Infusion of adipose-derived mesenchymal stem cells inhibits skeletal muscle mitsugumin 53 elevation and thereby alleviates insulin resistance in type 2 diabetic rats

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Abstract. It is widely accepted that infusion of mesenchymal stem cells (MSCs) ameliorates hyperglycemia by alleviating insulin resistance in rats with type 2 diabetes mellitus (T2D). However, the detailed underlying mechanisms are not clearly defined. Mitsugumin 53 (MG53) is an E3 ligase that has recently been implicated in the aggravation of insulin resistance by promoting the ubiquitinoylation of insulin receptor substrate-1 (IRS-1) in skeletal muscles. It was therefore hypothesized that MG53 may be involved in MSC-mediated therapeutic effects on insulin resistance. To test this hypothesis, in the present study, T2D rat models were induced by a high-fat diet combined with streptozotocin administration and MSC infusion was performed four times (once every 2 weeks for 8 weeks). The therapeutic effects of MSC infusion on insulin resistance were evaluated and the effect on the expression of MG53 and insulin receptor signaling elements in skeletal muscle was also investigated by immunofluorescence staining and western blotting. The results demonstrated that MSC infusion ameliorated hyperglycemia and insulin resistance in T2D rats. Furthermore, MSC infusion inhibited MG53 elevation and reversed the decreases in glucose transporter type 4, insulin receptor, IRS-1 and phosphorylated-AKT levels in the skeletal muscle of T2D rats. These results indicated that MSC infusion has therapeutic effects in rats and that MG53 in skeletal muscle may be a promising novel therapeutic target protein for MSC-mediated amelioration of insulin resistance in T2D.

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Introduction

Type 2 diabetes mellitus (T2D) is a major metabolic disease and is a risk to human health worldwide (1,2). T2D is characterized by reduced sensitivity of insulin receptors in target organs and absolutely or relatively insufficient secretion of insulin (3,4). These characteristics result in persistent hyperglycemia, which affects the heart, blood vessels, eyes, kidney and nerves, in addition to the wound-healing process, in diabetic patients (5-10). Although drugs or exogenous insulin administration can ameliorate hyperglycemia, the amelioration of peripheral insulin resistance in target tissues is not very effective.

Currently, cell-based therapy is being explored as a potential treatment strategy for diabetes (11,12). Mesenchymal stem cells (MSCs) possess various properties, including differentiation potential, local microenvironment modulatory and immunoregulatory effects, and the capacity to secrete various factors (13). These properties of MSCs make them excellent candidates for diabetes management. To date, numerous studies on diabetic animal models have demonstrated that infusion of MSCs ameliorates hyperglycemia (14-17). In addition, the majority of registered clinical trials on MSC-treated type 1 and/or type 2 diabetes in phase I/II have indicated that MSC administration exerts promising therapeutic effects in diabetic volunteers (18). Furthermore, our previous study demonstrated that MSCs alleviated hyperglycemia and insulin resistance by reversing the reduced expression of glucose transporter type 4 (Glut4) and insulin receptor substrate-1 (IRS-1), as well as AKT phosphorylation, in peripheral insulin target tissues of T2D rats, including the skeletal muscle, adipose and liver (19). These results concerning the beneficial effect of MSC infusion in alleviating insulin resistance have been widely verified by different research institutions (14,20-22). However, the precise underlying mechanisms require further investigation.

Insulin resistance is a key pathogenic factor that presents in several metabolic disorders, including obesity and T2D. In the peripheral insulin target tissues, skeletal muscle accounts

for 70-90% of insulin-stimulated glucose metabolism (23). The insulin resistance of skeletal muscle has been the focus of numerous studies worldwide. Recent studies have demonstrated that the muscle-specific TRIM family protein mitsugumin 53 (MG53; also termed TRIM72) is implicated in insulin resistance (24-27). In addition to acting as a key component of plasma membrane repair during normal cellular physiology (28,29), MG53 is also an E3 ligase that interacts with IRS-1 (24,25,27,29). Certain studies have demonstrated that MG53 expression is elevated in the skeletal muscle of rodents and humans with insulin resistance or metabolic disorders (24,27). In addition, it has been indicated that elevated MG53 in skeletal muscle may interact with and ubiquitinated IRS-1, thereby disrupting insulin signaling and inducing insulin resistance or metabolic disorders (24,27). Notably, the role of MG53 in metabolic disorders is controversial, as other studies have indicated that muscle samples derived from human diabetic patients and mice with insulin resistance exhibit normal expression of MG53 (25,26). Nevertheless, we recently reported that MG53 was elevated in the cardiac muscle of rats with diabetic cardiomyopathy (30). Therefore, the expression of MG53 in skeletal muscle during T2D should be investigated in greater detail. In addition, the aforementioned studies (19,24,27) identified that MSC infusion and MG53 may exert their effects via certain common molecules in the insulin signaling pathway, including IRS-1, AKT phosphorylation and Glut4, and our previous results indicated that MSC infusion inhibited MG53 elevation in the cardiac muscle of T2D rats (30). Therefore, it was hypothesized that MG53 in skeletal muscle may be a promising novel therapeutic target protein for MSC-mediated amelioration of insulin resistance in T2D.

To test this hypothesis and investigate the specific therapeutic mechanisms or targets involved in the beneficial effects of MSC infusion, a T2D rat model was generated and MSC infusion performed. The effects of MSC infusion on hyperglycemia, insulin resistance and MG53 expression in skeletal muscle, in addition to the expression of proteins associated with insulin signaling, were investigated. The results demonstrated that MSC infusion ameliorated hyperglycemia through improving insulin resistance. The underlying mechanisms may include the inhibition of MG53 elevation and reduced degradation of IRS-1 and phosphorylated-AKT (p-AKT) in skeletal muscle.

Materials and methods

Animals. A total of 40 adult (aged 8 weeks; weight, 210±12 g) and 20 immature (aged 4 weeks; weight, 80-100 g) male Sprague-Dawley rats were supplied by the Experimental Animal Center of the Chinese PLA General Hospital (Beijing, China). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Beijing, China) and were approved by the Animal Care and Use Committee of the Chinese PLA General Hospital. Animals were maintained in a room with filtered air and a 40-70% relative humidity, with 12 h light/dark cycle and an ambient temperature of 22-25˚C. Unless required to fast, animals had free access to food and water. At the end of experiments, rats were anesthetized with pentobarbital sodium intraperitoneally (60 mg/kg) and sacrificed by cervical dislocation.

Adipose-derived MSC isolation, culture and identification. Adipose-derived MSCs were isolated and purified from immature rats as described previously (17,19). Briefly, rats were anesthetized with pentobarbital sodium intraperitoneally (60 mg/kg) and sacrificed by cervical dislocation; adipose tissue isolated from the groin was digested using 0.05% trypsin and 0.1% collagen I. Following filtration and centrifugation at 600 x g for 10 min at room temperature, cells were cultured in low-glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (80 U/ml) and streptomycin (0.2 mg/ml) at 37˚C in an atmosphere of 5% CO_2 and a relative humidity of ~100%. MSCs were identified as previously described (17,19). In order to perform surface immunophenotype analysis, third passage (P3) MSCs were used for flow cytometry. Once an ~80% confluence was reached, cells were collected and counted, then cells were randomly divided into six groups (one group per antibody), each containing $1x10^6$ cells. Cells were then washed with PBS and incubated for 15 min at room temperature in the dark with the following antibodies: Allophycocyanin-conjugated CD90 (1:20; cat. no. 561409; BD Biosciences, San Jose, CA, USA), R-phycoerythrin-conjugated CD54 (1:20; cat. no. 554970; BD Biosciences), fluorescein isothiocyanate (FITC)-conjugated CD44 (1:20; cat. no. 550974; BD Biosciences), FITC-conjugated CD34 (1:20; cat. no. 11-0341-82; eBioscience; Thermo Fisher Scientific, Inc.), FITC-conjugated CD11b (1:20; cat. no. 554982; BD Biosciences) and FITC-conjugated CD45 (1:20; cat. no. 554877; BD Biosciences). Following incubation, cells were washed with PBS and then subjected to flow cytometry analysis, which was performed using a BD Accuri C6 software system (version 1.0.264.21; BD Biosciences).

In order to perform differentiation potential analysis, P3 MSCs were cultured in a six-well plate at a density of 10^4 cells/well at 37˚C in an atmosphere of 5% CO_2 and a relative humidity of ~100%. Once cells had reached a confluency of ~70 or ~100% for osteogenic or adipogenic differentiation, respectively; the medium was replaced with SD rat MSCs adipogenic (cat. no. RASMD-90031) or osteogenic (cat. no. RASMD-90021) differentiation medium (Cyagen Biosciences Inc., Guangzhou, China) and following this, the cells were subsequently cultured at 37˚C in an atmosphere of 5% CO_2 and a relative humidity of ~100% for 2 weeks. Following a fixation using 4% paraformaldehyde at room temperature for 30 min, adipogenic differentiation was identified by staining with 0.5% Oil red O (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 1 h, and osteogenic differentiation was identified via staining with 0.1% alizarin red S (pH 4.2; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. The cells were observed using an inverted microscope. The freshly harvested, early passage (P4) MSCs were used in all subsequent experiments.

Induction and treatment of T2D rat models. A high-fat diet (HFD) combined with streptozotocin (STZ) injection-induced T2D model was established as previously described (19).

Briefly, adult (8 weeks old) rats were provided with a HFD (40% fat, 41% carbohydrate and 19% protein) for 8 weeks. Subsequently, a low dose of STZ (20 mg/kg) was administrated intraperitoneally. At 1 week following STZ administration, the fasting blood-glucose (FBG) and refeeding blood-glucose levels were measured. In addition, 5 normal chow diet-induced rats and 15 HFD+STZ-induced rats were randomly chosen for oral glucose tolerance tests (OGTTs) and insulin tolerance tests (IPITTs) to verify the T2D model as previously described (19). For FBG, OGTTs and IPITTs, rats were fasted overnight prior to measurements or procedures. The verified T2D model rats were divided into a T2D group and a MSC-treated group (n=15 each). Each rat in the MSC-treated group was infused with $2x10^6$ MSCs suspended in 0.3 ml physiological saline via the tail vein once every 2 weeks, for a total of four infusions. Rats in the normal group (n=10) were provided with a normal chow diet and only infused with 0.3 ml physiological saline.

Determination of the effects of infused MSCs on hyperglycemia and insulin resistance in T2D rats. At 24 h (refed) and 48 h (fasted) following each MSC injection, blood glucose levels were detected with a glucometer (Accu-Chek Advantage Meter; Roche Diagnostics GmbH, Mannheim, Germany), and levels were monitored throughout the whole experiment. At 1 week following the completion of the final MSC infusion, 5 rats from each group were random chosen for the OGTTs and IPITTs assessment again. Blood samples were obtained from rats by squeezing the caudal vein. Following this, serum was isolated from whole blood samples via centrifugation at 800 x g for 10 min at 4˚C. The serum insulin (FINS) levels were measured using an ELISA assay kit (cat. no. EZRMI-13K; EMD Millipore, Billerica, MA, USA) 1 week following the final injection of MSCs. Additionally, at 1 week after the final MSC infusion, 5 rats from each group were random chosen for the hyperinsulinemic-euglycemic clamp studies to measure insulin sensitivity, as previously described (19). Briefly, rats were fasted overnight and 8 mU/kg/min insulin (Novo Nordisk Ltd., Bagsvaerd, Denmark) was intravenously administered. Blood glucose was monitored at 5 min intervals and exogenous glucose infusion rates (GIRs) were assessed until a steady blood glucose level was achieved. In addition, the homeostatic model assessment (HOMA) was used to assess changes in insulin resistance (HOMA-IR) and pancreatic β-cell function (HOMA-β, HBCI), calculated according to the following equations: HOMA-IR=(FBGxFINS)/22.5 and HOMA-β=(20xFINS)/(FBG-3.5) (19).

Immunofluorescence staining. Following the final hyperglycemia/insulin resistance readings that had been taken 1 week following final MSCs injection, rats were sacrificed and muscle samples were obtained. The localization of MG53 protein in rat tibialis anterior muscle was detected by immunofluorescence staining with 5-μm-thick frozen sections, which were permeabilized at room temperature for 15 min using 0.25% TritonX-100 (cat. no. 30632-2I; FARCO Chemical Supplies, Hong Kong, SAR, China) diluted in PBS. Following this, sections were incubated with a 1X blocking buffer (cat. no. 12411; Cell Signaling Technology, Inc., Danvers, MA, USA) at 37˚C for 1 h, and then initially incubated with rabbit polyclonal anti-MG53/TRIM72 antibody diluted in PBS (8 μg/ml; cat. no. ab118651; Abcam, Cambridge, UK) overnight at 4˚C, followed by incubation for 1 h at 37˚C with anti-rabbit IgG fragment antibody (1:200; Alexa Fluor 555-conjugated, red; cat. no. 4413; Cell Signaling Technology, Inc.). Nuclei were stained with DAPI (cat. no. H-1200; Vector Laboratories, Inc., Burlingame, CA, USA) for 15 min at room temperature. Negative controls were processed simultaneously by replacing the antibodies with PBS. The sections were examined by confocal microscopy using a Zeiss 780 system (Zeiss AG, Oberkochen, Germany).

Western blot analysis. Following the final hyperglycemia/insulin resistance readings that had been taken 1 week following final MSCs injection, rats were sacrificed and muscle samples were obtained. Whole cell lysate from the skeletal muscle was extracted using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) containing 1% protease inhibitor cocktail. The protein concentration of lysate was then determined using bicinchoninic acid assay (Applygen Technologies Inc., Beijing China). For electrophoresis, a total of 30 μg protein was loaded onto an 8 or 10% SDS-PAGE gel. Following transfer to polyvinylidene difluoride membranes, the membranes were blocked in 10% non-fat milk in TBS/Tween-20 (0.2%) for 1 h at 37˚C and then incubated with rabbit antibodies against MG53/TRIM72 (1:500; cat. no. ab118651; Abcam), Glut4 (1:500; cat. no. sc-7938; Santa Cruz Biotechnology, Inc.), Na^+K^+ATPase (1:100,000; cat. no. ab76020; Abcam), insulin receptor (1:2,000; cat. no. ab131238; Abcam), IRS-1 (1:500; cat. no. ab131487; Abcam), p-AKT (1:500; cat. no. 9271; Cell Signaling Technology, Inc.), AKT (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.) and β-actin (1:2,000; cat. no. sc-1616-R; Santa Cruz Biotechnology, Inc.) diluted in TBS/Tween-20 overnight at 4˚C. Following three washes in TBS-Tween-20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. TA130023; OriGene Technologies, Inc., Beijing, China) at a dilution of 1:2,000 in TBS-Tween-20 for 40 min at 37˚C. The immunoreactive bands were visualized using a western blotting luminol reagent (cat. no. TA100016; OriGene Technologies, Inc.) and captured on X-ray film. Densitometric analysis was performed using a Gel-Pro Analyzer 3.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) was used to process the data. All experiments were performed at least five times independently. Data are presented as the mean ± standard deviation. Student's t-test or one-way analysis of variance with Tukey's post-hoc analysis was used when data passed the test for normality and equal variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Development and validation of the T2D rat model. Rats were fed a HFD or normal chow diet for 8 weeks. No significant differences were observed in the blood glucose level between the two groups during this 8-week period (Fig. 1A and B). However, HFD induced a higher body weight compared with

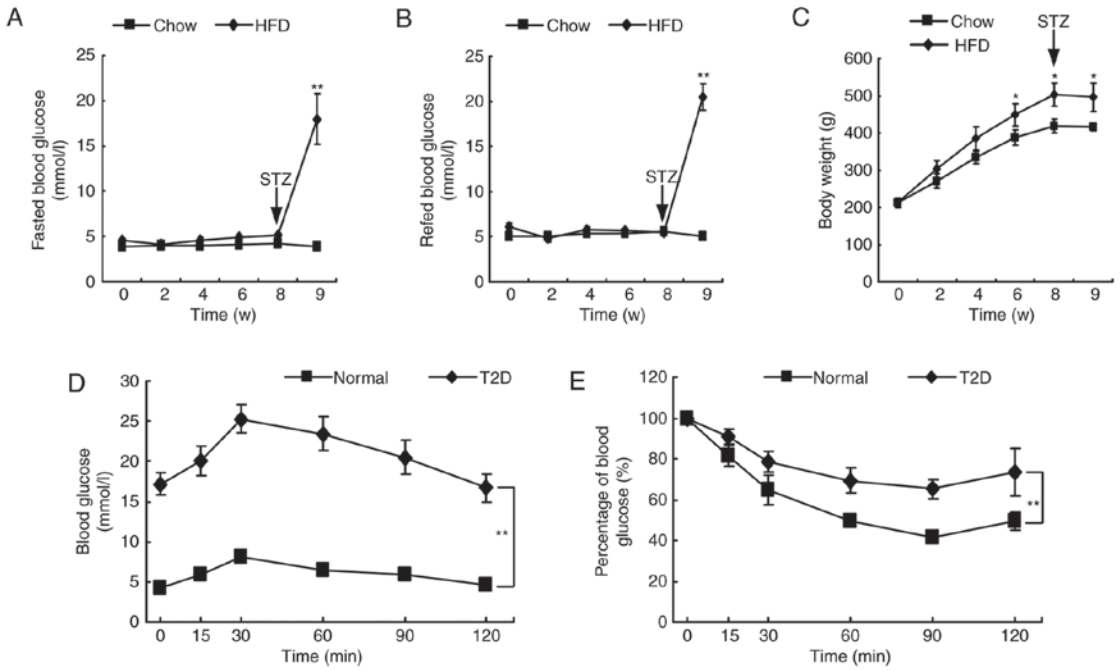

Figure 1. Characterization of the induced T2D rat model. Rats were fed a HFD or normal chow diet for 8 weeks. Subsequently, the HFD-induced rats received a single STZ injection intraperitoneally. Blood glucose level was determined consecutively in (A) fasted and (B) refed rats. (C) Body weight was also determined consecutively during the process. There were 10 rats in the chow diet group and 30 rats in the HFD group. At 1 week following STZ administration, the fasting blood glucose was measured. In addition, (D) individual glucose tolerance was assessed by oral glucose tolerance tests and (E) individual insulin tolerance was assessed by insulin tolerance tests. n=5 rats in normal group and n=15 rats in T2D group. *P<0.05 and **P<0.01 vs. chow diet or normal group. T2D, type 2 diabetes mellitus; HFD, high-fat diet; STZ, streptozotocin; w, week.

the normal chow diet (Fig. 1C). At 1 week following STZ administration in HFD rats, blood glucose levels significantly increased by 4-5 fold compared with normal control rats (Fig. 1A and B). In addition, the results of OGTT and IPITT experiments indicated a notable decrease in glucose tolerance and insulin sensitivity compared with normal control rats (Fig. 1D and E). These results confirmed that the T2D rat model had been established successfully.

MSC infusion alleviates hyperglycemia in T2D rats. Infused MSCs were identified in advance by their phenotypes and the potential for differentiating into adipocytes and osteoblasts (Fig. 2). For immunological phenotypes, the cells were positive for CD90, CD54 and CD44, and negative for CD34, CD11b and CD45 (Fig. 2A). Successful adipogenic and osteogenic differentiation was confirmed by oil red O and alizarin red staining, respectively (Fig. 2B). Each rat in the MSC-treated group was infused with MSCs four times (Fig. 3A). In different stage phases, untreated T2D rats demonstrated notable hyperglycemia and decreased body weight (Fig. 3). However, following the four continuous infusions of MSCs, the blood glucose demonstrated a marked decrease, almost returning to normal levels (Fig. 3B and C), and the body weight was significantly increased (Fig. 3D), compared with T2D rats without treatment. These results indicated that continuous infusion of MSCs improved the long-term regulation of blood glucose levels in T2D rats.

MSC infusion improves glucose metabolism and insulin sensitivity in T2D rats. To investigate the effects of MSC infusion on hyperglycemia alleviation in T2D rats, glucose metabolism and insulin sensitivity were compared, and hyperinsulinemic-euglycemic clamp studies performed. As demonstrated in Fig. 4, the results of OGTTs (Fig. 4A) and IPTTs (Fig. 4B) revealed a significant deterioration in glucose metabolism and insulin sensitivity in T2D rats, compared with normal control rats. In addition, the GIR was also significantly decreased in T2D rats compared with normal control rats (Fig. 5A). Consistent with hyperglycemia alleviation, MSC-treated rats demonstrated a significant improvement in glucose metabolism and were more sensitive to insulin compared with untreated T2D rats (Figs. 4 and 5A). The serum insulin levels were significantly increased in the T2D and MSC-treated T2D groups compared with the normal control group; however, no significant difference was observed between the T2D and MSC-treated T2D groups (Fig. 5B). Nevertheless, the HOMA results (IR and HBCI) indicated that insulin resistance and pancreatic β-cell function were notably improved in MSC-treated T2D rats (Fig. 5C and D). These results demonstrated that MSC-mediated hyperglycemia alleviation may be associated with improvements in target tissue insulin sensitivity.

MSC infusion inhibits MG53 elevation in the skeletal muscle of T2D rats. To further investigate the potential mechanisms of MSC-induced alleviation of insulin resistance and the potential association with MG53, MG53 protein expression in the skeletal muscle of T2D rats with or without MSC administration was assessed by immunofluorescence staining and western blotting. Immunofluorescence results demonstrated that MG53 expression was markedly elevated in the skeletal muscle of T2D rats compared with normal control rats, but MSC infusion markedly inhibited this MG53 elevation in T2D rats (Fig. 6A). The results

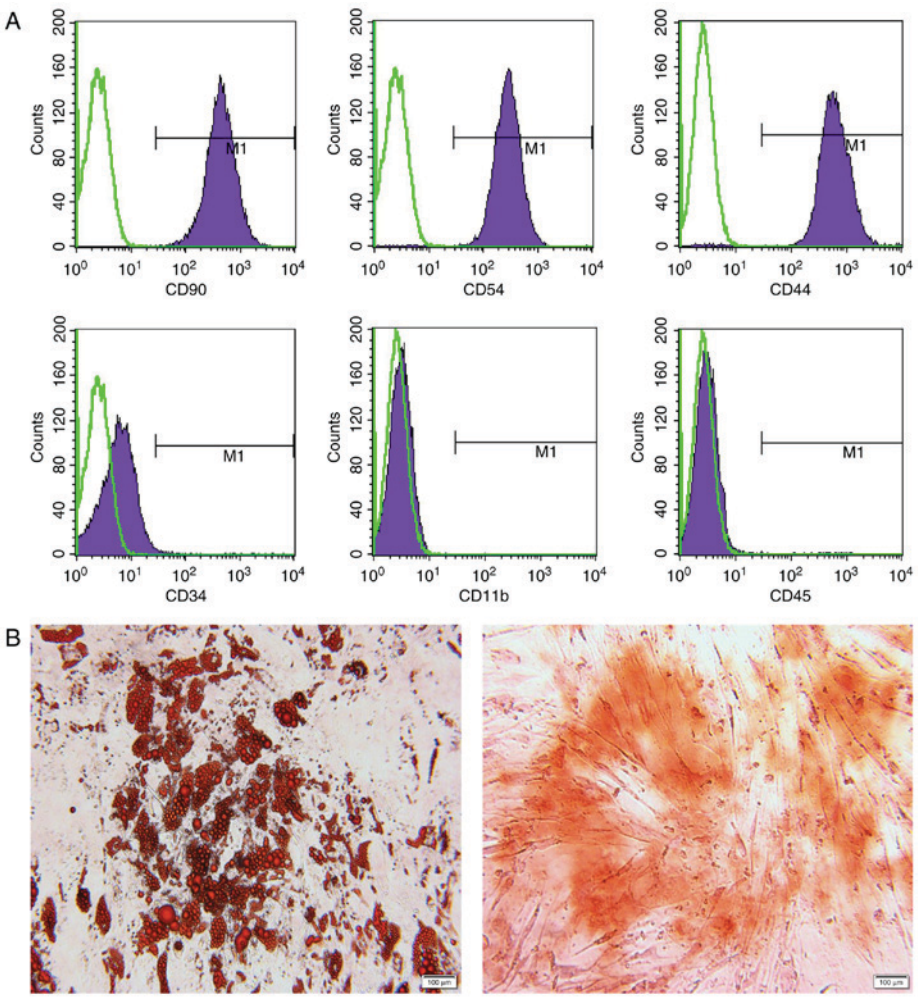

Figure 2. Characteristics of adipose-derived MSCs. The MSCs were identified by their phenotypes and the potential for differentiating into adipocytes and osteoblasts. (A) For immunological phenotypes, the isolated and cultured cells were positive for CD90, CD54 and CD44, and negative for CD34, CD11b and CD45. (B) Differentiation of cells into adipocytes and osteoblasts was confirmed by oil red O staining and alizarin red staining, respectively. Oil red O staining results are presented in the left image, while alizarin red staining results are presented in the right image. Scale bar, 100 μm. MSCs, mesenchymal stem cells.

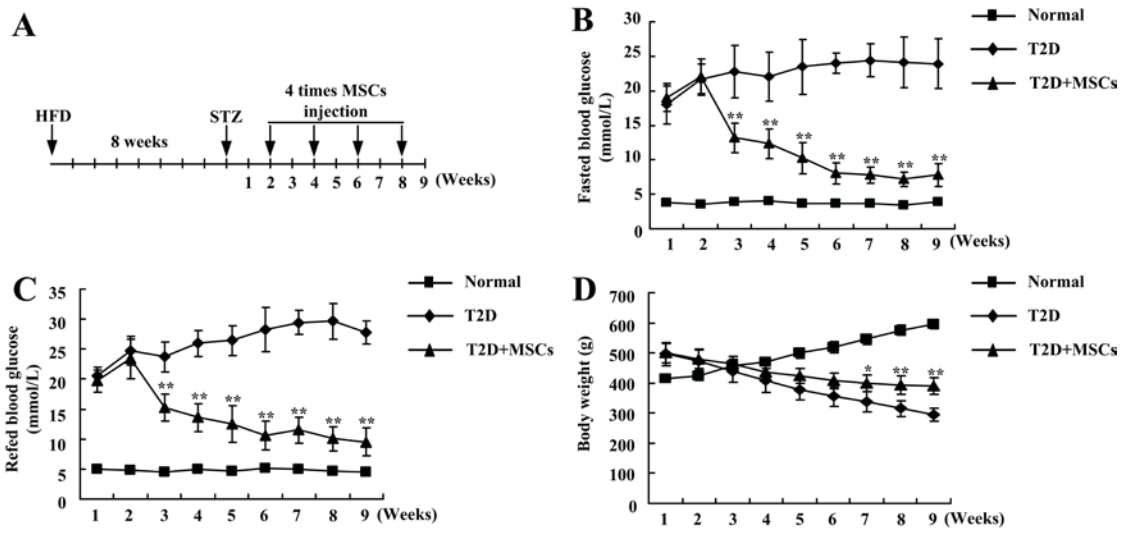

Figure 3. MSC infusion alleviated hyperglycemia in T2D rats. (A) Schematic representation of experimental design. Rats were fed a HFD for 8 weeks. Subsequently, HFD-induced rats received a single dose of 20 mg/kg STZ intraperitoneally. At 1 week following the STZ administration, the fasting blood-glucose was measured. The verified T2D rats were divided into a T2D group and an MSC-treated T2D group. Each treated rat was infused with $2x10^6$ MSCs suspended in 0.3 ml physiological saline via the tail vein four times. Normal rats received a standard chow diet for 8 weeks and received tail vein injections of 0.3 ml physiological saline only. The blood glucose levels were determined consecutively in (B) fasted and (C) refed rats during the treatment. (D) Body weight was also determined consecutively. n=10 in normal group, n=15 in T2D group and n=15 in T2D + MSCs group. *P<0.05 and **P<0.01 vs. T2D group. MSCs, mesenchymal stem cells; T2D, type 2 diabetes mellitus; HFD, high-fat diet; STZ, streptozotocin.

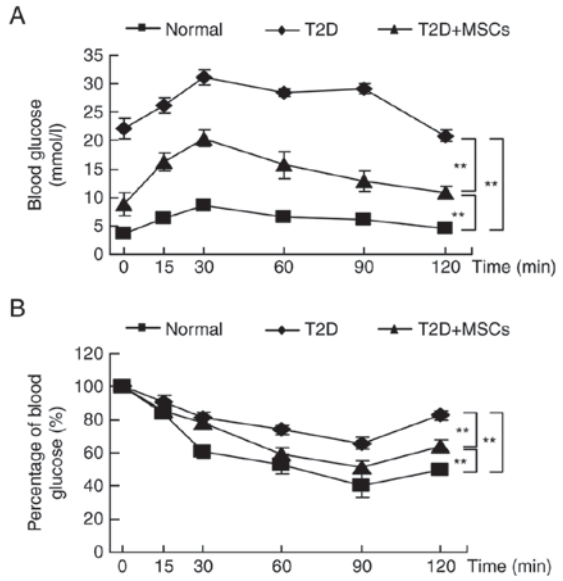

Figure 4. MSC infusion improved glucose metabolism in T2D rats. (A) At 1 week after the completion of the final MSC infusion, individual glucose tolerance was assessed by oral glucose tolerance tests through intragastric administration of glucose at a dose of 2 g/kg and determining blood glucose levels. (B) At 1 week after the completion of the final MSC infusion, individual insulin tolerance was assessed by insulin tolerance tests through intraperitoneal insulin administration at a dose of 1 U/kg. n=5 rats per group. **P<0.01, as indicated. MSCs, mesenchymal stem cells; T2D, type 2 diabetes mellitus.

were further confirmed by western blot analysis (Fig. 6B). These results were consistent with the amelioration of hyperglycemia and insulin sensitivity by MSC infusion, which indicates that MG53 in skeletal muscle may be a potential therapeutic target in the treatment of T2D with MSCs.

Insulin signaling elements in skeletal muscle are restored by MSC infusion in T2D rats. The IRS family of proteins serves a central role in insulin signal transduction. Studies have demonstrated that elevated MG53 in skeletal muscle may ubiquitinate IRS-1 and subsequently decrease AKT phosphorylation, which is considered to be one important mechanism of insulin resistance in T2D or metabolic disorders (24,27). Thus, in the present study, the protein expression levels of insulin receptor, IRS-1 and p-AKT in skeletal muscle were analyzed to further confirm whether skeletal muscle MG53 may be a potential therapeutic target during the treatment of T2D by MSC infusion. As demonstrated in Fig. 7A-D, consistent with the alleviation of insulin resistance and inhibition of MG53 elevation, reductions in feeding-induced expression of insulin receptor, IRS-1 and p-AKT in skeletal muscle were markedly restored in T2D rats by MSC infusion. In addition, the decrease in the expression of Glut4 in the skeletal muscle of T2D rats was also restored following MSC infusion. Therefore, these results indicate that MSC infusion in T2D may inhibit MG53 elevation, subsequently inhibiting insulin signaling element degradation and alleviating insulin resistance.

Discussion

Numerous studies and clinical trials have demonstrated that MSC infusion is able to alleviate hyperglycemia in diabetes mellitus and that MSCs are potential candidates for the treatment of T2D (11,14,18,19). Despite extensive research in this field, the specific underlying mechanisms remain poorly understood. Our previous study demonstrated that infusion of MSCs contributed to ameliorating hyperglycemia by improving peripheral insulin sensitivity in rats with T2D (19). These findings have been confirmed a number of times by different institutes (14,20-22). However, the precise mechanisms remain unclear.

The mechanism of insulin resistance is complex. In previous publications, it has been emphasized that defects in the glucose signaling pathway are a major obstacle, which typically manifest as decreased expression of Glut4 and defective Glut4 traffic to the surface membrane, and disruption of phosphatidylinositol 3-kinase (PI3K)-AKT phosphorylation (31,32). Our previous study demonstrated that infusion of MSCs contributed to improving peripheral insulin sensitivity in rats with T2D by reversing the reduced expression of Glut4 and IRS-1, as well as AKT phosphorylation, in peripheral insulin target tissues, including skeletal muscle, adipose and liver tissues (19). A recent study indicated a novel mechanism by which MSCs alleviated insulin resistance, which involved the regulation of M2 macrophage polarization and promotion of interleukin-6 production in adipose tissue (33). Another study indicated that MSCs improved hyperglycemia by regulating hepatic glucose metabolism in an AMP-activated protein kinase signaling pathway-dependent manner (17). However, in the peripheral insulin target tissues, skeletal muscle accounts for ~70-90% of insulin-stimulated glucose disposal, which serves an important role in the modulation of insulin resistance (23). Previous studies have demonstrated that muscle-specific MG53 may be implicated in insulin resistance (24-27). Other studies have demonstrated that in addition to acting as a key component of plasma membrane repair during normal cellular physiology (28,29), MG53 is elevated in the skeletal muscle of insulin resistance or metabolic disorder models, and that elevated MG53 may interact with and ubiquitinate IRS-1, thereby disrupting insulin signaling (24,27). By contrast, certain studies have indicated that muscle samples originating from human patients with diabetes and mice with insulin resistance did not exhibit an abnormal expression of MG53 (25,26). The key point of contention is whether MG53 expression is elevated in skeletal muscle in rodent or human metabolic disorders. Thus, further investigation is required to investigate MG53 expression and its effects on insulin resistance. We recently reported that MG53 was elevated in cardiac muscle in diabetic cardiomyopathy in rats (30). The present study indicated that MG53 was also elevated in the skeletal muscle of T2D rats. Therefore, it is hypothesized that MSC-mediated MG53 reduction in skeletal muscle may be a novel mechanism for alleviating insulin resistance. Until now, the association between MSCs and skeletal muscle MG53 expression, and the associated effects on insulin signaling, were unknown.

The results of the current study indicated that MSC infusion inhibited MG53 elevation in the skeletal muscle of T2D rats. These results are consistent with the hyperglycemia and insulin resistance alleviation effects of MSCs also observed in the present study. Thus, MG53 in skeletal muscle may be a promising novel therapeutic target protein of MSCs during their

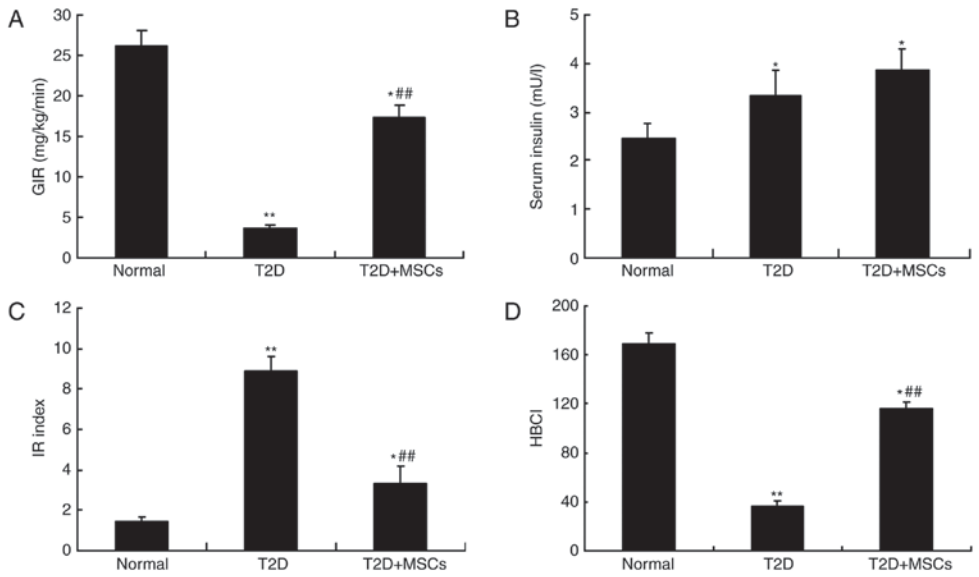

Figure 5. MSC infusion improved insulin sensitivity in T2D rats. At 1 week after the completion of the final MSC infusion, hyperinsulinemic-euglycemic clamp studies were used to measure the insulin sensitivity of each group. (A) Exogenous GIRs were assessed by infusion with 8 mU/kg/min insulin during the hyperinsulinemic clamp, n=5 rats per group. (B) Serum insulin levels were assessed by ELISA assay kits. The HOMA was used to assess alterations in (C) IR index and (D) pancreatic β-cell function, which were calculated according to the following equations: HOMA-IR=(fasting blood glucosexserum insulin)/22.5 and HOMA-β=(20xserum insulin)/(fasting blood glucose-3.5). n=10 in normal group, n=15 in T2D group and n=15 in T2D + MSCs group. *P<0.05 and **P<0.01 vs. normal group; $^{\#\#}$P<0.01 vs. T2D group. MSCs, mesenchymal stem cells; T2D, type 2 diabetes mellitus; GIRs, glucose infusion rates; HOMA, homeostatic model assessment; IR, insulin resistance; HOMA-β/HBCI, pancreatic β-cell index.

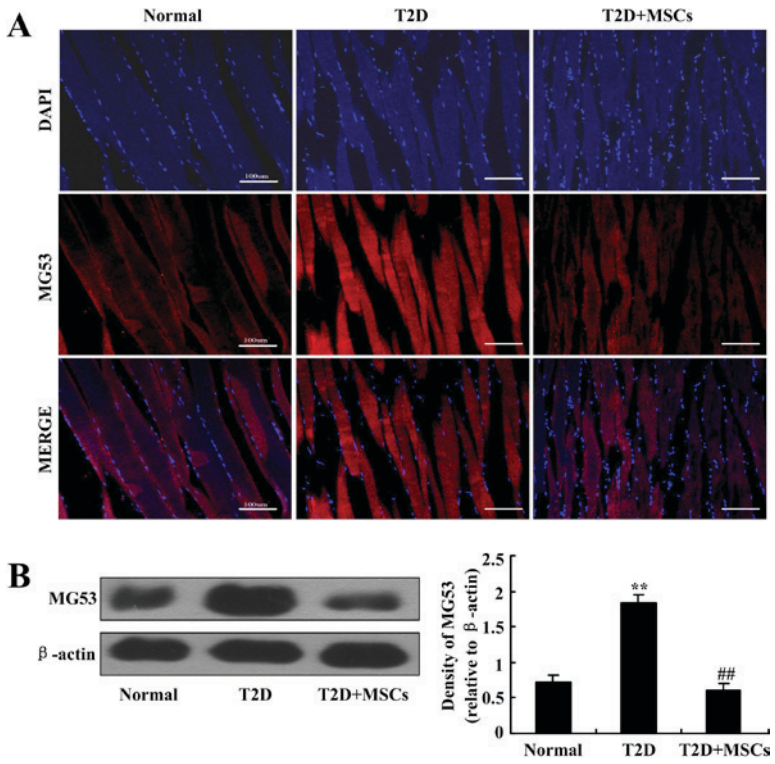

Figure 6. MSC infusion inhibited MG53 elevation in the skeletal muscle of T2D rats. (A) MG53 protein localization in the tibialis anterior muscle of normal, T2D and MSC-treated T2D rats detected by immunofluorescence staining. MG53 staining is indicated by red fluorescence, DAPI staining of nuclei is indicated by blue fluorescence. Scale bar, 100 μm. (B) Western blotting confirmed the immunofluorescence results and demonstrated that MSC infusion inhibited MG53 elevation in the skeletal muscle of T2D rats. MG53 expression in the skeletal muscle was normalized to β-actin. n=5 sections or independent experiments per group. **P<0.01 vs. normal group; $^{\#\#}$P<0.01 vs. T2D group. MSCs, mesenchymal stem cells; MG53, mitsugumin 53; T2D, type 2 diabetes mellitus.

alleviation of insulin resistance in T2D. To further verify this hypothesis, the protein levels of insulin receptor, IRS-1 and p-AKT, components of the insulin signaling pathway, were analyzed by western blotting. Notably, consistent with the alleviation of insulin resistance and inhibition of MG53 elevation, the decreased expression of insulin receptor, IRS-1 and p-AKT

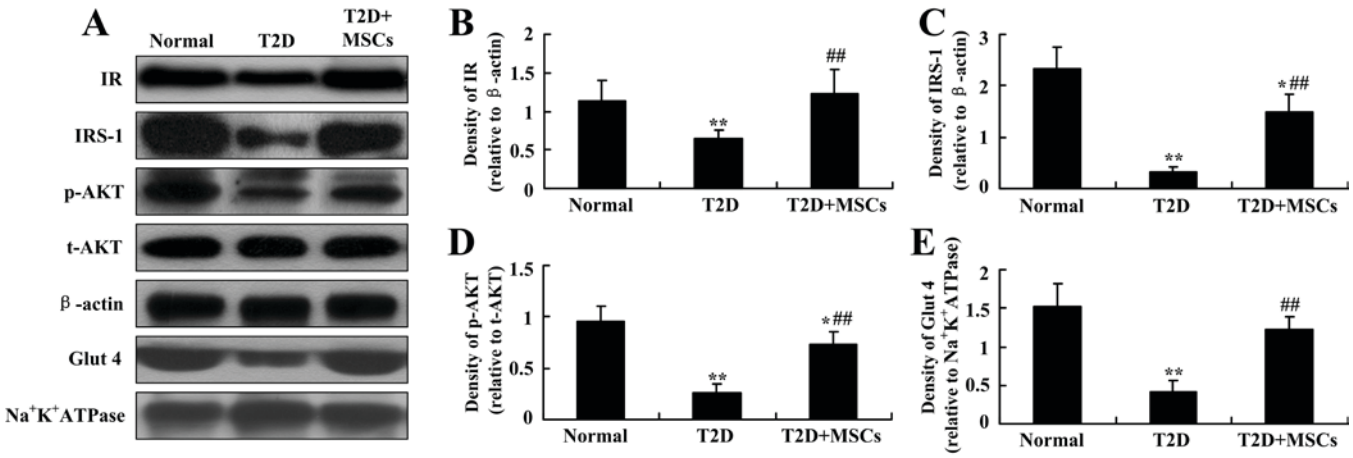

Figure 7. Protein expression of insulin signaling components in skeletal muscle was restored by MSC infusion in T2D rats. (A) Representative western blot bands for the protein expression of various components of the insulin signaling pathway. Densitometric analysis of western blotting results was performed to quantify the relative protein expression of (B) IR and (C) IRS-1, (D) feeding-induced p-AKT and (E) Glut4. IR and IRS-1 levels were normalized to β-actin in skeletal muscle, p-AKT levels were normalized to t-AKT expression in skeletal muscle and Glut4 expression in skeletal muscle was normalized to Na^+K^+ATPase expression. n=5 independent experiments per group. *P<0.05 and **P<0.01 vs. normal group; $^{\#\#}$P<0.01 vs. T2D group. MSCs, mesenchymal stem cells; T2D, type 2 diabetes mellitus; IR, insulin receptor; IRS-1, insulin receptor substrate-1; p-AKT, phosphorylated-AKT; Glut4, glucose transporter type 4; t-AKT, total-AKT.

in the skeletal muscle of T2D rats was markedly restored by MSC infusion. The IRS family of proteins (IRS-1-4) are important components in insulin signal transduction. Studies of single-gene knockout mice have demonstrated that the roles of IRS-1 and IRS-2 may be more distinctive and partially overlapping, while IRS-3 and IRS-4 do not appear to be as important in terms of the effects of insulin on glucose homeostasis (34,35). However, double knockout of IRS-1 and IRS-3 has been reported to induce severe phenotypes of diabetes, indicating a strong compensatory role of IRS-1 and IRS-3; and Laustsen *et al* (34) also demonstrated that the major factor in the development of this diabetic phenotype was the deficiency of IRS-1 and IRS-3 in adipose tissue and the associated decreased level of adipose-derived leptin. As IRS-3 is reported to be most abundant in adipocytes, with its mRNA also detected in the liver, heart, lungs and kidneys (34), the IRS-1 expression in skeletal muscle may be more important in regulating insulin resistance. MG53 is a muscle-specific E3-ligase that has been reported to ubiquitinate IRS-1 and subsequently inactivate the downstream PI3K-AKT signaling pathway to impair glucose homeostasis in skeletal muscle (25,27). Thus, MSC infusion may inhibit MG53 elevation and subsequently restore insulin receptor, IRS-1 and feeding-induced p-AKT levels in the skeletal muscle of T2D rats. This may explain why MSC infusion has been demonstrated to alleviate insulin resistance.

Furthermore, in the present study, the decreased expression of Glut4 in the skeletal muscle of T2D rats was also restored following MSC infusion. Previous studies have demonstrated that decreased levels of Glut4 are implicated in insulin resistance, acting as an obstacle for glucose disposal (31,32). Therefore, the results of the present study indicate that MSC infusion in T2D may inhibit MG53 elevation in skeletal muscle, subsequently inhibiting insulin signaling element degradation and alleviating insulin resistance. However, certain limitations remain that should be addressed in future studies, including the specific mechanisms involved in the negative regulation of MG53 expression by MSCs.

In conclusion, the results of the present study demonstrated that MSC infusion may ameliorate hyperglycemia by alleviating insulin resistance. The specific mechanisms involved may include inhibiting the elevation of skeletal muscle MG53 and the subsequent degradation of IRS-1 and p-AKT in skeletal muscle. These findings indicate that MG53 may be a potential therapeutic target in the treatment of T2D with MSCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZD, HX, WH and GC designed and conducted experiments, performed data analyses and contributed to the writing of the manuscript. JZ, CY, LJ, JL and HS conducted the experiments and performed data analyses. YS designed and conducted experiments, performed data analyses and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care

and Use Committee of the Chinese PLA General Hospital (Beijing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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