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# MSC attenuate diabetes-induced functional impairment in adipocytes via secretion of insulin-like growth factor-1

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ABSTRACT

The function of subcutaneous adipocytes in promoting wound healing is significantly suppressed in diabetic wounds. Recent studies have demonstrated the ability of mesenchymal stem cell (MSC) to ameliorate impaired diabetic wound healing. We hypothesized that MSC function may involve subcutaneous adipocytes. The abnormal function of subcutaneous adipocytes from STZ induced diabetic mice including glucose uptake and free fatty acid (FFA) secretion level were assessed. Then these cells were co-cultured with MSC via a transwell system to observe the changes of metabolic index and glucose transporter four (GLUT4) as well as phosphoinositide 3-kinase/protein kinase (PI3K/AKT) signaling pathway expression. The results of metabolic index suggest that MSC obviously attenuated the diabetes-induced functional impairment. Both mRNA and protein expression analyses showed that PI3K/AKT insulin signaling pathway and GLUT4 expression were up-regulated. These changes were substantially associated with a increased level of insulin-like growth factor-1 (IGF-1) secretion from MSC. These findings suggest that MSC could attenuate abnormal function of diabetic adipocytes by IGF-1secretion, which was more or less associated with the beneficial effects of MSC on improving diabetic wound healing.



## 1. Introduction

Diabetes mellitus is prevalent in modern society and can ultimately result in several serious complications. Delayed or impaired wound healing frequently occurs in patients with diabetes and is of intractability due to impaired cutaneous regeneration capabilities. Simultaneously, the function of subcutaneous adipocytes in promoting wound healing is significantly suppressed [1,2] on the background of insulin resistance. Although pathogenesis of diabetic wounds remains obscure, the influence of functional impairment of adipocytes is more or less associated with the improper wound healing process.

Accumulating evidence strongly suggests that mesenchymal stem cells (MSC), also known as multipotent mesenchymal stromal cells showed tremendous potential in promoting diabetic wound healing [3]. Thus, its effects of MSC on subcutaneous adipocytes are of interest, which may improve the innovative therapeutics and further resolve impaired wounds.

It has been shown that MSC, particularly MSC from bone marrow (BM-MSC), produce and secrete a broad variety of cytokines, chemokines, and growth factors that may potentially be involved in wound repair [4,5]. Furthermore, diabetes suppressed the production of several of these factors [6]. In addition, auto-/paracrine function of adipocytes appeared to be impaired in diabetic mice [7,8]. In this work, we initially examined whether subcutaneous adipocytes from STZ-induced diabetic mice were functional impaired, as shown by their glucose uptake function and free fatty acid (FFA) secretion level. To elucidate the paracrine

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effect of MSC, we co-cultured subcutaneous adipocytes from diabetic mice with MSC from wild type mice using a transwell system and investigated the amelioration of insulin resistant and expression of glucose transporter four (GLUT4). Then the activation of the insulin-IGF-1 receptor insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase/protein kinase (PI3K–Akt) pathways were investigated which is the first step in the activation of the insulin signaling cascade. Lastly, we investigated the effects of IGF-1 siRNA MSC, further confirming the role of MSC in ameliorating insulin resistant in adipocytes.

In the study reported here, we demonstrated that MSC could ameliorate the diabetes-induced function impairment in adipocytes by exerting paracrine actions, mainly involving the secretion of IGF-1.

## 2. Materials and methods

All animal procedures were approved under the guidelines of the Institutional Animal Care and Use Committee of Chinese PLA General Hospital (Beijing, China).

### 2.1. Diabetes murine model and preparation of conditioned medium (CM)

The male C57BL/6 wild-type mice (4–6 weeks old) were fed by high fat diet (HFD) one month [9,10], then intraperitoneal injection of streptozotocin (STZ) (40 mg/kg) (Sigma, USA) for 3 consecutive days [9]. And the control group mice were fed normal diet with intraperitoneal injection of physiological saline. Four weeks later, a model with metabolic characteristics of type 2 diabetes (T2D) was achieved. Then the diabetic and normal mice were performed a 8 mm diameter full-thickness wound on the back and the secreted fluid was collected from normal and diabetic mice wound after 3 days. The collected culture supernatant was stored at −20 °C after centrifugation and sterilization. Then the CM was used to supplement the adipocytes medium for our future experiment.

### 2.2. The adipocytes isolation and culture

The preadipocytes were obtained from STZ-induced diabetic mice as previously researches [11–13] and the preadipocytes were cultured and differentiated as described [14]. Differentiated cultures contained at least 80% adipocytes and these cells were identified by oil-red O.

### 2.3. Observation the effect of secreted fluid of wound on adipocytes metabolism

The STZ-induced diabetic mice adipocytes were fed in 6 well plates and divided randomly as follows ($n$ = 6/group): (A) treated with normal mice wound secreted fluid; (B) treated with diabetic mice wound secreted fluid; (C) no treatment (control). After 48 h, the number of apoptosis cells and glucose and fat metabolism levels were detected in different groups.

### 2.4. Detection the apoptosis of adipocytes with different treatment

A terminal deoxynucleotidyl transferased UTP nick-end labeling (TUNEL) staining was performed at 6, 12, 24, 48, 72 h after different treatment by using In Site Cell Death Kit (Roche, Mannheim, Germany) following to the protocol. And TUNEL-positive cells were counted by a blinded pathologist followed five high power field of vision and expressed as percent of cell count.

### 2.5. Glucose uptake assays

2-[3H]deoxyglucose (2-DOG) was obtained from PerkinElmer Life (Waltham, MA). Then the adipocytes were grown in gelatin-coated 6 well plates and glucose uptake were detected as previous reports [15]. The cells were washed 5 min in KRP (12.5 mM HEPES pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.4 mM $NaH_2$ $PO_4$, 0.6 mM $Na_2HPO_4$). Then Cells were treated or not with insulin or serum for 20 min and 50 mM cytochalasin B was added (assay background) as control assay. At last, the Cells were incubated for 5 min with 0.1 mM 2-[3H]deoxyglucose (0.28 m Ci/well), extensively washed with ice-cold phosphate-buffered saline (PBS) three times, and lysed the cells in 1 mmoL/L NaOH. And the cell was centrifuged 1000 r/min, 10 min; radioactivity was measured by scintillation counting.

### 2.6. The detection of FFA

After the adipocytes treated with the normal and diabetic mice wound secreted fluid 48 h, and the adipocytes normal culture without any treatment as control group. The culture solutions were harvested into centrifugal tubes. FFA levels in the supernatant were assayed with commercial FFA ELISA Kits (Abcam).

### 2.7. Isolation, expansion and characterization of MSC

MSC were extracted from the bone marrow of the tibiae and femurs of 6-week-old male C57BL/6 mice by Ficoll-paque density gradient, then these cells were identified as previous reports [16,17]. And the fourth-passage MSC were used for experiment.

### 2.8. MSC pretreated with IGF-1 knockdown via siRNA and IGF-1 was detected in vitro

IGF-1 is an important secretion factor, which exhibits similar homologies action to insulin, is also an important regulator of the healing process. To determine the potential contribution of secreted IGF-1, MSC were pretreated with a designed siRNA to inhibit the IGF-1 effect. The siRNA was examined by using NCBI BLAST and obtained from Genechem co. (Shanghai, China). MSC were prepared for transfection by seeding 5 × 105 cells into each well with complete media. After 24 h, the media was removed, the cells were washed with PBS, and the antibiotic free/low serum Optimem media (GIBCO Invitrogen) was added. SiRNA was then complexed with Lipofectamine 2000 (Invitrogen) in Optimem media and incubated at room temperature for 20 min. Complexes were then added to culture wells to yield a final siRNA concentration of 100 nM/well. The media and siRNA complexes were removed after 24 h, normal complete media was added, and the cells were allowed to incubate for an additional 72 h. MSC were transfected with Lipofectamine 2000 only (mock) or scrambled siRNA as control. At last, the cells growth cure was examined by Cell Counting Kit-8 (cck-8) (Beyotime institute of Biotechnology, China) and the blocking efficiency in each group was analyzed by qRT-PCR (IGF-1: 5′-GGTGGTTTATGAATGGTT-3′ and 5′-AGGGTG TGTCTAATGGAG-3′) and ELISA assays.

### 2.9. Adipocytes and MSC transwell co-culture

We have used transwell plants to observe the influence of MSC on regulating adipocytes metabolism. Adipocytes and MSC cultured alone in upper and lower transwell chambers respectively. Adipocytes were seeded above transwell 6 well inserts with a pore size of 0.4 μm for incubation in diabetic CM. And MSC cultured alone in lower transwell chambers. The experiment was divided 3 groups randomly as follows ($n$ = 6/group): (A) adipocytes with

diabetic CM (control); (B) adipocytes with diabetic CM + MSC; (C) adipocytes with diabetic CM + MSC (IGF-1 siRNA), After 48 h, the glucose and fat metabolism levels were detected again to observe the influence of MSC on adipocytes metabolism.

### 2.10. Total protein expression

Total protein isolated from the samples and 20 μg protein were dissolved in substrate-soluble buffer, and separated electrophoretically on a 8% SDS–polyacrylamide gel. Subsequently, proteins were transferred on to nitrocellulose membrane that was blocked with 5% bovine serum albumin 2 h. Then it was exposed to desired primary antibodies (PI3K, AKT, GLUT4, IRS-1 and β-actin) at 4 °C overnight. After washing, the membranes were incubated with HRP-connected IgG secondary antibody (Rockland, USA) at room temperature for 2 h and washed with TBST thrice, each 5 min. Then the protein bands were detected by chemiluminescence (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) and were taken photos in Universal gel imaging analysis system (Image Quant LAS4010, USA).

### 2.11. Total RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA was separated from cells after various treatments. The primers used for gene amplification were: GLUT4: 5′-ACTCTTGCCACACAGGCTCT-3′ and 5′-CCTTGCCCTGTCAGGTATGT-3′; PI 3K: 5′-AGGGAAGAGGTGAATGAGAG-3′ and 5′-TTGGACACAGGGTAGAGAAG-3′; IRS-1:5′-TCAACCACAGCCGCTCCATCCC-3′ and 5′-ATCG CTAGGCGAACCGGACACG-3′; AKT: 5′-ATCCCCTCAACAACTTCTCAGT-3′ and 5′-CTTCCGTCCACTCTTCTCTTTC-3′; β-actin: 5′-CCTGA GGCTCTTTTCCAGCC-3′ and 5′-TAGAGGTCTTTACGGATGTCAACGT-3′. Reactions were performed using One Step RT-PCR Kit Ver. 2 (Dye Plus) (Takara, Japan: www.takara-bio.co.jp) and the DNA ladders were taken photos by BIO-RAD LightCycler instrument and software (Bio-Rad, Hercules, CA, USA).

### 2.12. Statistical analysis

All values are expressed as mean ± SD. Statistical analysis was performed using SPSS13.0. Data were compared by One-way ANOVA, if the variance was homogeneous, the Least-significant Difference (LSD) was used between the groups comparison; and the variance was not homogeneous, the Dunnett T3 was used for the groups comparison. A probability ($P$) value <0.05 was considered significant.

## 3. Results

### 3.1. Observation the damage in adipocytes caused by diabetic wound environment

Our previous research revealed that the disorder metabolism and insulin resistance in diabetic mice wound. To observe the influence of the wound secretion composition on wound fat layer, the diabetic and normal wound secretion was added in STZ-induced mice adipocytes and the adipocytes normal culture as the control group. The results showed better cell morphology, more adipocytes (Fig. 1A) and fewer apoptosis cells (Fig. 1B) in adipocytes normal culture (control group) than other groups and the worst cell morphology, fewest adipocytes and more apoptosis cells in diabetic wound secretion treatment group. Also, the damaged effect on glucose intake and increased secretion of FFA on adipocytes treated with diabetic wound secretion composition (Fig. 1C–D), which showed weaken glucose utilize and increased insulin resistance. And normal wound secretion composition treated group, adipocytes exhibited the slightly weaker glucose and FFA metabolism compared with adipocytes with normal culture.

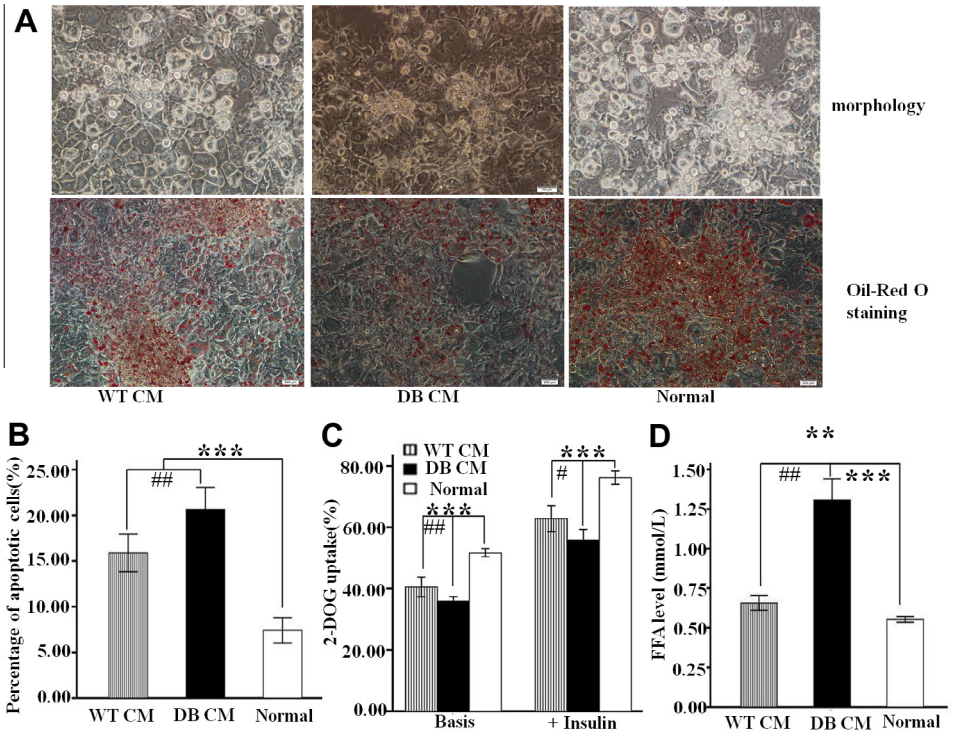


**Fig. 1.** The injured function of diabetic damage in adipocytes. (A) Morphology of differentiated of fourth passage preadipocytes and adipogenesis was stained by oil red O after induced 15 day. (B) Adipocytes cells apoptosis assays: adipocytes apoptosis was examined using TUNEL assay at 48 h after treatment with control (adipocytes normal culture), adipocytes + wild type CM or adipocytes + diabetic CM and the apoptosis index in the different groups. (C) Glucose uptake assays in each group of adipocytes. (D) The FFA level by adipocytes secretion in each group (scale bars = 100 μm) (data are expressed as means ± SD, $n = 6$, $^{**}P < 0.01$ and $^{***}P < 0.001$ and adipocytes + wild type CM group compared with adipocytes + diabetic CM group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$).

And our results showed the adipocytes function of 48 h exchanged more obviously than other time, and dates of the other time did not show.

### 3.2. Recognition features of MSC and transfection effects on MSC in vitro

MSC were isolated from bone marrow plastic adherence and spindle-shaped properties (Fig. 2A) and the cells showed osteogenic and adipogenic differentiationpotential in vitro (Fig. 2B and C); Flow cytometry analyses of these cultured cells at fourth passage showed expression of typical MSC markers such as CD105, CD29, Sca-1 but the absence of CD45 and CD34 (Fig. 2D). As our previous reports, it is known that IGF-1 secreted by MSC contributed to improvement on wound healing. The potential contribution of IGF-1 was examined by pretreating with IGF-1 siRNA, and the transfected side effects were detected by cell proliferation (cck-8 assay) (Fig. 2E). From the results: There were no significant differences of cell proliferation in Mock-transfected cells or cells transfected with the scrambled siRNA or IGF-1 siRNA, suggesting transfection had no obvious side effects on MSC. Additionally, transfected efficiency detected not only on gene level but protein levels and our data revealed that secreted IGF-1 was significantly downregulated by qRT-PCR and ELISA assays in MSC treated with IGF-1 siRNA group (Fig. 2F and G).

### 3.3. MSC paracrine proteins IGF-1 attenuate the effects of diabetic damage in adipocytes

IGF-1 was similar action to insulin, which could relive insulin resistance and in our experiment adipocytes was found to be damaged metabolism and insulin resistance suffering from diabetic environment. So we have seeded adipocytes and MSC in transwell system to observe whether IGF-1 secreted by MSC on the effects on reliving STZ-induced mice adipocytes damage via promoting metabolism (Fig. 3A). And the results showed that MSC had the function on reliving adipocytes damage suffering from diabetes via promoting glucose metabolism (Fig. 3B) and decreasing FFA secretion (Fig. 3C); at the same time, the improvement metabolism was weaker in adipocytes + MSC (IGF-1 siRNA) treatment group than adipocytes + MSC group, suggested the function of MSC on adipocytes might associate with secretion protein IGF-1 (Fig. 3A–C).

### 3.4. Insulin signaling pathway was up-regulated in ameliorative metabolism of damaged adipocytes

Figs. 1 and 3 have showed that MSC could relive adipocytes damage suffering from diabetes which was associated with the IGF-1 insulin similar action, and we have collected the adipocytes and extracted the protein and gene to analysis the possible mechanism. RT-PCR and western blot assay revealed that adipocytes with diabetic wound CM was obviously reducing the number of IRS-1 receptor reduced, insulin pathway PI3K/AKT was blocked and leaded to decrease GLUT4 expression, resulted in damaged metabolism and increased insulin resistance (Fig. 4A and B). MSC treated damaged adipocytes were significantly increased the number of IRS-1 receptor , promoted insulin similar action and insulin pathway protein PI3K and AKT expression and up-regulated GLUT4 expression not only in gene level but also in protein level (Fig. 4C and D). And higher expression appeared in MSC treated group than that in MSC (IGF-1 siRNA) treated group, suggested the modulatory function was associated with IGF-1secretion by MSC. All these results together implied reduced insulin receptor, increased insulin resistance and disorder metabolism might be associated with

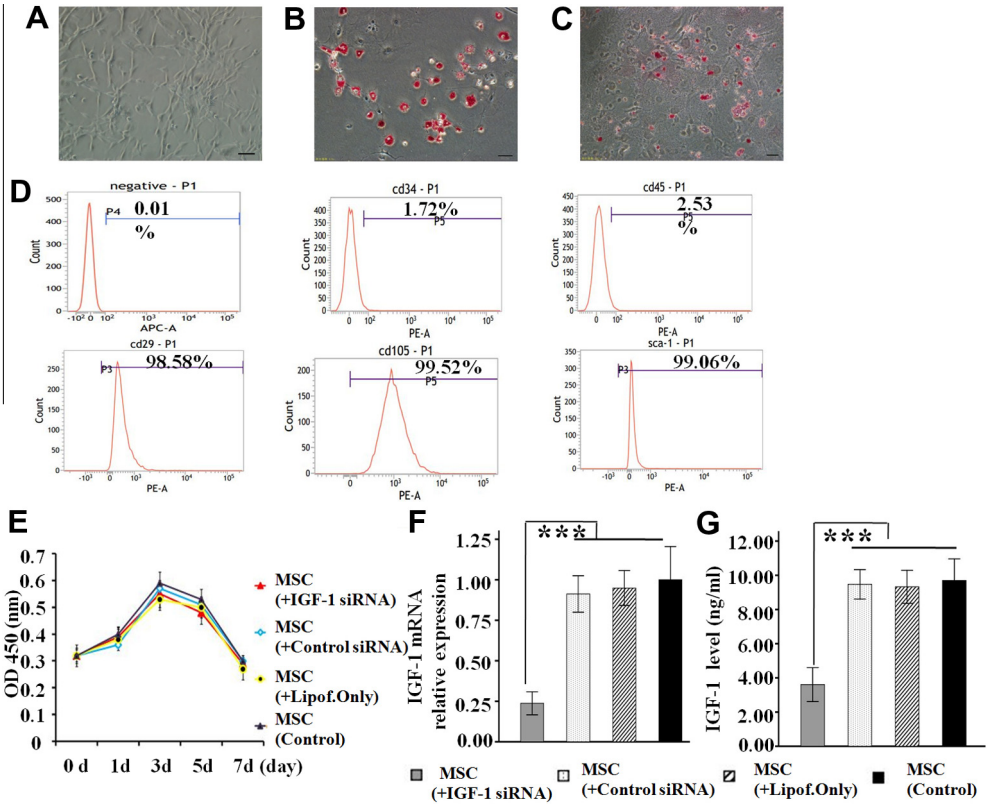


**Fig. 2.** Identification of the characterization of MSC and detection the effects of siRNA transfection on MSC. (A) Morphology of fourth passage MSC. (B) Adipogenesis was stained by oil red O after induced 15 day. (C) Osteogenesis was stained by alizarin red after induced 21 day. (D) Cell-surface markers were assessed by FACS. (E) Cell proliferation was assessed by cck-8 assay. (F–G) Comparison of the transfection efficient by PCR and ELISA assays (scale bars = 100 μm) (data are expressed as means ± SD, $n = 6$, $^{***}P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

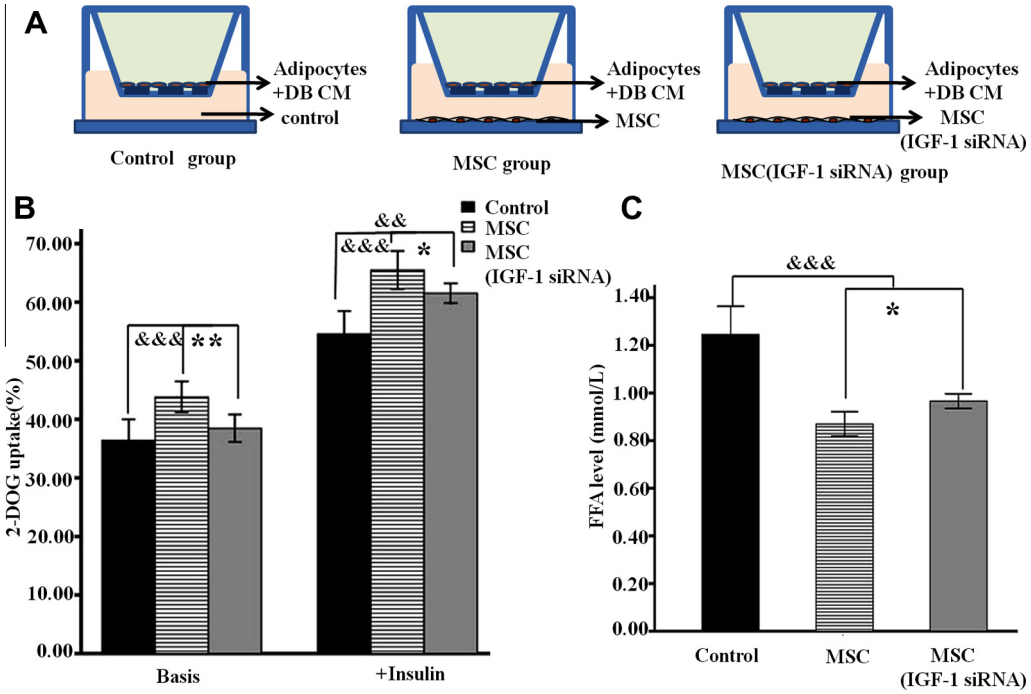


**Fig. 3.** MSC improved glucose and lipid metabolism of diabetic damage in adipocytes. (A) Transwell co-culture system in three groups: control group: adipocytes + medium added diabetic wound fluid (DB CM) in upper chamber, normal medium was in lower chamber; MSC group: adipocytes + medium added diabetic wound fluid (DB CM) in upper chamber, normal medium + MSC was in lower chamber; MSC (IGF-1 siRNA) group: adipocytes + medium added diabetic wound fluid (DB CM) in upper chamber, normal medium + MSC (IGF-1 siRNA) was in lower chamber. (B) Glucose uptake assays in each group. (C) The FFA level by adipocytes secretion in each group (data are expressed as means ± SD, $n = 6$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ and MSC group compared with MSC (IGF-1 siRNA) group, $^{*}P < 0.05$ and $^{**}P < 0.01$).

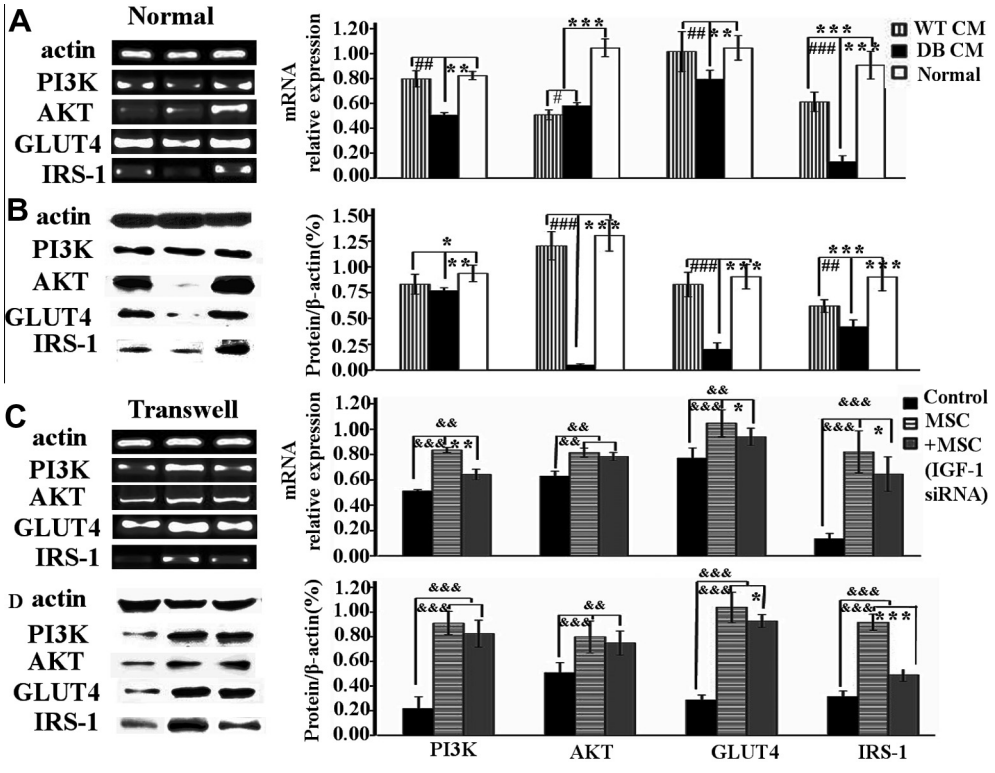


**Fig. 4.** MSC adminstration increased the expression of GLUT4 and insulin relative pathway expression. (A and C) mRNA levels of IRS-1, GLUT4 and PI3K/AKT expression by RT-PCR in observing the effect of different wound fluid on adipocytes and observing the effect of MSC on adipocytes metabolism via transwell co-culture. (B and D) Quantitative analysis of relative IRS-1, GLUT4 and PI3K/AKT expression levels normalized to β-actin in protein level in observing the effect of different wound fluid on adipocytes and observing the effect of MSC on adipocytes metabolism via transwell co-culture (all data are expressed as the mean ± SD, $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ and adipocytes + wild type CM group compared with adipocytes + diabetic CM group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$; $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ and MSC group compared with MSC (IGF-1 siRNA) group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{**}P < 0.001$).

damaged insulin relative function in adipocytes suffering from diabetic microenvironment.

## 4. Discussion

In our previous research and other reports [18,19], the delayed diabetic wound exhibited a well consequence by MSC administration and it might be associated with the improved metabolism conditions of local tissues. Given that the close relationship between cutaneous wound healing and the subcutaneous adipocytes, there has been considerable interest in determining whether MSC alter the imbalance metabolism conditions of subcutaneous adipocytes response to diabetes. The main goal of the present study was to understand whether MSC could ameliorate the function impairment in subcutaneous adipocytes induced by diabetes and whether this effect is related to paracrine actions of MSC. To the best of our knowledge, this study is the first to investigate the relation between MSC efficiency and the function impairment in diabetic adipocytes. More importantly, our finding that impaired metabolism function of subcutaneous adipocytes is associated positively with diabetic microenvironments and could be improved by paracrine mechanism of MSC provide a plausible explanation for the relation between metabolism of subcutaneous adipocytes and consequence of cutaneous wound healing in diabetic models. To evaluate the possible effect of MSC on metabolism of diabetic subcutaneous adipocytes, we established a T2D mouse model as previous reports [20] and observed the influence of MSC on biological efficiency and metabolism of adipocytes response to diabetic wound microenvironments. As demonstrated in Fig. 1, the biological efficiency and glucose metabolism was obviously damaged and FFA secreted was increased in adipocytes treated with diabetic CM. These findings exhibited increased diabetes-induced adipocytes damaged suffering from diabetic environment, which could explain why diabetic condition delayed the cutaneous wound healing process [21]. And from Fig. 3 we observed the effects of MSC on damaged adipocytes via transwell co-culture system in vitro, the results revealed that MSC could promote glucose uptake and reduce FFA secretion, which more or less attenuate diabetes-induced functional impairment in adipocytes.

It is noteworthy that we discovered IGF-1, secreted from MSC could play a direct modulatory role in GLUT4 activation to ameliorate glucose metabolism and insulin sensitivity, which is in accord with the data from previous reports involving positive effects of IGF-1 [22,23]. In addition, previous evidences showed that type 2 diabetes individuals treated with recombinant human IGF-I exhibited reduced insulin dose requirements, and decreased serum glucose levels [24]. However, previous studies did not clarify the relationship between MSC derived IGF-1 and insulin sensitivity. Furthermore, GLUT4 expression was an efficient index of insulin resistance and GLUT4 and PI3K/AKT were crucial for conferring insulin signaling transduction and glucose uptake in main target organs [25]. As demonstrated in Fig. 4, MSC administration increased GLUT4 and PI3K/AKT expression on damaged adipocytes suffering from diabetic environment. These findings demonstrated that MSC could ameliorate metabolism by modulating insulin sensitivity.

To future determine the contribution of IGF-1secretion by MSC, we have pretreated MSC with IGF-1 siRNA and compared the metabolism changes and insulin pathway protein expression with damaged adipocytes. Compared with MSC (IGF-1 siRNA), normal MSC treatment depressed glucose and lipid in damaged adipocytes, and the metabolism indexes was better than damaged adipocytes only with diabetic CM. At the same time, the insulin dependent signaling pathway PI3K/AKT and GLUT4 expression was matched with metabolism level, which also suggested that MSC played a direct modulatory role in insulin sensitivity via PI3K/AKT up-regulation GLUT4 expression. On the other hand, with MSC administration, the expression of IRS-1 receptor was obviously elevating in damaged adipocytes, which also implied that MSC could promote insulin sensitivity through IGF-1 connected to IRS and consequently promote the expression of receptors [26]. Our observation might explain the mechanism of MSC effects on ameliorating damaged adipocytes in diabetes-induced mouse, including promoting metabolism and relive insulin resistance.

In conclusion, our findings indicate that impaired metabolism function of adipocytes resulting from diabetes could be ameliorated by MSC derived IGF-1 increased GLUT4 expression in damaged adipocytes via PI3K/AKT pathway and attenuated the effects of diabetic damage in adipocytes, which was associated with reduced insulin resistance and accelerated metabolism.

## Competing interest statement

The authors declare that they have no competing financial interests.

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