Int J Clin Exp Pathol 2017;10(4):4009-4022
www.ijcep.com /ISSN:1936-2625/IJCEP0049089

*Original Article*

# Adipose-derived mesenchymal stem cells alleviating heart dysfunction through suppressing MG53 protein in rat model of diabetic cardiomyopathy

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Received January 17, 2017; Accepted February 22, 2017; Epub April 1, 2017; Published April 15, 2017

**Abstract:** Objective: To investigate the effects of mesenchymal stem cells on cardiac injury and heart dysfunction induced by metabolic disorders as well as the underlying mechanisms including the expression of MG53--muscle specific E3 ligase Mitsugumin 53 in diabetic cardiomyopathy (DCM) in rats. Methods and results: We used a high-fat diet combined with low dose of streptozotocin administration to induce the diabetic cardiomyopathy model in rats, and performed four infusions of adipose-derived mesenchymal stem cells (ADMSCs). Here, we found that the hyperglycemia and cardiac dysfunction weresignificantly alleviated in the ADMSCs treated group compared to DCM group, as manifested by reduced blood glucose, improved systolic and diastolic heart function, inhibited interstitial fibrosis and myocardial hypertrophy, and prevented cardiomyocytes injury. DCM rats showed a significantly increased expression of MG53, and decreased insulin receptor (IR), insulin receptor substrate 1 (IRS1), and serine phosphorylation of Akt (p-Akt), which exhibited opposite changes to the ADMSCs treated group. In vitro, we simulated cardiac injury via 33 mmol/L glucose, used adipose-derived mesenchymal stem cells culture-medium to interfere with primary cardiomyocytes induced by 33 mmol/L glucose. The ADMSCs-CM treated group exhibited decreased expression of MG53, increased IR, IRS1, and p-Akt protein levels, with the differences being significant. Conclusions: Our results indicate that hyperglycemia is an independent risk factor for DCM, and MG53 plays a crucial role in the cardiac injury in the diabetic rat model. Furthermore, ADMSCs can alleviate the cardiac injury and improve heart dysfunction of DCM rats, involving their ability to downregulate the overexpression of MG53, and further elevate IR, IRS1, and p-Akt protein levels, which could improve insulin sensitivity and cardiac metabolism.

**Keywords:** Diabetic cardiomyopathy, adipose-derived mesenchymal stem cells (ADMSCs), MG53 protein, cardiac dysfunction

## Introduction

Diabetes mellitus is a major global threat to public health, with an estimated dramatic prevalence of 336 million cases expected among the worldwide population in 2030 [1]. The main features of diabetes include absolutely or relatively insufficient secretion of insulin and insulin resistance of target organs leading to a series of metabolic disorders. These characteristics cause hyperglycemia and dyslipidemia. This pathophysiological change has been regarded as an independent risk factor for diabetic cardiomyopathy (DCM) [2]. It has been proposed that hyperglycemia and abnormal lipid deposition change the cardiac structure, which contribute to the myocardial hypertrophy and fibrosis. Clinically, the early dysfunctional alteration has included an abnormal ejection fraction (EF) and diastolic dysfunction [4]. Heart failure as a major complication of DCM, if not properly treated, is the dominating cause of morbidity and mortality for diabetic patients [3].

However, the specific mechanisms of DCM are still not well studied. It has been accepted that insulin resistance is an additional risk factor in the pathological changes of cardiovascular disease in type 2 diabetes [5]. Cao et al. reported that tripartite motif protein 72 (TRIM 72), whichis also defined as MG53, and specifically

expresses in cardiomyocytes and skeleton muscle [6], plays a critical role in the progress of DCM in recent studies [7] via modulating insulin signal proteins and capability of triggering insulin resistance. Recent studies have suggested that transgenic mice with overexpression of MG53 develop a typical DCM [8]. Song et al. proposed that the upregulation of MG53, acting as an E3 ligase on the degradation of insulin receptor (IR) and insulin receptor substrates 1 (IRS1) can lead to the downregulation of IR and IRS1, suppressing insulin signal transduction and resulting in the cardiac myocytes insulin resistance, glucose and lipid metabolic disorders, and further leading to the cardiac hypertrophy, fibrosis, and cardiac dysfunction. The Sprague-Dawley (SD) rat with high-fat diet (HFD) also increases the expression level of MG53 and reduce the IR and IRS1 protein levels similar to upregulation of MG53 [7]. These findings indicate that the downregulation of MG53 may be a crucial therapeutic target for the DCM.

According to cell-based clinical trials, mesenchymal stem cells (MSCs) have shown a promising therapy for diabetic mellitus and DCM. Recent studieshave shown that MSCs can reduce the secretion of various injury cytokines to improve the microenvironment of cardiomyocytes [9]. MSCs primarily include bone-marrow mesenchymal stem cells, adipose-derived stem cells, and umbilical cord mesenchymal stem cells. With the multiple differentiation potentials, modulating microenvironment, and secretion of various factors, MSCs can ameliorate hyperglycemia, relieving cardiac dysfunction and ventricular remodeling induced by HFD and streptozotocin (STZ) [10]. Furthermore, MSCs can improve insulin resistance via increasing the expression of IR and IRS1 in the diabetes rat model [11]. MG53 and MSCs have the common targets on insulin signaling, resulting in reverse effects on DCM. To further understand the therapeutic mechanism of MSCs and the relationship between MG53 and insulin signaling related proteins, we induced the DCM rat model via HFD combined with low doses of STZ administration. We then performed four infusions of ADMSCs and detected the effect of ADMSCs on the cardiac function and the protein level of MG53 to further understand why the ADMSCs could effectively prevent DCM from developing into heart dysfunction. This experiment finally provided the experimental evidence for the clinical application of ADMSCs in the prevention and treatment of DCM.

## Materials and methods

### *Animals*

All animals were from the PLA Experimental Animal Center and all procedures were approved by PLA Basic Research Institute and experimented on in accordance with the protocols of the committee. The study was carried out in compliance with the Guide for the Care and Use of Laboratory Animals, which was the publication of National Institutes of Health.

### *Induction of diabetic cardiomyopathy rat*

Male Sprague-Dawley (SD) rats weighting 160-180 g were randomly divided into three groups: ① normal group (Normal), ② DCM group, and ③ ADMSCs treated group (DCM+ADMSCs). The rats were fed with HFD (DCM group, ADMSCs treated group) (40% fat, 41% carbohydrate, and 19% protein) or normal chow (Normal) diet for 4 weeks. Type 2 diabetes was induced by intraperitoneally injecting 20 mg/kg STZ to HFD-fed rats at 4th, 6th, 10th week. One week after STZ injection every time, fasting blood-glucose (FBG) and postprandial blood-glucose (PBG) were obtained. The successful establishment of type 2 diabetes was determined by FBG (FBG >16.7 mmol/L), oral glucose tolerance tests (OGTTs) and intraperitoneal insulin tolerance tests (IPITTs). Then all the rats were given a normal chow diet for 8 weeks and the echocardiograph was performed to confirm the DCM rat model.

### *Cell culture*

*Isolation, cell culture, identification and administration of ADMSCs and primary cardiomyocytes:* ADMSCs were isolated from abdominal fat located in groin. The adipose was digested using 0.05% trypsin and 0.1% collagen I in a shaker at 37°C for 45 minutes. The cells were collected by centrifugation at 1000 r/min and cultured in low glucose Dulbecco's Modified Eagle's Medium (L-DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, incubated at 37°C, 5% $CO_2$. After 48 h, the medium was replaced and the cell was sub-cultured with 0.25% trypsin-EDTA.

Passage 4 ADMSCs were used in all experiments at a density of $5\times10^6$/mL, and the supernatant was collected at -80°C. ADMSCs were identified by flow cytometry as previously described [12]. $2\times10^6$ ADMSCs were suspended in 0.2 ml physiological saline for each rat and infused through the tail vein. The ADMSCs treated group was injected at the week after the establishment of the DCM model and performed the same infusion once a week in the next 3 weeks. The normal group and DCM group were infused with 0.2 ml physiological saline. After a continuous four-infusion of ADMSCs, tissues and blood were collected.

*Isolation, culture and identification of primary cardiomyocytes:* 1-to 2-day-old Sprague-Dawley rats were prepared for the isolation of primary neonatal ventricular cardiomyocytes. An isolated heart removed from the atrial tissues and blood vessels was perfused using phosphate-buffered saline (PBS) for 3 times. The heart tissue was minced in 0.08% collagenase II for 5 minutes, discarding the supernatant fluid. The remaining tissue then was treated with 0.08% collagenase II for 5 minutes following slow agitation at 37°C. The supernatant fluid was added into the DMEM medium containing 10% fetal serum. Repeating the digestion procedure 2 times until the tissue disappeared within 30 minutes. The collected supernatant was filtered using 200 mesh sieve and cells were harvested by centrifugation at 1900 r/min for 8 minutes. The cells were resuspended in DMEM with 15% fetal serum and 1% penicillin/streptomycin for 90 min and incubated at 37°C, 5% $CO_2$ to allow the attachment of non-cardiomyocytes. The supernatant added into 0.1 mmol/L 5-Brdu was re-plated in 6 well plates at a density of $5\times10^4$/ml at 37°C in a humidified incubator equilibrated with 95% air and 5% $CO_2$. The cells were cultured in DMEM containing 15% fetal bovine serum and 1% penicillin- streptomycin in a humidified incubator with 5% $CO_2$ at 37°C. Cardiomyocytes were identified by immunofluorescence according to reference [13].

*Determination of the effects of ADMSCs on DCM rats*

*Blood glucose examination and determination of insulin resistance:* FBG and PBG were obtained one week after ADMSCs injection every time. Serum insulin, OGTTs and IPITTs were detected after the completion of four ADMSCs infusions. The blood glucose level and serum insulin was measured by ACCU-CHEK Integra (Germany) and ELISA (Rat insulin ELISA Kit, R&D) using blood from the tail vein. For OGTTs and IPITTs, rats were fasted overnight and orally given glucose (2 g/kg) or intraperitoneally injected insulin (1 u/kg), and blood glucose was measured every 30 minutes up to 120 minutes. HOMA-IR and HOMA-β were calculated based on the FBG and serum insulin representing the insulin resistance.

*Gross structure changes:* Heart tissues were obtained and blood was dried with filter paper. Left interventricular inner diameter and heart weight were measured to show changes of general structure.

*Histology staining:* Heart tissues were perfused with 4% paraformaldehyde and fixed in neutral formalin. HE, Masson, oil O red staining were performed according to the instructions. The histological sections were observed under fluorescent microscope.

*Blood biochemical detection for cardiac injury and remodeling:* Blood was collected from tail vein and centrifuged at 3000 r/min for 20 min to extract the serum. 100 μl serum were applied to determine the levels of AST, LDH, CK, TnT, BNP using Cobas 8000 (made in German).

*Echocardiography evaluation for heart function:* Echocardiography was performed after the forth infusion of ADMSCs. Animals were lightly anesthetized with inhaled isoflurane (1%) during the whole procedure in a supine position. Transthoracic echocardiography was equipped with a 40-MHz mechanical transductor (Vevo77, Visual Sonics). The M mode of the parasternal long and short-axis scans at both the papillary muscle and apex levels were used to access the LV systolic function. LVID-S and LVID-D, representing the LV inner end-systolic and end-diastolic diameter, respectively. Left ventricular posterior wall at diastole (LVWP-D) indicate the changes of cardiac remodeling. Left ventricular ejection fraction (EF) and fractional shortening (FS) express the heart ejection and fractional shortening. Doppler measurements of apical four chamber images of LV were obtained and maximal early (E) and late (A) transmitral velocities were tested to assess diastolic function.

**Table 1.** Myocardial cell surface and pulse frequency (mean ± SEM)

| Group ($n$=15) | Cell surface ($cm^2$) | Pulse frequency (times/min) |
|---|---|---|
| Normal | 18.63±0.61 | 89.6±4.39 |
| HG | 39.85±0.78** | 57.8±7.86** |
| HG+ADMSCs-CM | 25.46±0.80## | 76.4±2.97## |

**$P$<0.01, vs normal group; ##$P$<0.01, vs HG group.

*Determination of the effects of ADMSCs-CM on the cardiomyocytes injury in vitro induced by high glucose*

Neonatal SD rats were prepared for the isolation of primary cardiomyocytes and cells were divided into three groups randomly: ① normal group (Normal) (25 mM glucose 72 h); ② high glucose group (HG) (33 Mm glucose 72 h); ③ high glucose and ADMSCs-CM (HG+ADMSCs-CM) group (33 mM glucose+ADMSCs-CM 2 mL). The morphology and cell surface of cardiomyocytes were observed under phase contrast microscope. The mRNA level of ANP, BNP and β-MHC was tested by real-time PCR to determine the degree of myocardial hypertrophy. On the basis of three, establishing ADMSCs-CM gradient groups (2 mL, 1 mL, 0.5 mL, 0.25 mL) for the next research. The protein level of MG53, IR, IRS1 and p-Akt were examined by Western blot.

*Immunofluorescence for MG53 and cardiac Troponin*

The tissues and cells were ice-colded with 4% paraformaldehyde for 15 min, and washed with PBS for 15 min. The tissues and cells were incubated with anti-cardiac Troponin (cells 1:400, tissues 1:200) and anti- TRIM72 (MG53, 1:60) at 4°C overnight, washed 2 times with PBS, incubated with secondary antibody (1:200) for 1 h at 37°C, washed 2 times with PBS, incubated with DAPI (1:3000) for 5 min and observed using phase contrast microscope.

*Western blot analysis for MG53, IR, IRS1 and p-Akt*

Ventricular tissue and cardiomyocyteswere lysed with RIPA buffer. The protein concentration of each extraction was measured using the BCA method. Equal quantities containing 100 μg of protein from each tissue lysate were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 4% skim milk for 90 min, incubated with primary antibodies (MG53 1:500, IR 1:5000, IRS1 1:500, p-Akt 1:7000, t-Akt 1:1000) overnight at 4°C, washed with TBS-T buffer for 3 times (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), and incubated with each secondary antibody for 90 min at 37°C. The proteins were visualized using enhanced chemiluminescence. The protein grey analyses were performed by Image Plos.

*Real-time PCR examination for ANP, BNP and β-MHC*

Total RNA was isolated with Trizol reagent (Invitrogen) and 4 μl was reverse-transcribed into cDNA using ReverTra Ace qPCR RT Master Mix Kit with gDNA Remover (Torobo). The process was operated as introduction. (PCR; Stepone Plus Real-Time PCR System, Applied Biosystems). Relative mRNA levels were calculated by controllingthe GAPDH level. The primers used are listed in Supplementary Table 1.

*Statistical analysis*

All numerical data are presented as means ± SEM. One-Way ANOVE and Repeated Measures were used to compare the variation among multiple groups. SNK method was adopted to compare the variation between every two groups. Spearman correlation was used to correlation analysis. $P$<0.05 was considered statistically significant. Numerical data were calculated and analyzed using SPS 17.0 (SPSS Inc., Chicago, Ill., USA).

## Results

*Successful establishment of DCM rat model*

Success of the fat-fed, STZ-induced DM rat model was confirmed by conducting blood glucose tests, OGTTs, and IPITTs, respectively. After three injections of STZ combined with HFD, the FBG and PBG in the DCM group increased to fourfold that of the normal group (Supplementary Figure 1A, 1B). For the DCM group, OGTTs showed that during 0 min to 120 min, the blood glucose maintained a high level compared to the normal group. IPITTs revealed that at 120 min, the percentage of blood glu-


**Figure 1.** The whole procedure of establishment of DCM rat model and infusions of ADMSCs.

cose was still higher than 50% (Supplementary Figure 1C and 1D). The DCM rat model was confirmed by echocardiography, suggesting that both diastolic and systolic functions were impaired in the model group (Supplementary Figure 2A-D). DCM group showed a significant decrease in LVWP-D, EF, as well as FS, while a significant increase in LVID-D and LVID-S compared to the normal group. Hemodynamic of transmitral studies suggested that there were statistically significant decrease in the parameters of E between the two groups meanwhile a significant increase in A.After the success of the DCM, the FBG and PBG in the DCM group with a lower weight increased to fivefold than that of the control group (**Figure 2A**, **2B**). The entire procedure is represented below (**Figure 1**).

*The effect of ADMSCs on blood glucose and insulin resistance*

To determine the effect of infused ADMSCs on hyperglycemia, we carried out a continuous four-injection of ADMSCs in DCM rats in total. ADMSCs we used in our experiment were obtained as previously described, and their characteristics were tested by flow cytometry (Supplementary Figure 4A) andmultiple differentiation potentials into adipocytes and osteoblasts (Supplementary Figure 4B). After the first infusion of ADMSCs, the blood glucose level showed a mild decrease in ADMSCs treated group. After the second and third injection, the fasting blood-glucose and postprandial blood-glucose level showed a dramatic decrease, and after the forth infusion, the blood glucose is almost back to normal in DCM treated group, while the DCM group showed persistent hyperglycemia (**Figure 2A** and **2B**). In ADMSCs treated group, OGTTs showed that during 0min to 120 min, the blood glucose level was lower than DCM group, reaching a peak value at 30min, almost returning back to normal at 120 min (**Figure 2C**). IPITTs revealed that the percentage of blood glucose was lower than DCM group but still higher than 50% at 120 min (**Figure 2D**). Additionally, the improvement of glucose metabolism was also determined by a decreased HOMA-IR index value and an increased HOMA-β index value in the ADMSCs treated group compared to DCM group (**Figure 2E-G**). These results suggested that ADMSCs infusion ameliorated hyperglycemia and improved insulin resistance in DCM rats.

*The effect of ADMSCs on cardiomyocytes remodeling and injury*

To determine the effect of ADMSCs on the pathological changes and cardiac injury, we collected the heart tissue and blood after the forth infusion of ADMSCs. At 10 weeks of HFD diet, the body weight of every group was not significantly different. However, the body weight of DCM group showed a significant decrease at 22 weeks after the three times infusion of STZ (**Figure 2H**). The interventricular inner diameter and the ratio of heart weight to body weight of DCM group were overtly enlarged combined with significant high level of BNP (brain natriuretic peptide) in blood respectively, compared with normal group, while these were significantly decrease in ADMSCs treated group (**Figure 3A-E**). The DCM group showed significant myocardial lipid accumulation and stromal fibrosis evidenced by oil o red and Massonstaining; the situation was improved in ADMSCs treated group (**Figure 3F-H**). In addition, the analysis of blood AST, LDH, CK, TnT, revealed higher secretion in DCM group, together with lower secretion in ADMSCs treated group (**Figure 4A-C**). The results above showed that ADMSCs infusion inhibited cardiac interstitial fibrosis, hypertrophy, injury, which could contribute to the improvement of cardiomyocytes remodeling in DCM rats.

*The effect of ADMSCs on heart dysfunction*

To determine the effect of infused ADMSCs on heart function, we performed echocardiography after the fourth infusion of ADMSCs. After the fourth infusion of ADMSCs, significant improvement was seen in LVWP-D, EF and FS between the treated group and DCM group.

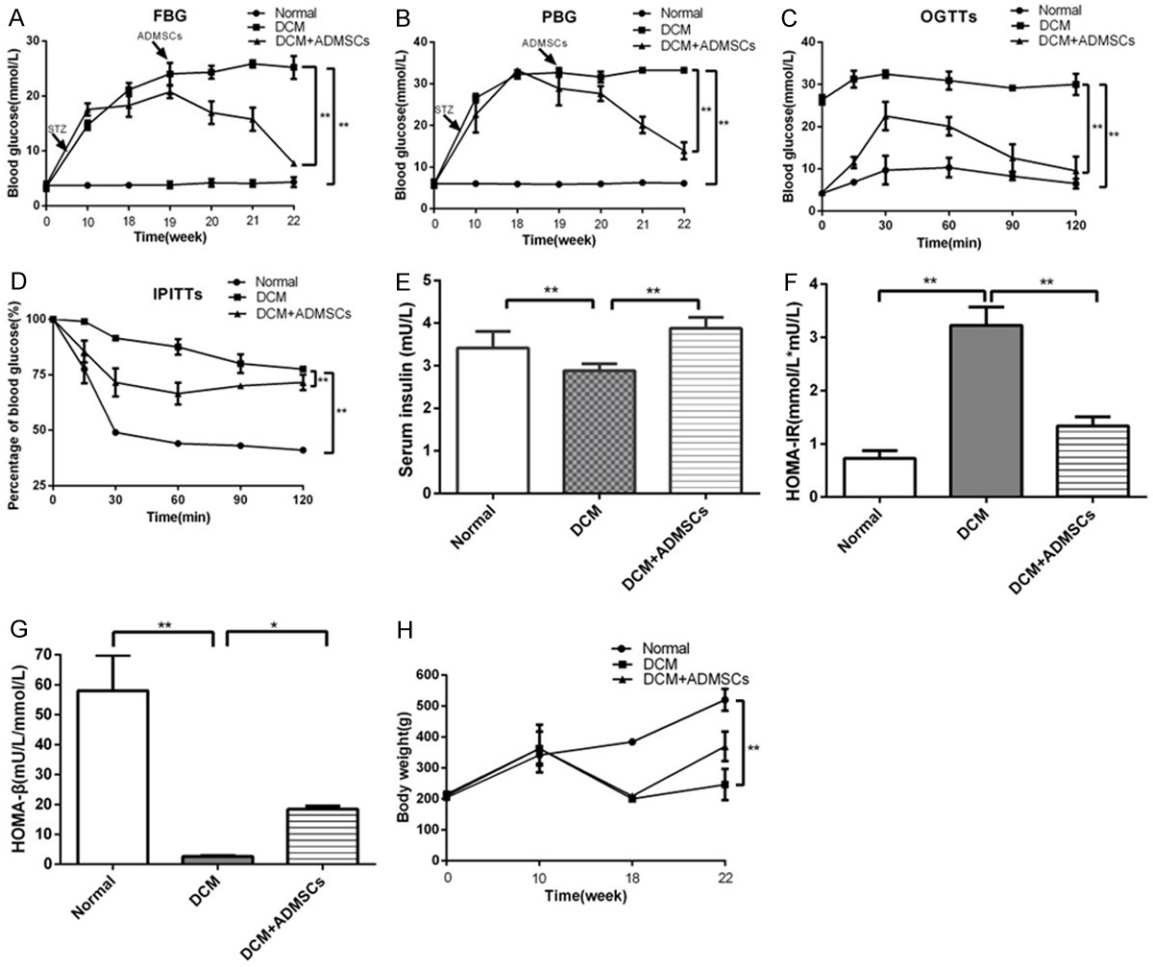


**Figure 2.** Infusion of ADMSCs ameliorate hyperglycemia in DCM rats. (A and B) The fastening blood glucose and postprandial blood-glucose levels were determined by a glucometer. (C and D) Individual glucose tolerance was determined by OGTTs, and individual insulin tolerance was assessed by IPITTs. (E) Serum insulin. (F) Insulin resistance was evaluated by HOMA-IR index (FBG [in mmol/L]*FINS [in units/L])/22.5). (G) The function of β-cells was estimated by HOMA-β (20*FINS [in units/L])/(FBG [in mmol/L]-3.5). (H) The body weight of rats in each group during the whole procedure. Data in (A-H) are means ± SEM, n=8 for each group, $*P<0.05$, $**P<0.01$.

Moreover, a significant decrease occurred in LVID-D and LVID-S, and the diastolic function also significantly was improved between these two groups, as verified by increased E/A (**Figure 5D-G**). These results indicated that ADMSCs infusion improves the heart dysfunction in DCM rats.

*The effect of ADMSCs on the expression of MG53, IR, IRS1, p-Akt*

To determine the pathways relevant to ADMSCs ameliorating DCM injury, we examined the expression of MG53 and insulin signaling proteins. Immunofluorescence for MG53 show significantly higher number in DCM group compared to that in normal group while the decrease in ADMSCs treated group (**Figure 6A-C**). Western blot for the expression of MG53, IR, IRS1 and p-Akt in every group showed that the expression of MG53 was significantly upregulated accompanied by IR, IRS1, p-Akt downregulated in DCM group and ADMSCs treated group showed the opposite changes (**Figure 6D** and **6E**).

To obtain mechanistic insight in a defined in vitro setting, we establish the DCM myocardial injury in vitro. The identification of cardiomyocytes were tested by immunofluorescence of cardiac troponin (Supplementary Figure 3A and 3B). We use 33 mmol/L glucose stimulates the cultured ventricular myocytes. The pulse frequency of cardiomyocytes was significantly

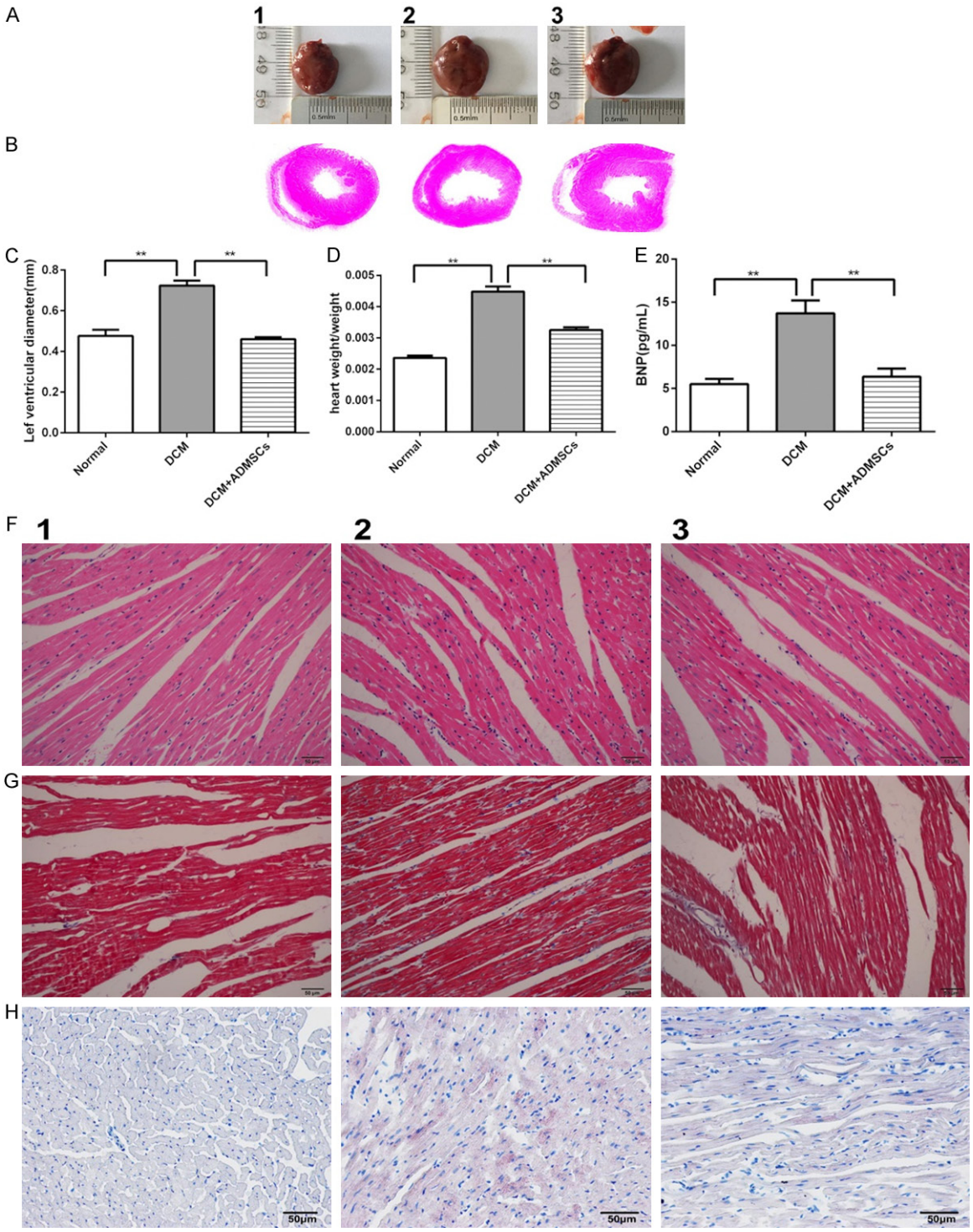


**Figure 3.** Morphology changes after ADMSCs infusion relative to DCM group. (A and B) Representative heart specimens and shorter axes of the heart showing left ventricular dimensions and wall thickness (1: normal group, 2: DCM group, 3: DCM+ADMSCs group). (C and D) The left ventricular diameter and the ratio of heart weight to body weight. (E) BNP in each group. (F) HE staining showed the tissue injury degree of heart. (G) Oil red O staining indicated triglyceride deposition in heart. (H) Masson staining revealed the heart fibrosis. (1: normal group, 2: DCM group, 3: DCM+ADMSCs group). Scale bar in (F-H) 50 μm. Data in (C-E) are mean ± SEM, n=5-8 for each group, $^{*}P<0.05$, $^{**}P<0.01$.

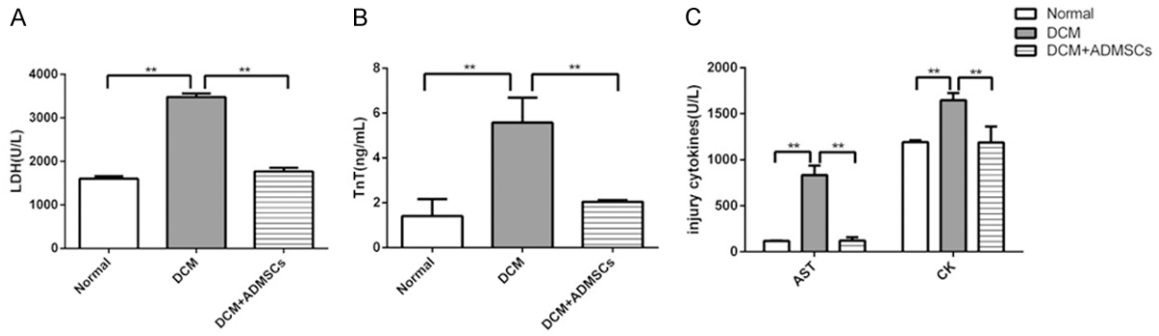


**Figure 4.** The injury cytokines in blood after infusion of ADMSCs in each group. Data in (A-C) are mean ± SEM, n=5-8 for each group, $^{*}P<0.05$, $^{**}P<0.01$.

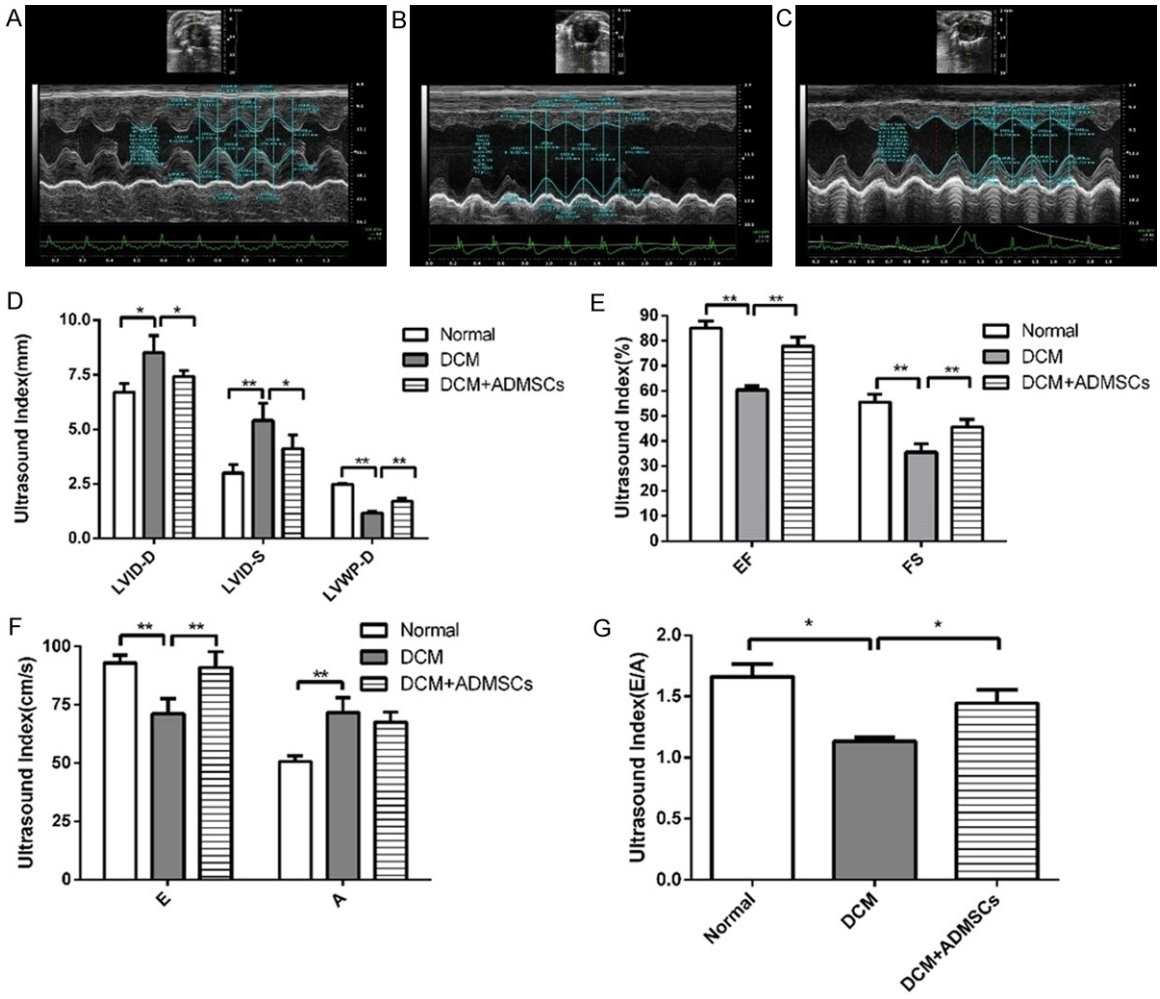


**Figure 5.** Infusions of ADMSCs improve the heart dysfunction. (A-C) Echocardiographic representative images of left ventricular (A: Normal group, B: DCM group, C: DCM+ADMSCs group). (D-G) Ultrasound indexes showing cardiac systolic and diastolic dysfunction. Data in (D-G) are means ± SEM, n=5-8 for each group, $^{*}P<0.05$, $^{**}P<0.01$.

decreased (**Table 1**), while the myocardial cell surface and mRNA level of BNP, ANP, β-MHC were significantly increased in HG group (**Figure 7A-C**). In HG+ADMSCs-CM group, the pulse frequency was significantly increased, while the myocardial cell surface and mRNA level of BNP, ANP, β-MHC were significantly decreased (**Figure 7D-F**).

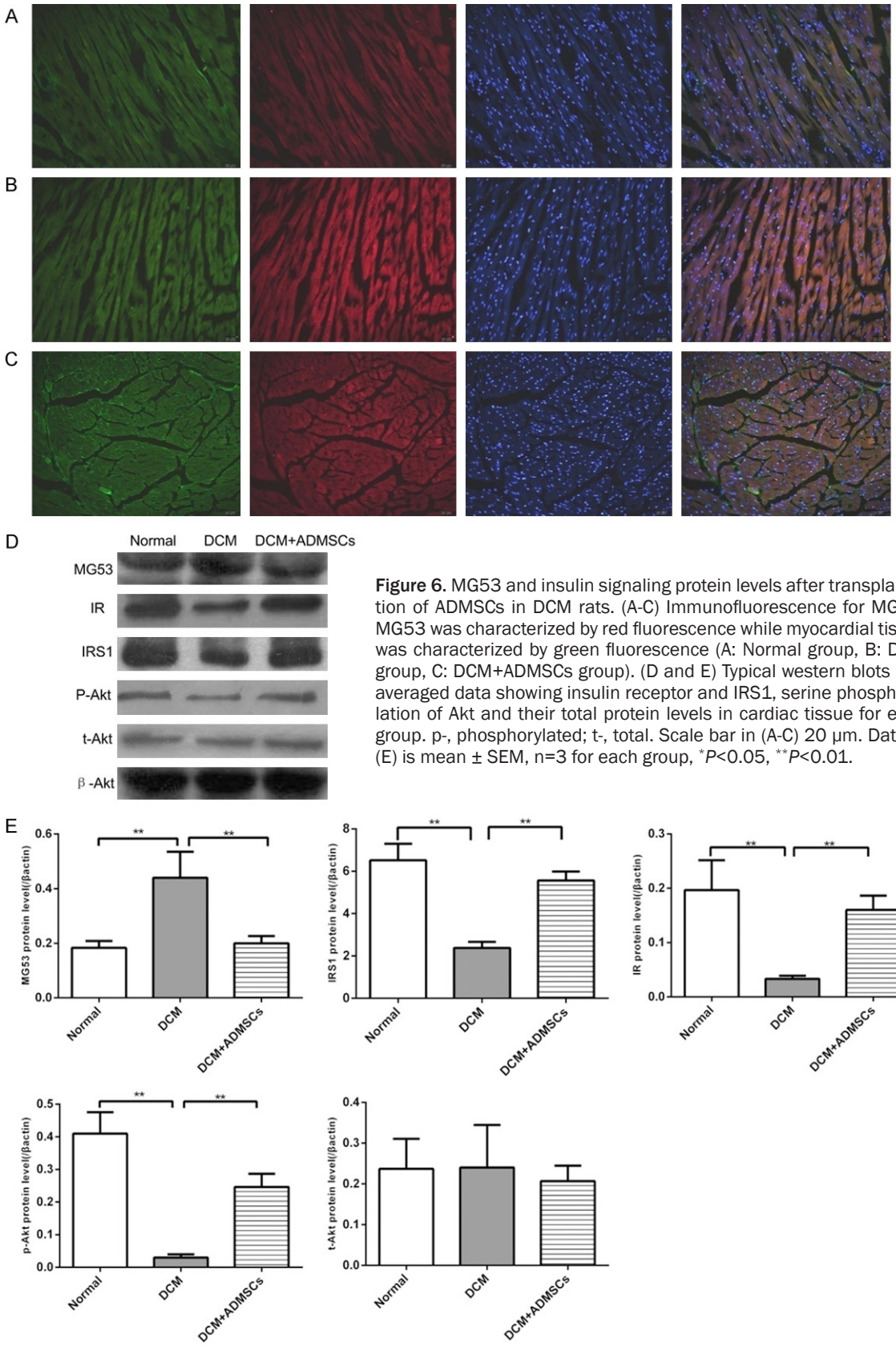


**Figure 6.** MG53 and insulin signaling protein levels after transplantation of ADMSCs in DCM rats. (A-C) Immunofluorescence for MG53. MG53 was characterized by red fluorescence while myocardial tissue was characterized by green fluorescence (A: Normal group, B: DCM group, C: DCM+ADMSCs group). (D and E) Typical western blots and averaged data showing insulin receptor and IRS1, serine phosphorylation of Akt and their total protein levels in cardiac tissue for each group. p-, phosphorylated; t-, total. Scale bar in (A-C) 20 μm. Data in (E) is mean ± SEM, n=3 for each group, $^{*}P<0.05$, $^{**}P<0.01$.

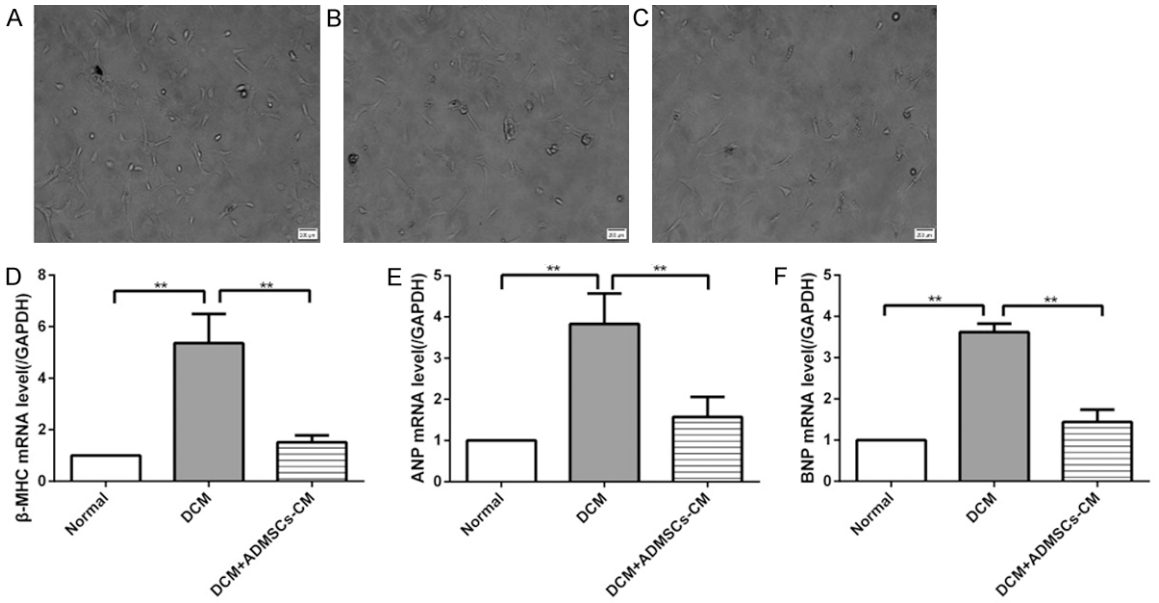


**Figure 7.** The morphology changes of cardiomyopathy induced by high glucose. (A-C) The morphology was tested under phase contrast microscope for each group. (D-F) The mRNA level of BNP, ANP, and β-MHC for each group. Scale bar in (A-C) 200 μm. Data in (D-F) are mean ± SEM, n=9 for each group, $^{*}P<0.05$, $^{**}P<0.01$.

After the successful establishment of myocardial injury in vitro, we use ADMSCs-CM gradient volume (2 ml, 1 ml, 0.5 ml, 0.25 ml) to interfer with the cardiomyocytes stimulated by high glucose. The expression of MG53 has been significantly suppressed with the increase volume of ADMSCs-CM, while, IR, IRS1 and p-Akt were all completely upregulated. The expression of MG53 was negatively correlated with IR, IRS1 and p-Akt (r=-0.94, -0.75, -0.84) (**Figure 8A** and **8B**).

## Discussion

Diabetic cardiomyopathy was first described in 1972 as a heart failure with cardiac remodeling and dysfunction and in the absence of hypertension, coronary artery disease, and valvar or congenital heart disease [14]. Metabolic disorders changed the cardiac structure and function by increasing myocardial fibrosis, oxidative stress, inflammation and apoptotic cell death, impairing mitochondrial respiratory capacity and $Ca^{2+}$ handling, and altering cardiac insulin signaling, which contributed to the development of DCM [15]. MG53 was originally defined as a protective factor of membrane repair and cardiac ischemia-reperfusion injury [16, 17]. However recent studies showed that overexpression of MG53 in cardiomyocytes could be sufficient to trigger DCM involving downregulation of IR, IRS1, p-Akt and upregulation of peroxisome proliferation-activated receptor, which could lead to cardiac metabolic disorders representing the cardiac hypertrophy and lipid toxicity [8]. The rat with HFD was more easily subjected to the high level of MG53, which was necessary to trigger hyperglycemia and insulin resistance. These phenotypes were not found in $MG53^{-/-}$ mice [7]. A number of recent studies and clinical trialshave indicated that transplanted bone marrow mesenchymal stem cells could reduce glucose levels [18-20], which have been conducted to II clinical trials, and ameliorate rat cardiac function with DCM [21]. The underlying mechanism was mainly considered to be that MSCs could regulate the cardiac inflammation via paracrine and upregulation of insulin receptor and IRS1 to improve the insulin resistance, which show the reverse effect of MG53. However, the relationship between MSCs and the expression of MG53 remained indistinctly understood. Our study was aimed at exploring the effect of adipose-derived mesenchymal stem cells on heart function and its underlying mechanism.

Our study first established rat model of DCM via HFD combined with STZ administration. Key characteristics of DCM are hyperglycemia, insulin resistance and heart dysfunction. Given the important role of hyperglycemia and insulin resistance on the pathogenesis of DCM, we evaluated the possible effects of MSCs on DCM

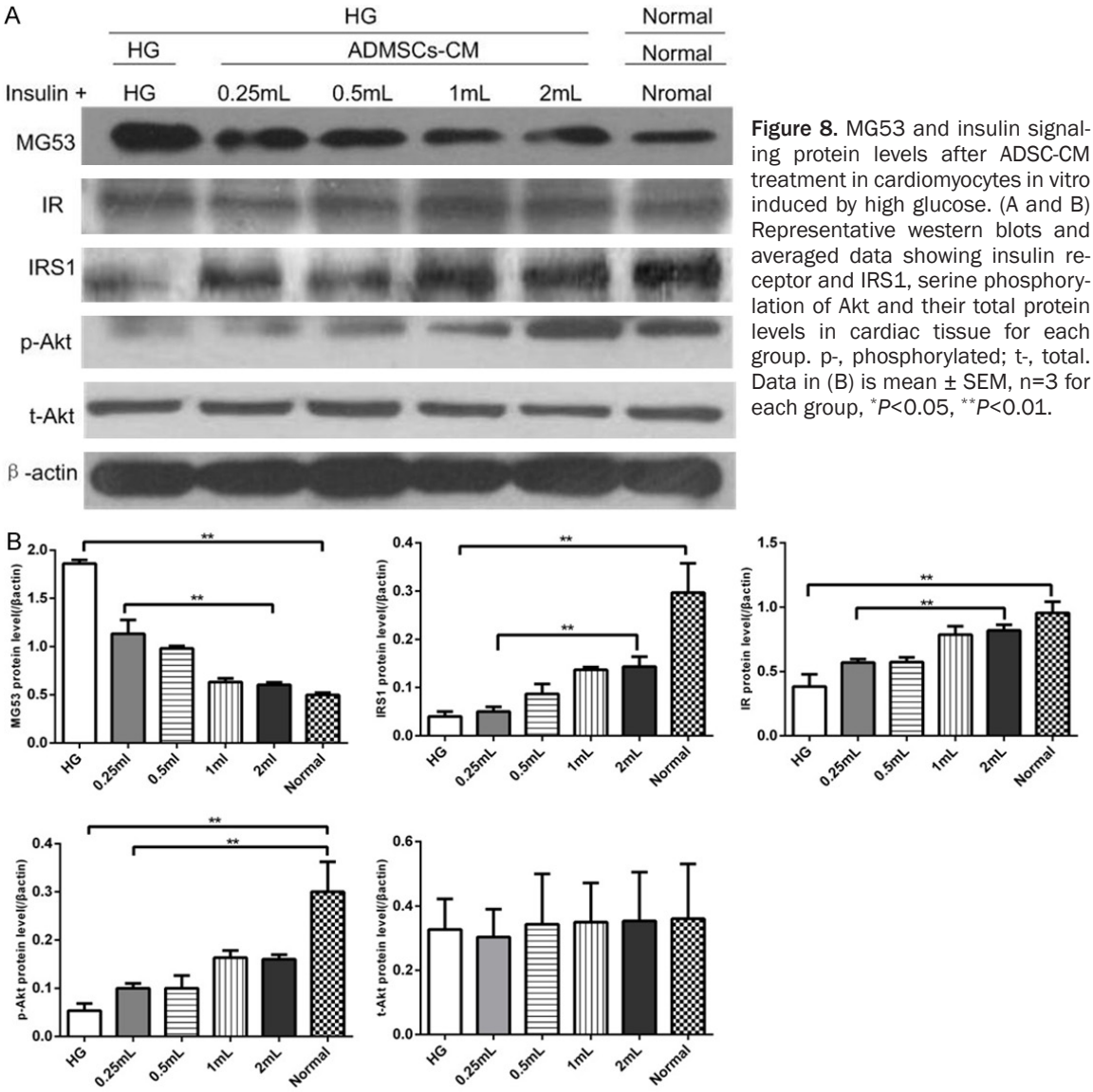


**Figure 8.** MG53 and insulin signaling protein levels after ADSC-CM treatment in cardiomyocytes in vitro induced by high glucose. (A and B) Representative western blots and averaged data showing insulin receptor and IRS1, serine phosphorylation of Akt and their total protein levels in cardiac tissue for each group. p-, phosphorylated; t-, total. Data in (B) is mean ± SEM, n=3 for each group, $^{*}P<0.05$, $^{**}P<0.01$.

rat glucose level. The results of blood glucose levels, OGTTs and IPITTs indicated that glucose metabolism and insulin sensitivity were significantly improved after the infusion of ADMSCs. Consistent with the previous findings [7, 8], our study demonstrated that MSCs treatment could reduce hyperglycemia, and improve insulin sensitivity and the function of β-cells. We observed that a single infusion of MSCs would not efficiently reduce the blood glucose compared to the four continuous infusions. These findings demonstrated that MSCs could ameliorate hyperglycemia and modulate the insulin sensitivity, especially with the use of continuous infusions.

It has been accepted that BNP is an indicator of heart failure representing the cardiac remodeling and AST, TnT, LDH and CK are the biomarkers of cardiac injury representing the hyperglycemia effect on the cardiomyopathy [23]. Our study showed that DCM rats possessed severe cardiomyocytes remodeling and injury as verified by increased interventricular inner diameter, the ratio of heart weight to body weight, BNP level, cardiac injury cytokines, cardiac hypertrophy and interstitial fibrosis. In addition, echocardiography results indicatedthat the heart function of DCM rats was impaired significantly. These results provided evidence that the mainly pathological changes of DCM includ-

ed cardiac interstitial fibrosis and hypertrophy, which were sufficient to trigger cardiac injury, further leading to the cardiac remodeling and heart dysfunction.

Because the main pathological changes of DCM include cardiac hypertrophy and stromal fibrosis, we further examined the effect of MSCs transplantation on cardiomyocytes phenotype changes and injury induced by DCM. Our histological findings suggested that MSCs could relieve cardiac hypertrophy and stromal fibrosis. After the four infusions of ADMSCs, the interventricular inner diameter, the ratio of heart weight to body weight and echocardiography indexes of cardiac diastolic and systolic function were significantly improved as previously reported [24]. Meanwhile, we found a decrease in BNP, AST, TnT, LDH and CK after transplantation of ADMSCS, which suggested that MSCs could improve DCM heart function and prevent cardiac remodeling. These findings demonstrated that MSCs could improve cardiomyocytes remodeling and heart function via inhibiting cardiac hypertrophy interstitial fibrosis, and injury in DCM rats.

Based on the crucial role of MG53 in the progress of DCM mentioned above, we examined the expression of MG53 and insulin signal proteins to explore the mechanism of MSCs working on diabetic cardiomyopathy. Insulin signaling pathway is one of the major pathways regulating cellular energy metabolism [24, 25] and induction of insulin resistance may be a key factor in metabolic derangement subjected to MG53 overexpression in hearts [7]. Upon insulin stimulation, IR and IRS1 are the pivotal factors which, in turn, activates phosphorylation of Akt to regulate the glucose homeostasis [26]. Our study showed that a HFD diet combined with STZ administration elevated MG53 expression level and simultaneously downregulated insulin receptor, IRS1 and p-Akt levels. On the contrary, MSCs could downregulate the high expression of MG53, and then upregulate the above protein levels. MG53 overexpression impaired insulin signaling in cardiomyocytes, which could lead to DCM, while MSCs are sufficient to suppress the overexpression of MG53 and further improve the insulin resistance.

To further investigate the relationship between MSCs and MG53, we conducted experiments in vitro. It has been reported that MSCs could secrete various cytokines to improve the microenvironment [27]. MSCs-CM as a kind of conditioned medium contained a variety of cell factors and has a therapeutic effect on tissue damage even in the absence of MSCs without considering its immunological rejection. Sun et al. proposed that MSCs-CM could upregulate the protein level of IR and IRS1 similar to MSCs to improve insulin sensitivity [28]. We successfully induced the diabetic myocardial injury through 33 mmol/L high glucose, which was evidenced by increasing cell surface and high mRNA level of ANP, BNP and β-MHC. In addition, we observed that MSCs-CM could relieve this injury. When given ADMSCs-CM gradient intervention, the expression of MG53 was suppressed, while, IR, IRS1 and p-Akt were all completely upregulated in a dose-dependent manner. The expression of MG53 was negatively correlated with IR, IRS1 and p-Akt. These results suggested that high glucose could simulate cardiac injury in vitro, which was an independent risk factor for DCM similar to hyperglycemia. MSCs-CM could alleviate cardiomyocytes injury induced by high glucose by downregulating the overexpression of MG53, thus elevating IR, IRS1 and p-Akt protein levels. Our study use MSCs-CM to dispose the injured cardiomyocytes, which further indicated that MSCs could ameliorate cardiac injury induced by high glucose through paracrine effects, but the underlying mechanism was unknown.

In summary, our study demonstrated that hyperglycemia was an additional risk factor for DCM, and MG53 played a crucial role in the cardiac injury in a DCM rat model. Importantly, ADMSCs could alleviate the cardiac injury and improve heart dysfunction of DCM rats, involving their ability to downregulate the overexpression of MG53, further elevate IR, IRS1 and p-Akt protein levels, which could improve insulin sensitivity and cardiac metabolism.

## Acknowledgements

This study was supported by the National Natural Science Foundation of China (8147-1052) and the Institute of Basic Medical Science, PLA General Hospital. The authors thank Professer Xu in Capital Medical University, Ms Feng Jie, Wang Hong for excellent technical assistance.

## Disclosure of conflict of interest

None.

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## References

[1] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27: 1047-1053.

[2] Calligaris SD, Conget P. Inravenous administration of bone marrow-derived multipotent mesenchymal stromal cells has a neutral effect on obesity-induced diacetic cardiomyopathy. Biol Res 2013; 46: 251-5.

[3] Wei LM, Ma BQ. Research progress of diabetic cardiomyopathy. Foreign Medical Sciences. Geriatrics 2002; 23: 18-21.

[4] Bugger H, Abel ED. Molecular mechanisms of diabetic cardiomyopathy. Diabetologia 2014; 57: 660-671.

[5] Patel TP, Rawal K, Bagchi AK, Akolkar G, Bernardes N, Dias Dda S, Gupta S, Singal PK. Insulin resistance: an additional risk factor in the pathogenesis of cardiovascular disease in type 2 diabetes. Heart Fail Rev 2016; 21: 11-23.

[6] Cao CM, Zhang Y, Weisleder N, Ferrante C, Wang X, Lv F, Zhang Y, Song R, Hwang M, Jin L, Guo J, Peng W, Li G, Nishi M, Takeshima H, Ma J, Xiao RP. MG53 constitutes a primary determinant of cardiac ischemic preconditioning. Circulation 2010; 121: 2565-74.

[7] Song R, Peng W, Zhang Y, Lv F, Wu HK, Guo J, Cao Y, Pi Y, Zhang X, Jin L, Zhang M, Jiang P, Liu F, Meng S, Zhang X, Jiang P, Cao CM, Xiao RP. Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. Nature 2013; 494: 375-9.

[8] Liu F, Song R, Feng Y, Guo J, Chen Y, Zhang Y, Chen T, Wang Y, Huang Y, Li CY, Cao C, Zhang Y, Hu X, Xiao RP. Upregulation of MG53 induces diabetic cardiomyopathy through transcriptional activation of peroxisome proliferatin-activated receptor α. Ciculation 2015; 131: 795-804.

[9] Volarevic V, Arsenijevic N, Lukic ML, Stojkovic M. Concise review: mesenchymal stem cell treatmentg of the complications of diabetes mellitus. Stem Cells 2011; 29: 5-10.

[10] Dong X, Zhu F, Liu Q, Zhang Y, Wu J, Jiang W, Zhang L, Dong S. Transplanted bone marrow mesenchymal stem cells protects myocardium by regulating 14-3-3 protein in a rat model of diabetic cardiomyopathy. Int J Clin Exp Pathol 2014; 7: 3714-23.

[11] Si Y, Zhao Y, Hao H, Liu J, Guo Y, Mu Y, Shen J, Cheng Y, Fu X, Han W. Infusion of mesenchymal stem cells ameliorates hyperglycemia in type 2 diabetic rats. Diabetes 2012; 61: 1616-25.

[12] Wang J, Hao H, Huang H, Chen D, Han Y, Han W. The effect of adipose-derived stem cells on full-thicness skin grafts. Biomed Res Int 2016; 2016: 1464725.

[13] Wang L, Lin GS, Yang B, et al. Co-culture of cardio myocytes induces mesenchymal stem cells to differentiate into cardiomyocyte-like cells. Basic and Clinical Medicine 2005; 10: 906-9.

[14] Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW, GrishmanA. New type of cardiomyopathy associated with diabetic glomerulosclerosis.Am J Cardiol 1972; 30: 595-602.

[15] Bugger H, Abel ED. Molecular mechanisms of diabetic cardiomyopathy. Diabetologia 2014; 57: 660-671.

[16] Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, Ko JK, Lin P, Thornton A, Zhao X, Pan Z, Komazaki S, Brotto M, Takeshima H, Ma J. MG53 nucleates assemble of cell membrane repair machinery. Nat Cell Biol 2009; 11: 56-64.

[17] Liu J, Zhu H, Zheng Y, Xu Z, Li L, Tan T, Park KH, Hou J, Zhang C, Li D, Li R, Liu Z, Weisleder N, Zhu D, Lin P, Ma J. Cardioprotection of recombinant human MG53 protein in a porcine model of ischemia and reperfusion injury. J Mol Cell Cardiol 2015; 80: 10-19.

[18] Ezquer FE, Ezquer ME, Parrau DB, Carpio D, Yañez AJ, Conget PA. Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. Biol Blood Marrow Transplant 2008; 14: 631-640.

[19] Jiang R, Han Z, Zhuo G, Qu X, Li X, Wang X, Shao Y, Yang S, Han ZC. Transplantation of placenta-derived mesenchymal stem cells in type 2 diabetes: a pilot study. Front Med 2011; 5: 94-100.

[20] Abdi R, Fiorina P, Adra CN, Atkinson M, Sayegh MH. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. Diabetes 2008; 57: 1759-1767.

[21] Dong S, Ma W, Hao B, Hu F, Yan L, Yan X, Wang Y, Chen Z, Wang Z. microRNA-21 promotes cardiac fibrosis and development of heart failure with preserved left ventricular ejection fraction by up-regulating Bcl-2. Int J Clin Exp Pathol 2014; 7: 565-74.

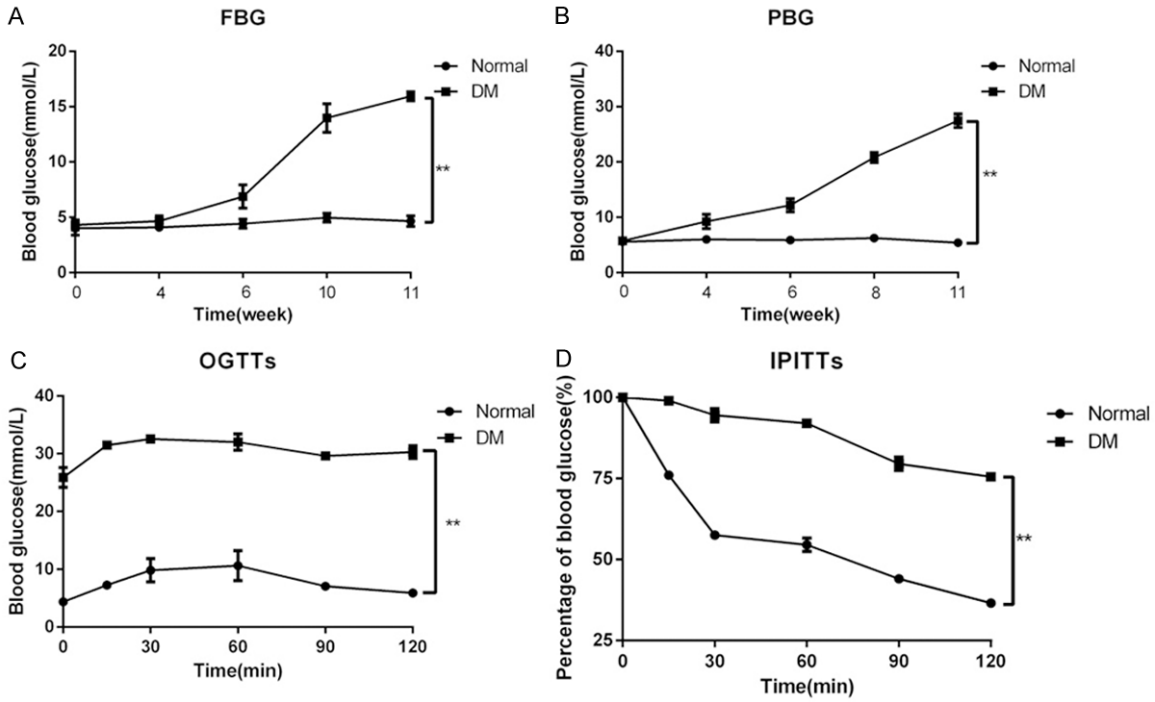
[22] Liew A, O'Brien T. The potential of cell-based therapy for diabetes and diabetes-related vascular complications. Curr Diab Rep 2014; 14: 469-79.

[23] Osmancik P, Louckova A. Biomarkers of apoptosis, inflammation and cardiac extracellular matrix remodeling in the prognosis of heart failure. Kardiol Pol 2016; [Epub ahead of print].

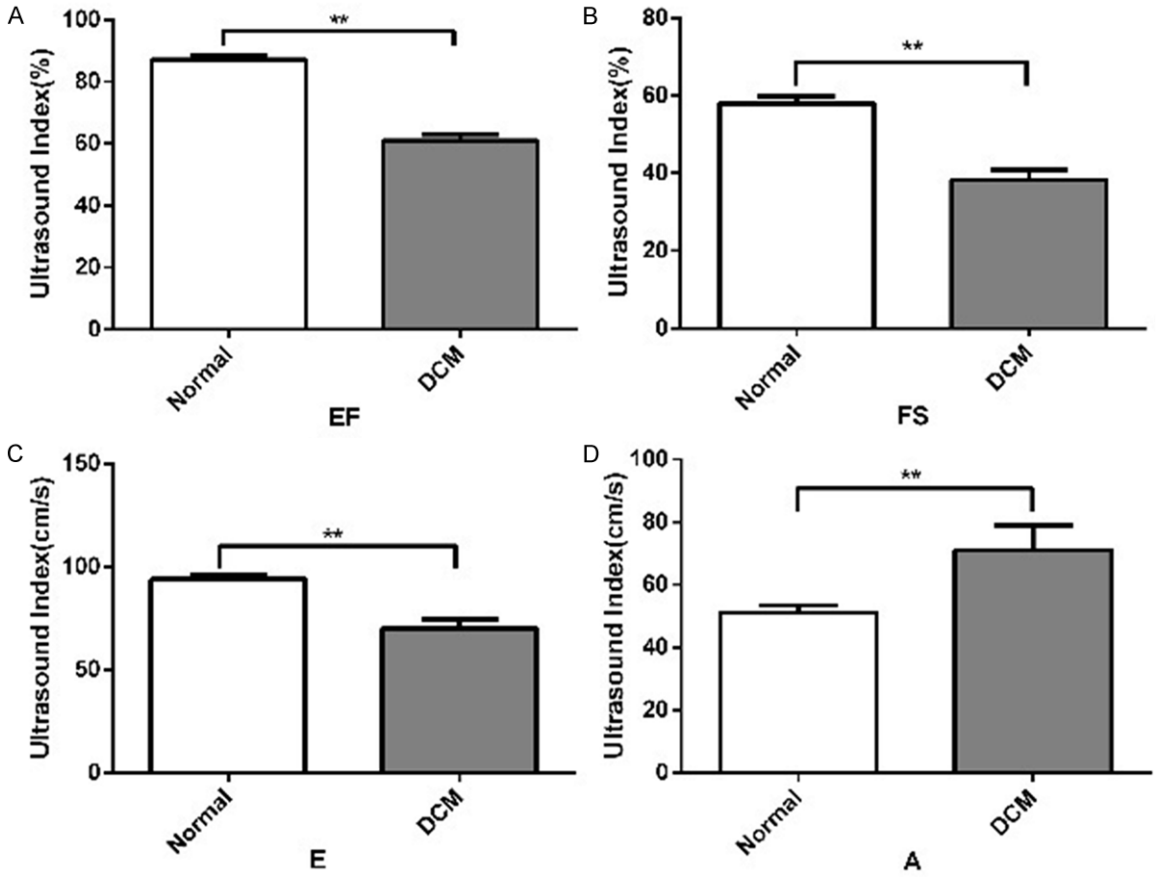
[24] Khan I, Ali A, Akhter MA, Naeem N, Chotani MA, Mustafa T, Salim A. Preconditioning of mesenchymal stem cells with 2,4-dinitrophenol improves cardiac function in infarcted rats. Life Sci 2016; 162: 60-9.
[25] Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995; 378: 785-789.
[26] Kohn AD, Kovacina KS, Roth RA. Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. EMBO J 1995; 14: 4288-4295.
[27] Thirone AC, Huang C, Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends Endocrinol Metab 2006; 17: 72-78.
[28] Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, Prockop DJ. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci U S A 2006; 103: 17438-17443.
[29] Sun X, Hao H, Han W, Mu Y. Effects of conditioned media for rat bone marrow-derived mesenchymal stem cells on palmitic acid-induced insulin resistance in HepG2 cells. Zhonghua Nei Ke Za Zhi 2015; 54: 439-444.

**Supplementary Table 1.** Primers of proteins for mRNA

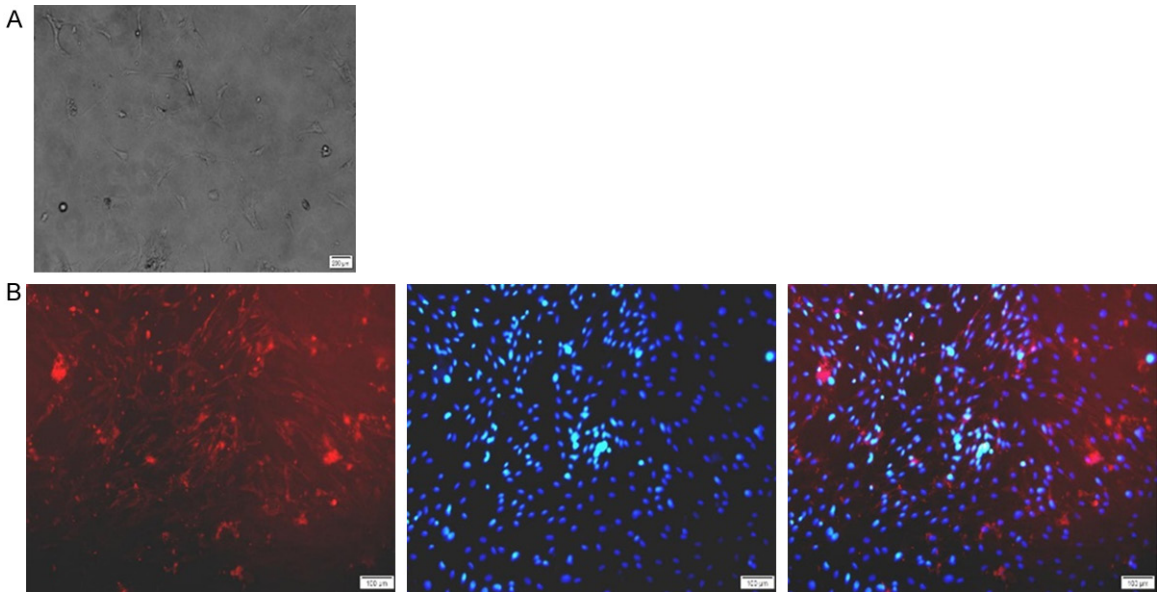
| Genes | Primers (5'-3') |
|---|---|
| MG53 | F-CGAGCAGGACCGCACACTT |
| | R-CCAGGAACATCCGCATCTT |
| ANP | F-CTCCGATAGATCTGCCCTCTTGAA' |
| | R-GGTACCGGAAGCTGTTGCAGCCTA |
| BNP | F-TTGGGCAGAAGATAGACCGGAT |
| | R-GGTCTTCCTAAAACAACCTCA |
| β-MHC | F-AACCTGTCCAAGTTCCGCAAGGTG |
| | R-GAGCTGGGTAGCACAAGAGCTACT |
| GAPDH | F-GACATCAAGAAGGTGGTGAAGC |
| | R-TGTCATTGAGAGCAATGCCAGC |



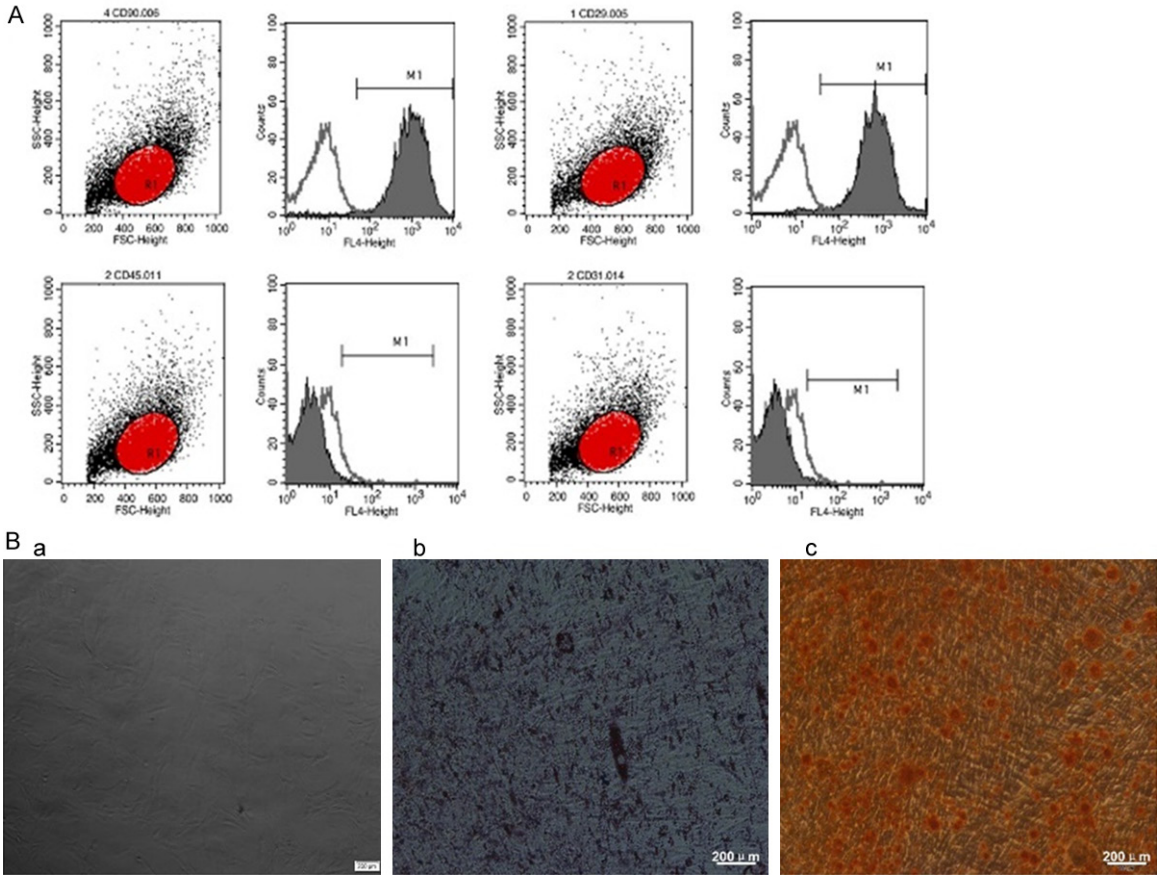

**Supplementary Figure 1.** Identification of diabetic rat model. (A and B) We monitored fastening and postprandial blood glucose before and after every administration of STZ. (C and D) At the 11th week, IPGTTs and IPITTs were performed. Data in (A-D) are mean ± SEM, n=8 for each group; $^{*}P<0.05$, $^{**}P<0.01$.




**Supplementary Figure 2.** Identification of diabetic cardiomyopathy rat model. (A-D) At the 18th week after the induction of DCM rat model, we performed echocardiography. Representative echocardiographic indexes showing cardiac dysfunction. Data in (A-D) are means ± SEM, n=5-8 for each group, $^{*}P<0.05$, $^{**}P<0.01$.




**Supplementary Figure 3.** Identification of cardiomyocytes. (A) Normal cultured cardiomyocytes. (B) Immunofluorescence for cardiomyocytes. Cardiac troponin was characterized by red fluorescence. Scale bar in (A, B) 100 μm.




**Supplementary Figure 4.** Identification of adipose-derived mesenchymal stem cells. (A) Normal cultured ADMSCs. (B) Immunologic phenotypes of ADMSCs. (A) ADMSCs used in our experiments were stained with CD90-APC, CD29-APC, CD45-PE, CD31-PE, then were analyzed by flow cytometry. (Ba) Normal cultured ADMSCs. (Bb) Adipocytes differentiation were stained by Oil red O staining. (Bc) Osteoblasts differentiation were stained by erythroskyrine. Scale bar in (B) 200 μm. Data are representative of three independent experiments.