more than three groups were statistically analyzed by ANOVA with Bonferroni's post-hoc tests at 95% CI. Different levels of statistic significance were represented by different alphabetical letters. Letter “a, b” represent a statistically significant difference between level “a” and “b”. In Figure 1, 2A and 2B, four characters were shown in each time point, and “a” “a” “a” “a” meant no statistical difference among the four groups at the same time point, while “a” “a” “b” “b” represented 2 of the 4 with level “a” at the same statistical level and
the other 2 with level “b” were at statistical level higher than “a”. Error bars shown in all figures represented the standard deviation of the means.

**Results**

**Characterization of MSCs**

MSCs were fibroblast-like, adherent cells. The osteogenic, chondrogenic and adipogenic differentiation capacities have been tested as previously reported (29). Immuno-phenotypically, these cells were negative for CD34, CD133, CD31, CD106, CD146, CD45, CD14, CD117 and human leukocyte antigen (HLA)-DR, and were highly expressed CD29, CD49b, CD49e, CD44, CD49d, CD58, CD90, CD105, and HLA-ABC (29).

**Blood sugar more sensitive to systemic MSC transplantations than body weight changes**

Fasting blood sugar and body weight of mice were regularly recorded every 2 weeks. In ND-fed group, systemic MSC transplantation did not alter blood sugar levels (Fig. 1A, ND vs. ND+MSCs). In ND animals, MSC transplantation slightly decreased the body weight after the second MSC transplantation (Fig. 1B, ND vs. ND+MSCs). In HFD-fed groups, the first dose of MSCs given at week 12 abrogated the progression of hyperglycemia for 8 weeks (Fig. 1A, HFD vs. HFD+MSCs); and the second dose of MSCs given at week 20 further dropped the blood sugar level (Fig. 1A, ND vs. HFD+MSCs). Significant HFD-induced body weight gain was noted after week 8, and single MSC transplantation did not alter the body weight till the second dose of MSCs given at week 20 (Fig. 1B, HFD vs. HFD+MSCs).

**Improvement of glucose tolerance correlated with localization of MSCs in liver and skeletal muscles**
Although the fasting blood sugar level in HFD-fed mice was still higher than that in ND-fed mice after two doses of MSC transplantations (Fig. 1A, ND vs. HFD+MSCs), systemic MSC transplantation effectively corrected HFD-induced glucose impairment (Fig. 2A) after each episode of MSC transplantation (Fig. 2B and C). One month after the second dose of MSC transplantation, quantitation of DNA from human MSCs by human-specific house keeper gene hβ2M versus DNA from tissue cells by human/mouse house keeper gene 18s rRNA was measured. In ND mice, human DNA was detectable in the mouse pancreas, liver, fat, skeletal muscles, heart, lungs, spleen, and kidneys, but the intensity of hβ2M was relatively low (< 0.00005) compared to 18s rRNA (Fig. 2D). Moreover, a type 2 diabetic environment enhanced the bio-distribution of MSCs in insulin-responsive tissues such as the liver and skeletal muscles, but not adipose tissues (Fig. 2D).

**Differentiation of MSCs into insulin-producing cells in type 2 diabetic pancreas**

In control mice, MSC transplantation did not alter the histological appearance of pancreatic islets (Fig. 3A, ND v.s. ND+MSCs). After HFD feeding, mice with persistently high blood glucose levels showed a decrease in islet size (Fig. 3A, HFD); while transplanted MSCs promoted islet growth (Fig. 3A, HFD+MSCs). Immuno-histochemical stained for mouse insulin and human insulin in two consecutive sections of pancreas in HFD-fed mice revealed that only mouse and no human insulin-expressing cells were found in any single islet of the diabetic pancreas (Fig. 3B, HFD, mouse insulin v.s. human insulin). In diabetic pancreas after MSC transplantations, 32 islets were counted in the tissue sections and 25% (8/32) islets showed consisting of both mouse and human insulin-producing cells (Fig. 3B, HFD+MSCs, mouse insulin v.s. human insulin). Before MSC transplantation, human insulin was non-detectable (N.D.); after MSC transplantations, human insulin could be detected in the circulation, and serum level of human insulin in diabetic mice was higher than in normal mice (Fig. 3C).
Cytoprotection of pancreas islet by MSCs

Next, we measured the expression of IL-1 and TNF-α, two critical pro-inflammatory cytokines related to the development of diabetes, in pancreas. It was found that MSC transplantation significantly down-regulated the expression of IL-1α, IL-1β, and TNF-α in pancreas elevated by HFD (Fig. 3D). After two MSC transplantations, human (h) IDO (hIDO), hIL-10, hsTNF RII, but not hIL-1 RA mRNA expressions were detectable in pancreas; however, only hsTNF RII was up-regulated by an HFD (Fig. 3E).

Prevention of fatty liver development and restoration of hepatic glycogen storage by MSCs

We previously reported that persistent HFD feeding resulted in fatty liver, and over-expression of Cpt 1A as well as decrease in glycogen storage ability in hepatocytes accounted for liver-related glucose impairment and insulin resistance (30). Here, MSCs did not alter the morphology (Fig. 4A, ND v.s. ND+MSCs), glycogen storage (Fig. 4B, ND v.s. ND+MSCs), or Cpt 1A level (Fig. 4C, ND v.s. ND+MSCs) in the liver. Systemic MSC transplantation prevented the development of HFD-induced fatty liver (Fig. 4A, HFD v.s. HFD+MSCs) and reduced HFD-induced overexpression of Cpt1A (Fig. 4C, HFD v.s. HFD+MSCs). Therefore, HFD-induced loss of glycogen storage ability in those foamy hepatocytes could be restored by MSCs (Fig. 4B, HFD v.s. HFD+MSCs). However, long-term engraftment of MSCs in the liver was not found regardless of whether mice were fed with ND or HFD (data not shown).

IL-1 RA produced by MSCs associated with glucose uptake enhancement in diabetic skeletal muscles

In skeletal muscles, neither MSCs nor the HFD induced significant pathological changes (Fig. 5A). After MSC transplantation, hIDO, hIL-10, hIL-1 RA and hsTNF RII in skeletal muscle
were detectable, and only hIL-1 RA was responsive to an HFD (Fig. 5B). Similar to hIL-1 RA, Glut4 level in skeletal muscle after MSC transplantation was also enhanced in response to an HFD (Fig. 5C). Long-term skeletal muscle engraftment of MSCs was neither observed in mice fed with ND nor HFD (data not shown).

Regulation of adipokines by MSC transplantation

In mice fed with ND, the numbers of adipocytes and the morphology in the fat tissues remained unchanged after systemic MSC transplantation (Figs. 6A and B, ND v.s. ND+MSCs). HFD feeding resulted in inflammatory cell infiltration into fat tissues and also increased adipocyte numbers; while systemic MSC transplantation reduced the inflammation in fat tissues but had no effect on adipocyte numbers (Figs. 6C and D, HFD v.s. HFD+MSCs). Two doses of systemic MSC transplantations did not alter serum adiponectin levels in mice with HFD feeding (Fig. 6C). However, serum leptin level was effectively decreased by MSCs after the first transplantation (Fig. 6D). After two MSC infusions, both circulating TNF-α (Fig. 6E) and IL-1 (Fig. 6F) levels were reduced in mice fed the HFD.

Discussion

In this study, we discover that systemic MSC transplantation improves the glucose tolerance in type 2 diabetes through a combined mechanism in a tissue-specific manner. The majority of transplanted MSCs in the pancreas support islet growth by mitigating the cytotoxicity from IL-1 and TNF-α. Some transplanted MSCs differentiate into insulin-producing cells in the diabetic pancreas to increase circulating insulin level. In type 2 diabetes, MSCs increase the biodistribution in the liver and skeletal muscles without long term engraftment. MSCs improve insulin sensitivity in the liver by preventing fatty liver formation as well as restoring glycogen
storage in hepatocytes. In the skeletal muscle, MSCs increase IL-1 RA production and enhance the glucose uptake from circulation. MSCs have no effect on reducing the number of adipocytes induced by an HFD. Nevertheless, transplantation of MSCs modulates adipokines such as leptin and TNF-α contributing the inhibition of inflammation in adipose tissues. Body weight reduction in HFD-induced obesity is also achieved by systemic transplantation of MSCs after metabolic balance.

Si et al. report that islet size is decreased in an insulin-independent diabetic pancreas, and systemic MSC transplantation preserves the islet size without the differentiation (32). According to our results, it is clearly demonstrated that the fate of transplanted MSCs depends on the status of islets in pancreas. In our previous study of type 1 diabetic model, pancreatic islets were totally depleted by streptozocin, and the environment of pancreas was not favorable for MSC engraftment; so we observed that the liver served as an alternative niche for β-cell differentiation of MSCs (28). In type 2 diabetes, although the islets were relatively atrophic and dysfunctional (Figs. 3A and B, HFD), such pancreatic environment preserved a niche for MSCs differentiation into insulin-secreting cells in the islets (Figs. 3B and C).

In this study, only 25% of islets in diabetic pancreas possessed the ability to provide human insulin after human MSC transplantation (Fig. 3B), and the level of human insulin in the serum was much lower than that in type 1 diabetes we reported in the previous study (28), indicating that differentiation into insulin producing cells is one of the therapeutic mechanisms of MSC transplantation for type 2 diabetes. We reason that paracrine support of islet growth and immune-modulation in insulin-sensitive tissues are critical for blood sugar regulation (Figs. 3-6). MSCs had been reported to ameliorate hyperglycemia in type 2 diabetic rats (32, 33) through islet protection and regulation of insulin sensitivity in peripheral insulin-sensitive tissues (32). In this
study, we explored that the mechanism of islet protection and insulin-sensitive tissues regulation by MSCs were niche-dependent (Figs. 3-6).

Free fatty acids in an HFD induce macrophage-mediated inflammation via releasing TNF-α and IL-1, which leads to apoptosis of islet cells (7-9) and initiates a series of inflammatory responses in vivo (34). It is known that release of soluble immune-modulatory factors from MSCs is induced by TNF-α, IL-1, and interferon (IFN)-γ (35,36). Recently, we and others report that MSCs exert their anti-inflammatory action via producing IDO, IL-10, IL-1 RA and sTNF RII by themselves and reducing TNF-α and IL-1 level from macrophages (27, 37). In a co-culture study, MSCs show their protective effect on both islet cells and β-cells against IFN-γ, TNF-α, and IL-1β-induced apoptosis (38). Herein, we demonstrated that MSCs abrogated HFD-induced elevation of IL-1α, IL-1β, and TNF-α in the pancreas (Fig. 3D), and which protected islet cells from IL-1- and TNF-α-mediated tissue injury and inflammation in vivo. Soluble TNF RII is a secreting receptor binding to TNF-α for abrogating the effect from receptor-ligand interaction (39). The expression of hsTNF RII was response to a diabetic environment in pancreas (Fig. 3E), suggesting that MSCs ameliorate the cytotoxicity of TNF-α toward islet cells through both the secretion of a soluble neutralizing receptor and the inhibition of TNF-α production.

In an HFD-induced fatty liver, a massive amount of saturated fatty acids accumulated in hepatocytes results in insulin resistance via inhibiting insulin signaling through a c-Jun N-terminal kinase-dependent mechanism (9,40) and turning off glucose metabolism in hepatocytes with active β-oxidation (30). Systemic MSC transplantation restored insulin sensitivity in the liver by preventing fatty liver formation induced by an HFD (Fig. 4A), and subsequently resumed the ability of hepatocytes to metabolize/storage glucose (Fig. 4B and C).
Skeletal muscle cells play a crucial role in regulating blood sugar levels by taking glucose up from the circulation into cells via Glut4 (41). Free fatty acids induce macrophage-mediated subclinical inflammation in skeletal muscles through TNF-α, which results in insulin resistance and reduced Glut4 expression (9,42,43). Although circulating levels of TNF-α and IL-1 in HFD-fed mice decreased with systemic MSC transplantation (Figs. 6E and F), MSC-enhanced Glut4 expression was associated with IL-1 RA production in skeletal muscles (Figs. 5B and C). IL-1 RA has been found to be a diabetogenic modulator produced by MSCs (25). In this study, the level of hIL-1 RA in pancreas was undetectable (Fig. 3E) but strongly increased by HFD in the skeletal muscle (Fig. 5B), suggesting that IL-1 RA modulates diabetic development by targeting on the skeletal muscles instead of the pancreas.

The effect of MSCs on adipose tissues was the amelioration of HFD-induced inflammation (Fig. 6A), which was associated with reduced leptin production triggered by an HFD (Fig. 6D). Leptin is an adipokine mainly secreted by white adipose tissues, and its circulating level is proportional to the total amount of fat in the body (44). Circulating leptin not only acts on the hypothalamus to regulate food intake and energy expenditure (45), but also acts as a pro-inflammatory and mitogenic factor for immune cells (46,47). Therefore, leptin is a marker of adipose tissue-exerted inflammation (46, 47). In this study, the change in leptin by transplantation of MSCs (Fig. 6D), similar to glucose tolerance (Figs. 3B and 3C), was more consequential to TNF-α and IL-1 regulation (Figs. 6E and F) and body weight control (Fig. 1B), indicating that correction of HFD-induced obesity by MSCs is the result of inflammation inhibition and the achievement of metabolic balance.
In general, the doses of MSCs in animal studies are higher than those used in clinical trials, as the volume of distribution of mice is very different from that of human. In future clinical trials, carefully designed dose escalation study will be performed to decide the optimal therapeutic dosage.

Taken together, systemic MSC transplantation improves glucose tolerance and metabolic balance in type 2 diabetes through multi-systemic regulation with tissue-specific mechanisms. The unique abilities of MSCs to achieve intricate multi-systemic regulation strongly support further investigation of the feasibility to use these cells to enhance, maintain and correct blood sugar homeostasis and the relevant metabolic balance in patients suffering from such problems.
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Author contributions

A.T.J. researched data and wrote the manuscript; Y.C.C. researched data; Y.J.F. researched data; O.K.L contributed to study design and reviewed/edited the manuscript; J.H.H. designed the study and wrote the manuscript.

Conflict of Interest Statement

No potential conflicts of interest relevant to this article were reported.

Guarantor Statement

Dr. J.H.H is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Figure legends:

**Figure 1. Change in blood sugar more sensitive than change in body weight under systemic mesenchymal stem/stromal cell (MSC) regulation**

(A) A single systemic MSC transplantation abrogated the progression of high-fat diet induced hyperglycemia, and the second dose of MSCs decreased the hyperglycemia. (B) Continued HFD feeding increased the body weight of mice, and MSCs gradually stabilized the body weight after the second transplantation. (ANOVA, Bonferroni's post-hoc test with 95% CI at the same time point, n=6 in A and B)

**Figure 2. Improvement of glucose tolerance correlated with enhanced distribution of MSCs in liver and skeletal muscle**

(A) Persistent HFD intake induced impairment of glucose tolerance. In normal conditions, MSCs did not alter the blood sugar level in response to glucose challenging, but significantly improved the impairment of glucose tolerance induced by HFD with one (B) and two doses (C) of MSCs. (ANOVA, Bonferroni's post-hoc test with 95% CI at the same time point, n=6 in A-C). (D) One month after the second MSC transplantation, the ratio of human DNAs probed by human beta2 microglobulin (hβ2M) versus housekeeper genes (18s rRNA for both mouse and human species) was less than 0.0001, and a diabetic environment caused the transplanted MSCs to enhance their distribution in the liver and skeletal muscles but decrease it in fat tissues (black bar). (Student’s t-test; *p<0.05, n=4)

**Figure 3. Islet protection and β-cell differentiation of MSCs in a diabetic pancreas**

(A) Systemic MSCs did not alter the morphology of islets in a healthy pancreas, and promoted islet growth in a diabetic pancreas (white arrows) (n=6). (B) Decrease in islet size and no human insulin-expressing cells were noted in a HFD-induced diabetic pancreas, while mixed mouse-
and human insulin-producing cells were found in some islets of a diabetic pancreas after MSC transplantation (n=3). (C) Human insulin was non-detectable (N.D.) before transplantation, and could be detected after MSC transplantations, and serum level of human insulin in diabetic mice was higher than in normal mice. (Student’s t-test; * p<0.05, n=6) (D) MSC transplantation abrogated IL-1α, IL-1β, and TNF-α release from mouse cells triggered by the HFD. (ANOVA, Bonferroni’s post-hoc test with 95% CI, n=4) (E) After MSC transplantation, human indoleamine 2,3-dioxygenase (hIDO), human (h)IL-10, and human soluble TNF receptor II (hsTNF RII) expressions were detectable in pancreases, and hsTNF RII in pancreas was up-regulated by the HFD. (Student’s t-test; * p<0.05, n=4)

Figure 4. Prevention of fatty liver and maintenance of glycogen storage by MSCs
(A–C) Systemic MSC transplantation neither altered the morphology nor liver function in a normal condition. (A) MSCs prevented HFD-induced foamy change in hepatocytes (n=6). (B) Periodic acid-Schiff (PAS) staining of glycogen demonstrated that loss of glycogen storage ability in those foamy hepatocytes, and which could be rescued by MSCs (n=3). (C) MSCs significantly reduced HFD-induced the carnitine palmitoyltransferase 1A (Cpt 1A) protein expression in the diabetic liver. (ANOVA, Bonferroni’s post-hoc test with 95% CI, n=3).

Figure 5. Up-regulation of interleukin-1 receptor antagonist (IL-1 RA) by MSCs associated with enhanced glucose uptake in diabetic skeletal muscles
(A) Neither MSCs nor a HFD induced significant pathological change in skeletal muscles (n=6). (B) Human IDO, hIL-10, hIL-1 RA and hsTNF RII were detectable in skeletal muscles and hIL-1 RA was strongly responsible for the HFD. (Student’s t-test; * p<0.05, n=4) (C) MSC transplantation significantly enhanced Glut4 production in diabetic skeletal muscles compared to the normal control. (Student’s t-test; * p<0.05, n=3)
Figure 6. Amelioration of adipose-derived inflammation via adipokine regulation by MSCs
(A, B) After systemic MSC transplantation, fat tissues maintained a normal appearance (A) and normal adipocyte numbers (B) in mice fed a ND, while a HFD triggered inflammatory cell infiltration into fat tissues (A) and increased adipocyte numbers (B). MSCs reduced HFD-induced inflammation in fat tissues (A) but had no effect on adipocyte number reduction (B). (ANOVA, Bonferroni's post-hoc test with 95% CI, n=6 in A and B) Systemic MSC transplantation did not alter serum adiponectin level (C), but effectively decreased serum leptin level (D) in diabetic mice. After two MSC infusions, circulating TNF-α (E) and IL-1 (F) levels were reduced in HFD-fed mice. (Student’s t-test; * p<0.05 at the same time point, n=6 in C-F)
Table 1. Primers for Real-Time Reverse Transcription-Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
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<th>Reversed</th>
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β2M: beta2 macroglobulin; IDO: indoleamine 2,3-dioxygenase; IL-10: interleukin-10; sTNF RII: soluble tumor necrosis factor receptor II; IL-1α: interleukin-1 alpha; IL-1β: interleukin-1 beta; TNFα: tumor necrosis factor alpha; 18s rRNA: 18s ribosomal ribonucleic acid.
Figure 1. Change in blood sugar more sensitive than change in body weight under systemic mesenchymal stem/stromal cell (MSC) regulation

(A) A single systemic MSC transplantation abrogated the progression of high-fat diet induced hyperglycemia, and the second dose of MSCs decreased the hyperglycemia. (B) Continued HFD feeding increased the body weight of mice, and MSCs gradually stabilized the body weight after the second transplantation. (ANOVA, Bonferroni’s post-hoc test with 95% CI at the same time point, n=6 in A and B)
Figure 2. Improvement of glucose tolerance correlated with enhanced distribution of MSCs in liver and skeletal muscle

(A) Persistent HFD intake induced impairment of glucose tolerance. In normal conditions, MSCs did not alter the blood sugar level in response to glucose challenging, but significantly improved the impairment of glucose tolerance induced by HFD with one (B) and two doses (C) of MSCs. (ANOVA, Bonferroni’s post-hoc test with 95% CI at the same time point, n=6 in A-C). (D) One month after the second MSC transplantation, the ratio of human DNAs probed by human beta2 microglobulin (hβ2M) versus housekeeper genes (18s rRNA for both mouse and human species) was less than 0.0001, and a diabetic environment caused the transplanted MSCs to enhance their distribution in the liver and skeletal muscles but decrease it in fat tissues (black bar). (Student’s t-test; * p<0.05, n=4)
Figure 3. Islet protection and β-cell differentiation of MSCs in a diabetic pancreas

(A) Systemic MSCs did not alter the morphology of islets in a healthy pancreas, and promoted islet growth in a diabetic pancreas (white arrows) (n=6). (B) Decrease in islet size and no human insulin-expressing cells were noted in a HFD-induced diabetic pancreas, while mixed mouse- and human insulin-producing cells were found in some islets of a diabetic pancreas after MSC transplantation (n=3). (C) Human insulin was non-detectable (N.D.) before transplantation, and could be detected after MSC transplantations, and serum level of human insulin in diabetic mice was higher than in normal mice. (Student’s t-test; * p<0.05, n=6) (D) MSC transplantation abrogated IL-1α, IL-1β, and TNF-α release from mouse cells triggered by the HFD. (ANOVA, Bonferroni’s post-hoc test with 95% CI, n=4) (E) After MSC transplantation, human indoleamine 2,3-dioxygenase (hIDO), human (h)IL-10, and human soluble TNF receptor II (hsTNF RII) expressions were detectable in pancreases, and hsTNF RII in pancreas was up-regulated by the HFD. (Student’s t-test; * p<0.05, n=4)
Figure 4. Prevention of fatty liver and maintenance of glycogen storage by MSCs

(A-C) Systemic MSC transplantation neither altered the morphology nor liver function in a normal condition. (A) MSCs prevented HFD-induced foamy change in hepatocytes (n=6). (B) Periodic acid-Schiff (PAS) staining of glycogen demonstrated that loss of glycogen storage ability in those foamy hepatocytes, and which could be rescued by MSCs (n=3). (C) MSCs significantly reduced HFD-induced the carnitine palmitoyltransferase 1A (Cpt 1A) protein expression in the diabetic liver. (ANOVA, Bonferroni's post-hoc test with 95% CI, n=3).
Figure 5. Up-regulation of interleukin-1 receptor antagonist (IL-1RA) by MSCs associated with enhanced glucose uptake in diabetic skeletal muscles
(A) Neither MSCs nor a HFD induced significant pathological change in skeletal muscles (n=6). (B) Human IDO, hIL-10, hIL-1 RA and hsTNF RII were detectable in skeletal muscles and hIL-1 RA was strongly responsible for the HFD. (Student’s t-test; * p<0.05, n=4) (C) MSC transplantation significantly enhanced Glut4 production in diabetic skeletal muscles compared to the normal control. (Student’s t-test; * p<0.05, n=3)
Figure 6. Amelioration of adipose-derived inflammation via adipokine regulation by MSCs
(A, B) After systemic MSC transplantation, fat tissues maintained a normal appearance (A) and normal adipocyte numbers (B) in mice fed a ND, while a HFD triggered inflammatory cell infiltration into fat tissues (A) and increased adipocyte numbers (B). MSCs reduced HFD-induced inflammation in fat tissues (A) but had no effect on adipocyte number reduction (B). (ANOVA, Bonferroni's post-hoc test with 95% CI, n=6 in A and B) Systemic MSC transplantation did not alter serum adiponectin level (C), but effectively decreased serum leptin level (D) in diabetic mice. After two MSC infusions, circulating TNF-α (E) and IL-1 (F) levels were reduced in HFD-fed mice. (Student’s t-test; * p<0.05 at the same time point, n=6 in C-F)