

Original Article

Bone marrow mesenchymal stem cells accelerate wound healing in diabetic mice via inhibiting the expression of microRNA-155 to up-regulate Sirt1 in endothelial cells

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Abstract: Background: Bone marrow mesenchymal stem cells (BMSCs) have been widely explored as a useful modality in chronic wounds. Moreover, research data on miRNAs in wound healing are sparse and the role of miRNAs in the wound healing of diabetic foot are not yet known. Methods: A mouse model of diabetic foot wound was established and the wound was covered with collagen containing BMSCs (BMSCs group) or collagen alone (control group). The mRNA level of miR-155, miR-21, miR-126, miR-29b, miR-143, miR-196a and Sirt1 were detected by qRT-PCR. In HUVEC-12 cells, miR-155 mimic or inhibitor were used to investigate the effect of miR-155 on Sirt1. Furthermore, MTT assay and transwell chamber experiments were performed to examine HUVEC-12 proliferation and migration treated with BMSCs and miR-155 mimic or si-Sirt1. Results: In this study, we observed that BMSCs accelerated wound healing in diabetic mice. BMSCs decreased the expression of miR-155 and enhanced the expression of Sirt1 in wound tissue. In HUVEC-12 cells, miR-155 down-regulated the expression of Sirt1. Moreover, miR-155 mimic and si-Sirt1 could reverse the effect of BMSCs on HUVECs proliferation and migration. Additionally, miR-155 mimic worsened the wound healing in diabetic mice treated with BMSCs. Conclusion: We found that BMSCs accelerated wound healing in diabetic mice via inhibiting the expression of miR-155 to up-regulate Sirt1.

Keywords: Bone marrow mesenchymal stem cells, diabetic foot, miR-155, Sirt1, wound healing

Introduction

Diabetic foot, as an intractable and high rate complication in patients with diabetes mellitus, may lead to amputation in the end [1]. In fact, diabetic foot is a typical chronic wound characterized by absence of proliferation and migration of cells, associated with narrowing of blood vessels within the wound edge [2, 3]. Current studies indicate that diabetic foot disease mainly results from diabetic peripheral vasculopathy, peripheral neuropathy, and regional ischemic ulcer [4]. However, the pathogenesis of wound healing disorders in diabetic patients is poorly understood.

In recent years, bone marrow mesenchymal stem cells (BMSCs) have been found to enhance healing of difficult wounds [5]. BMSCs

can differentiate into a number of cell types, including muscle, fibroblasts, brain cells and bone [6]. BMSCs also produce numerous cytokines, growth factors and angiogenic factors that are involved in the repair of injured tissues. Several preliminary researches suggest that implantation of BMSCs can significantly accelerate wound closure and enhance re-epithelialization and wound vascularity in diabetic mice [7-9]. Other than differentiation, one of the basic mechanisms by which MSCs are suggested to alter the host microenvironment might be that MSCs possibly transfer vesicular components containing mRNA, microRNA and proteins [10, 11].

Recent studies have showed the vital role of microRNAs (miRNAs) in the regulation of gene expression in various cells of the skin, including

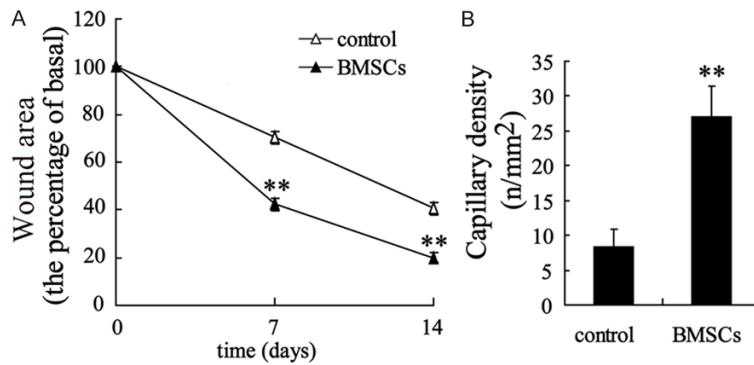


Figure 1. BMSCs accelerated wound healing in diabetic mice. The diabetic foot model was produced after four weeks of diabetes induction. The wounds were covered with collagen alone or collagen containing 2×10^4 BMSCs. A. The wound healing rate of mice in the control and BMSCs group. B. The capillary density in the control and BMSCs group. Control group: the wounds were covered with collagen alone; BMSCs group: the wounds were covered with collagen containing 2×10^4 BMSCs. The values are the mean \pm SE. **VS control, $P < 0.01$.

immune cells, stem cells and keratinocytes [12]. It has been proposed that changes in the expression of specific miRNAs are involved in wound healing process, and that aberrant regulation of miRNAs play a key role in the abnormal healing of chronic wounds [2, 13]. However, research data on miRNAs in wound healing are sparse and the role of miRNAs in the wound healing of diabetic foot are not yet known.

In this scenario, we first found the differential expression of miR-155 in BMSCs-treated diabetic wound healing through detecting the expression of several miRNAs. Then we highlighted the role of miR-155 in BMSCs accelerating wound healing in diabetic mice.

Materials and methods

Animal experiments

Five-to 8-week-old male C57BL/6J mice from the Laboratory Animal Center in Henan province people's hospital were injected with streptozotocin (STZ, Sigma) at 40 mg/kg for 5 days to induce diabetes. After 4 weeks of experiments, diabetic mice were successfully generated and then used for the diabetic foot model, according to the previously procedures [4, 14]. Full-thickness excisional wound (5×5 mm) was created in the dorsal thigh skin of both legs after proximal femoral artery ligation. To determine the healing potential of BMSCs in the model of diabetic foot, the wounds were cov-

ered with collagen alone (control group, $n=10$) or collagen containing 2×10^4 BMSCs (BMSCs group, $n=10$). All the procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals and were approved by the ethics committee of Henan province people's hospital.

Cell culture and experimental groups

Human umbilical vein endothelial cells (HUVECs) and Green fluorescent protein (GFP)-transfected BMSCs were purchased from Cyagen Biosciences (Guangzhou,

China). BMSCs were used between passage 4 and passage 6 in experiments. The cells were routinely cultured according to the instructions of the supplier. To detect the effect of miR-155 overexpression on the function of BMSCs to HUVECs proliferation and migration, HUVECs were divided into four groups; one group (control group) was cultured in a high-glucose medium (30 mM glucose), and the other three groups were cultured in a high-glucose medium with BMSCs, or BMSCs and pre-NC, or BMSCs and miR-155 mimic. To detect the effect of si-Sirt1 on the function of BMSCs to HUVECs proliferation and migration, HUVECs were divided into four groups; one group (control group) was cultured in a high-glucose medium (30 mM glucose), and the other three groups were cultured in a high-glucose medium with BMSCs, or BMSCs and si-control, or BMSCs and si-Sirt1.

Wound healing effects

After animal surgery, the images of the wounds were taken on days 0, 7 and 14 and the wound areas were analyzed using IPP software. On day 14 postoperative, the animals were sacrifice and the wound areas were harvested for immunohistochemistry. Capillary densities of the wounds were recognized for histological quantitative analysis in a blind manner [14].

Western blot analysis

The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis

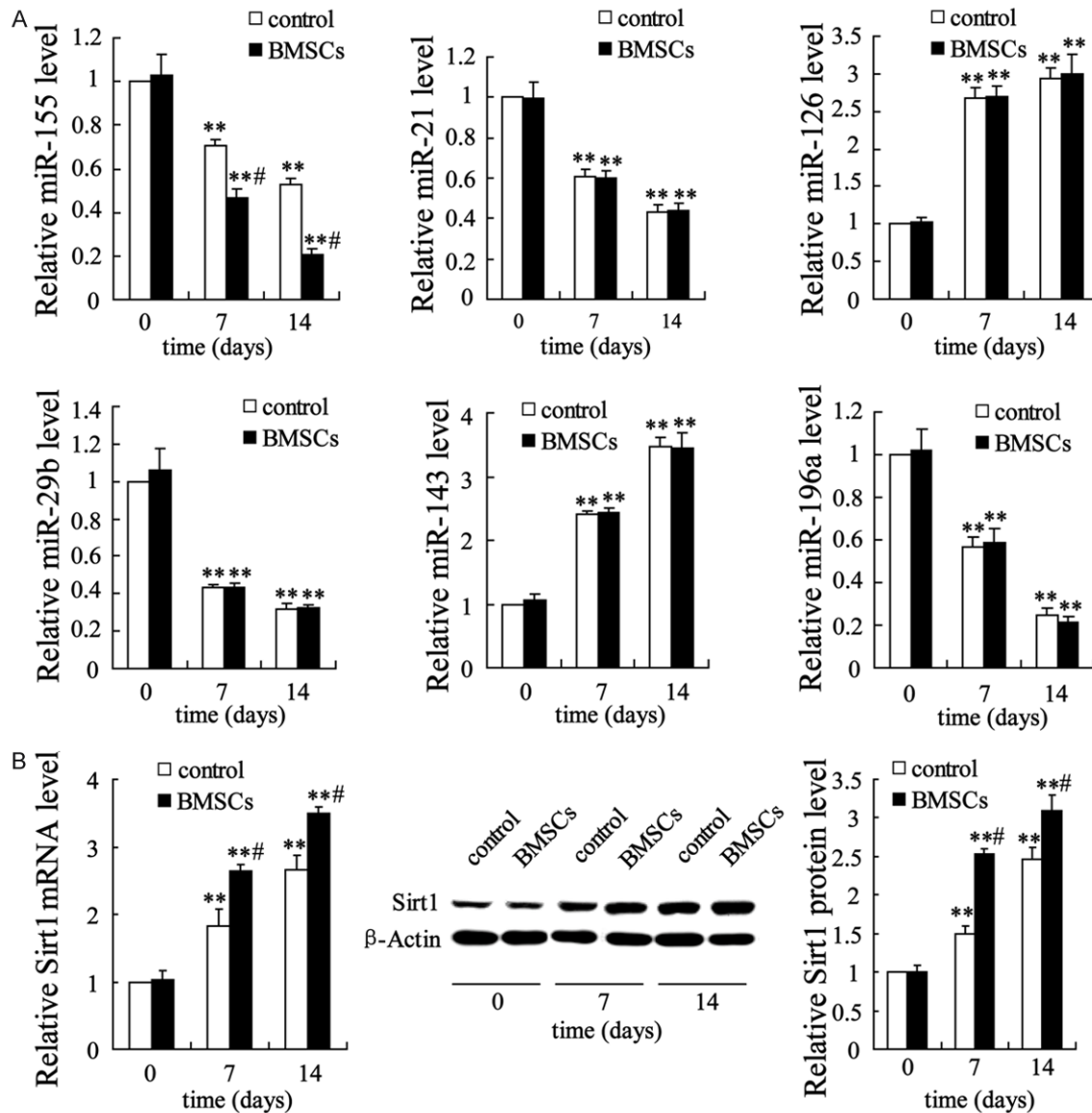


Figure 2. BMSCs decreased the expression of miR-155 and enhanced the expression of Sirt1 in wound tissue. A. The mRNA level of miR-155, miR-21, miR-126, miR-29b, miR-143 and miR-196a of mice in the control and BMSCs group. B. The mRNA and protein level of Sirt1 of mice in the control and BMSCs group. Control group: the wounds were covered with collagen alone; BMSCs group: the wounds were covered with collagen containing 2×10^4 BMSCs. The values are the mean \pm SE. **VS 0 days, $P < 0.01$; #VS control, $P < 0.05$.

(SDS-PAGE) and electrotransferred to nitrocellulose membranes (Takara, Dalian, China). After blocking in 5% non-fat dried milk for 1 hour, the membranes were incubated with polyclonal rabbit anti-Sirt1 antibodies at 4°C overnight. Then the membrane was incubated with HRP-conjugated secondary antibody (1:5,000) for 1 hour at room temperature. Band intensities were normalized to β -Actin.

Quantitative real-time PCR

Total RNA was extracted from cultured cells or tissues using Trizol. Reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. mRNA levels of target genes were quantified using SYBR Green Master Mix (Takara, Dalian, China). Changes in

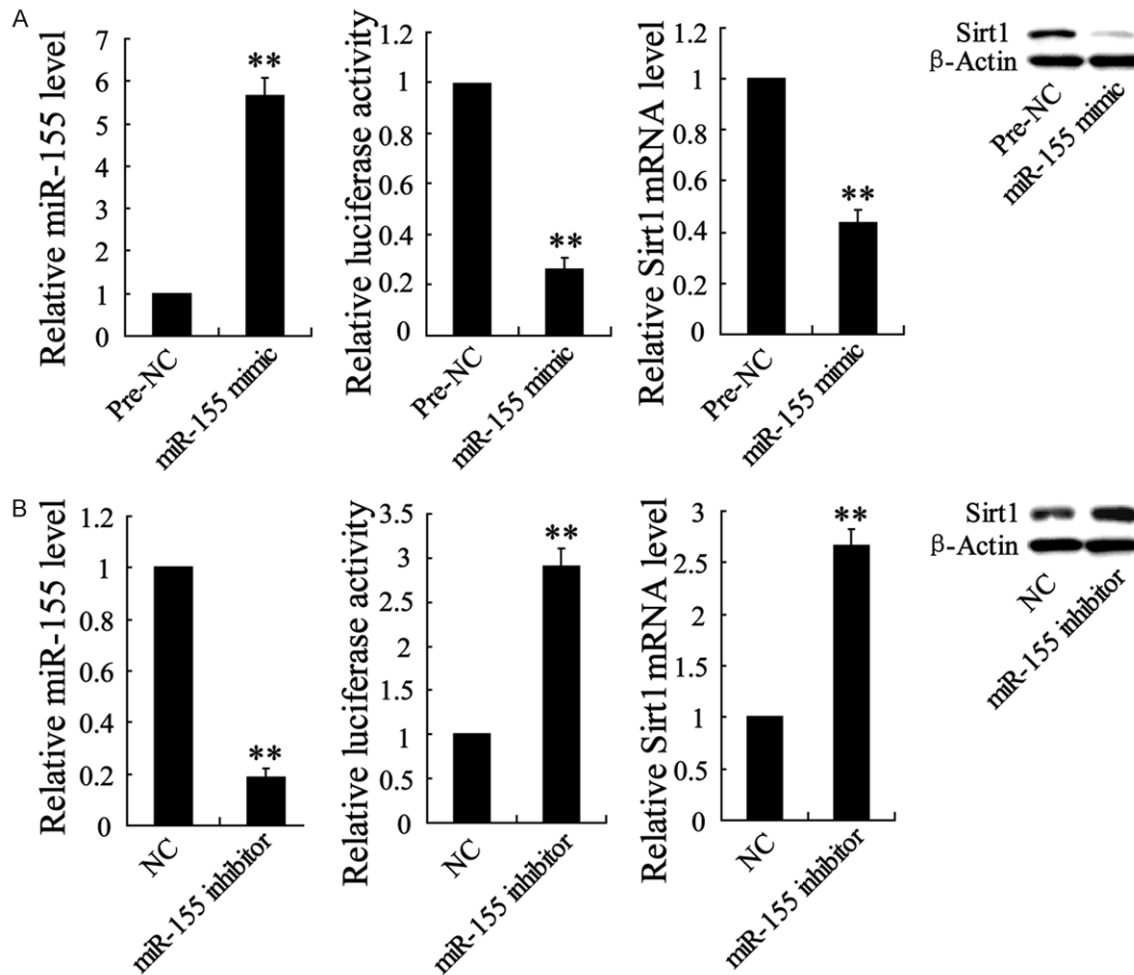


Figure 3. MiR-155 regulated the expression of Sirt1 in HUVEC-12. A. The expression of miR-155 and Sirt1 in HUVEC-12 transfected with miR-155 mimic. B. The expression of miR-155 and Sirt1 in HUVEC-12 transfected with miR-155 inhibitor. The values are the mean \pm SE. **VS control, ** $P < 0.01$.

relative gene expression of miR-155 were normalized to U6 levels and Sirt1 to GAPDH, which were determined using the relative threshold cycle method.

Cell transfection and luciferase reporter assay

The 3'-untranslated regions (3'-UTR) of Sirt1 generated by PCR amplification and were cloned into the pGL3-luciferase reporter plasmid (Promega). Transfection of miR-155 inhibitor or miR-155 mimic or si-Sirt1 was conducted using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. For luciferase reporter assay, cells (1×10^4) were seeded in triplicate in 48-well plates and allowed to settle for 24 h. Luciferase reporter plasmids or miR-155 inhibitor, miR-155 mimic, si-Sirt1 were transfected into cells

using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Luciferase signals were measured using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

MTT assay

The 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect HUVEC proliferation. HUVECs were cultured in the different media plated in 6-well plates at 5×10^5 cells/well at 37°C and 5% CO₂ for 48 h. MTT (5 mg/ml, 20 μ l Sigma) were added to each well for 4 h at 37°C. The supernatant was removed, 150 μ l dimethyl sulfoxide/well (DMSO, Sigma) was added, and the samples were shaken for 10 min. Emax micro-

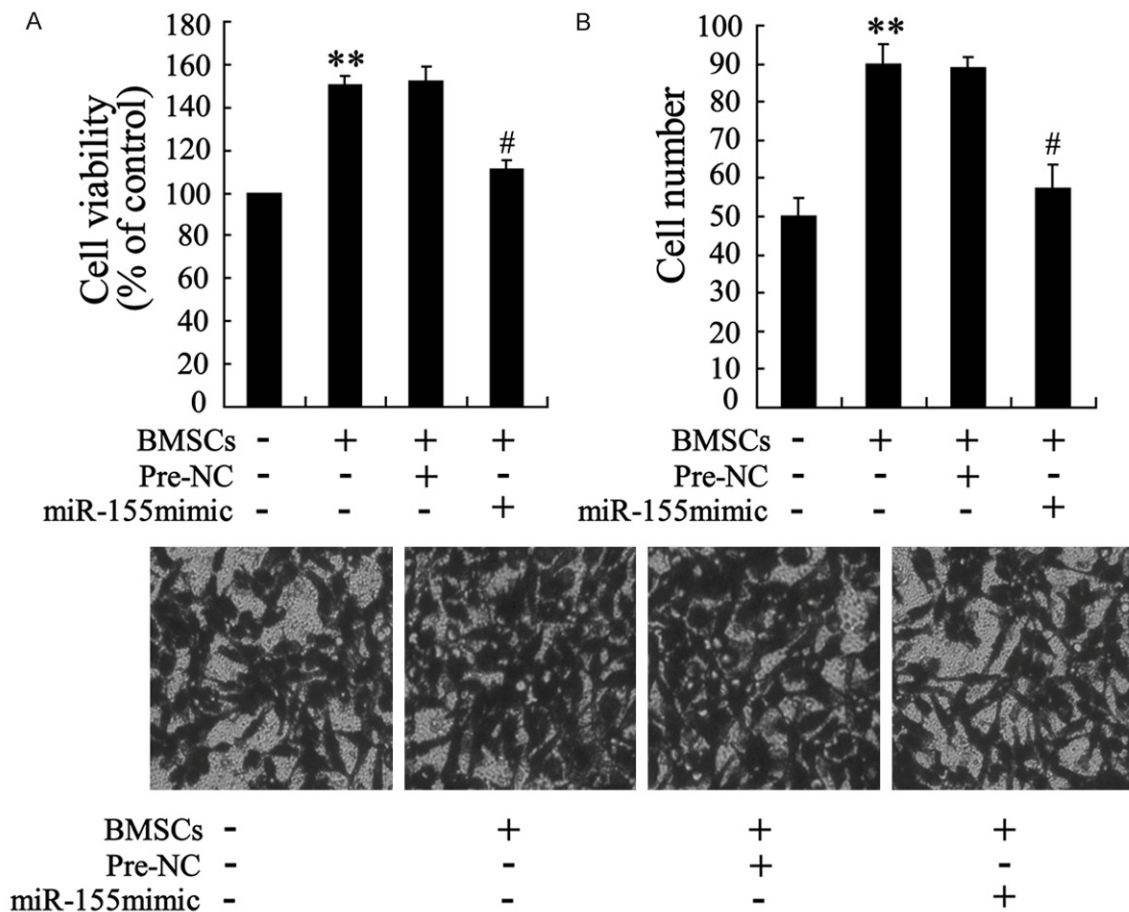


Figure 4. Overexpression of miR-155 reversed the effect of BMSCs on HUVECs proliferation and migration. HUVECs were cultured in BMSC-conditioned medium. A. Overexpression of miR-155 reversed the effect of BMSCs on HUVECs proliferation. B. Overexpression of miR-155 reversed the effect of BMSCs on HUVECs migration. The values are the mean \pm SE. **VS control, $P < 0.01$; #VS BMSCs + Pre-NC, $P < 0.05$.

plate reader (Molecular Devices, CA, USA) was used to measure the absorbance value (OD) of each well at 492 nm.

Transwell chamber experiments

HUVECs were cultured with 0.1% FBS high-glucose medium for 24 h, and the cells were loaded into 25 mm, 8.0 μ m transwell cell chambers (Corning) in the upper chamber at 5,000 cells/well. The BMSC, BMSCs and pre-NC, or BMSCs and miR-155 mimic, or si-control, or BMSCs and si-Sirt1 were added to the lower chamber. The cells were incubated at 37°C in 5% CO₂ for 6 h. Cells that migrated to the other side of the membrane were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

Statistics

Each experiment was repeated at least three times and the results were expressed as the

mean \pm SE. The experimental data were analyzed by SPSS 18.0, t-test and variance analyses were performed and. All p values were two-tailed, and p value < 0.05 was considered statistically significant.

Results

BMSCs accelerated wound healing in diabetic mice

To determine the effect of BMSCs on the wound healing of diabetic foot, we used a mouse model of diabetic foot wound that was covered with covered with collagen containing 2×10^4 BMSCs (BMSCs group) or collagen alone (control group). The wound area was measured on days 0, 7 and 14 after the operation. It has been shown that the BMSCs group had significantly accelerated diabetic foot healing and that the wound healing rate for the control

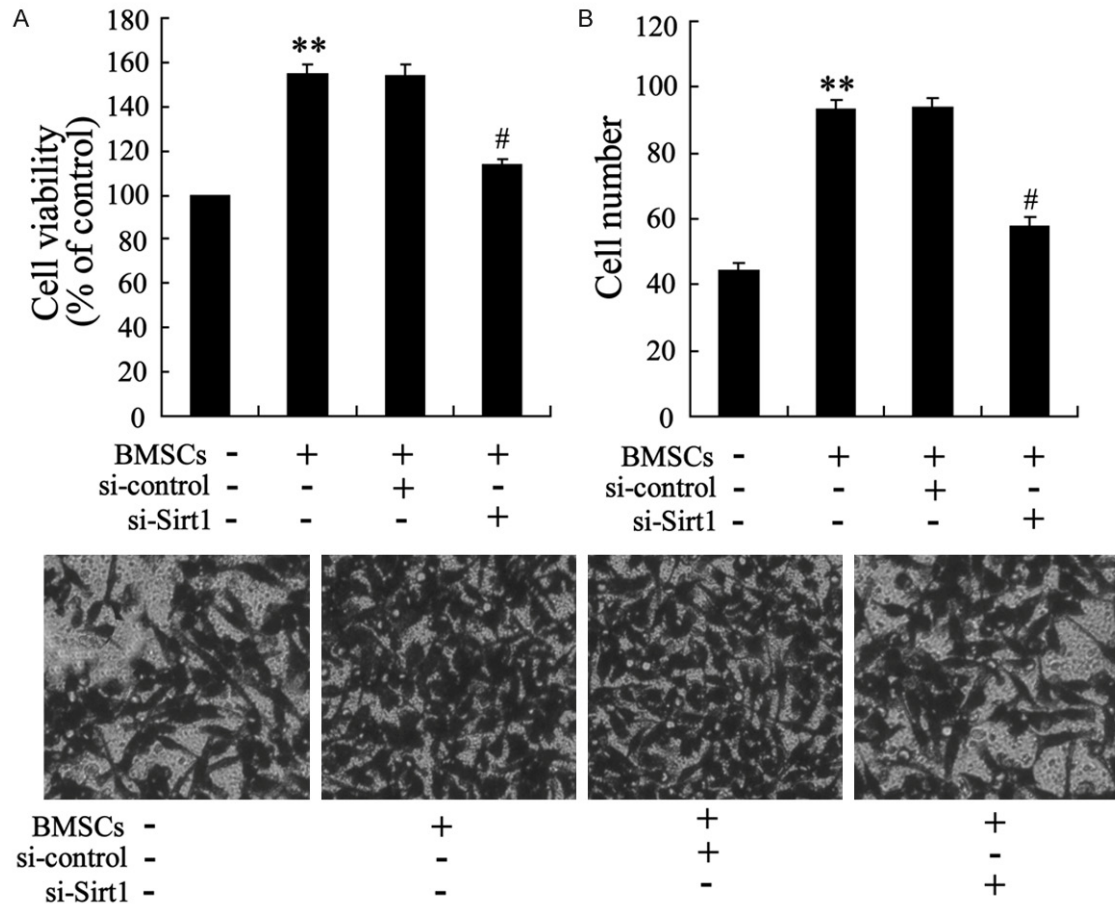


Figure 5. Si-Sirt1 reversed the effect of BMSCs on HUVECs proliferation and migration. HUVECs were cultured in BMSC-conditioned medium. A. Si-Sirt1 reversed the effect of BMSCs on HUVECs proliferation. B. Si-Sirt1 reversed the effect of BMSCs on HUVECs migration. The values are the mean \pm SE. **VS control, $P < 0.01$; #VS BMSCs + si-control, $P < 0.05$.

group was significantly slower than that of the BMSCs group (**Figure 1A**). To examine the function of BMSCs to vascular angiogenesis and repair, the capillary density was analyzed. As demonstrated in **Figure 1B**, the capillary density in BMSCs group was significantly higher than that in control group. These data suggest that BMSCs effectively accelerated diabetic foot wound healing.

BMSCs decreased the expression of miR-155 and enhanced the expression of Sirt1 in wound tissue

To investigate the role of miRNAs in the promoted function of BMSCs to diabetic foot wound healing, the mRNA level of miR-155, miR-21, miR-126, miR-29b, miR-143 and miR-196a were detected in wound tissue treated with BMSCs or none. The differential expression

between BMSCs group and control group was only observed in the expression of miR-155, and there were no changes in the expression of miR-21, miR-126, miR-29b, miR-143 and miR-196a between BMSCs group and control group. In addition, the mRNA level of miR-155 in BMSCs group was downregulated in a time-dependent manner (**Figure 2A**). Next, we detected the mRNA and protein level of Sirt1 in wound tissue treated with BMSCs or none. As shown in **Figure 2B**, the mRNA and protein level of Sirt1 were enhanced in a time-dependent manner in BMSCs group.

MiR-155 regulated the expression of Sirt1 in HUVEC-12

To examine the effect of miR-155 on the expression of Sirt1, HUVEC-12 cells were transfected

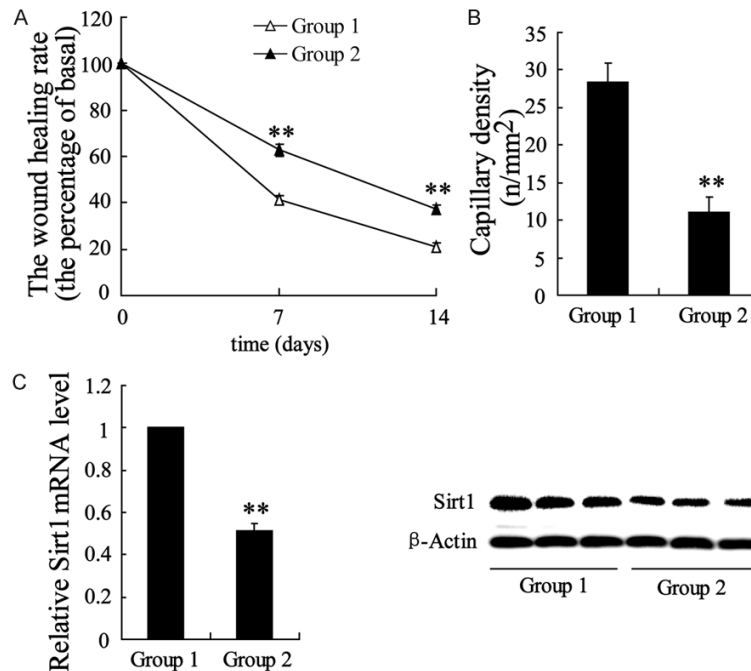


Figure 6. MiR-155 mimic worsened the wound healing in diabetic mice treated with BMSCs. Diabetic mice treated with BMSCs were transfected with NC (Group 1) or miR-155 mimic (Group 2). A. The wound healing rate of mice in the two groups. B. The capillary density of mice in the two groups. C. The expression of Sirt1 in mice of the two groups. The values are the mean \pm SE. **VS Group 1, $P < 0.01$.

with miR-155 mimic or miR-155 inhibitor or the corresponding control to overexpress miR-155 or inhibit the its expression. qRT-PCR and luciferase reporter assay revealed that miR-155 mimic highly increased the expression of miR-155 and down-regulated the activity of 3'-UTR of Sirt1. Moreover, it also decreased the mRNA and protein level of Sirt1 (Figure 3A). On the other hand, miR-155 inhibitor enhanced the activity of 3'-UTR of Sirt1 and the mRNA and protein level of Sirt1 in HUVEC-12 cells (Figure 3B). These findings indicated that miR-155 regulated the expression of Sirt1 in HUVEC-12.

Overexpression of miR-155 reversed the effect of BMSCs on HUVECs proliferation and migration

The proliferation and migration of endothelial cells play critical roles for vascular repair and regeneration. Herein, we detected the proliferation of HUVECs with the conditioned medium of BMSCs through MTT assay (Figure 4A). Moreover, the migration of HUVECs was verified through a transwell chamber experiment (Figure 4B). In MTT assay, the cell viability of

BMSCs group was 150% of the control value, and it indicated that BMSCs can improve HUVECs proliferation. In addition, the cell viability of BMSCs + miR-155 mimic group was significantly lower than that of BMSCs + pre NC group (Figure 4A). In transwell chamber experiment, BMSCs also significantly promoted HUVECs migration. The migration rate of BMSCs + miR-155 mimic group descended sharply compared with BMSCs + pre NC group (Figure 4B). These observations suggest that BMSCs can promote the proliferation and migration of HUVECs and miR-155-overexpressing BMSCs reversed this function.

Si-Sirt1 reversed the effect of BMSCs on HUVECs proliferation and migration

To further examine the role of Sirt1 in HUVECs proliferation and migration, HUVECs were cultured in medium containing BMSCs or BMSCs + si-Sirt1. As shown in Figure 5A, the cell viability of HUVECs in BMSCs + si-Sirt1 group was greatly lower than that in BMSCs + si-control group. In respect to the migration, si-Sirt1 also inhibits the migration of HUVECs in BMSCs + si-Sirt1 group (Figure 5B). These results suggest that si-Sirt1 could also reverse the effect of BMSCs on HUVECs proliferation and migration.

MiR-155 mimic worsened the wound healing in diabetic mice treated with BMSCs

Through the above experiments, we concluded that miR-155 was connected with the function of BMSCs in diabetic foot mice. To elucidate its role in wound healing of diabetic mice treated with BMSCs, diabetic mice treated with BMSCs were divided into two groups: treated with NC (Group 1) and treated with miR-155 mimic (Group 2). It has been shown that the wound healing rate was significantly decreased in Group 2 comparing to Group 1 (Figure 6A). In addition, the capillary density in Group 2 was

significantly lower than that in control group (**Figure 6B**). In Group 2, the mRNA and protein level of Sirt1 were also descended (**Figure 6C**).

Discussion

Chronic diabetic wounds pose a clinical challenge and are a major burden to the health care system. The wound healing process contains three overlapping phases, which are acute inflammation, proliferation and remodeling. Several different cell types are involved in it, including cells of the immune system, keratinocytes, endothelial cells and fibroblasts, as well as many different molecular factors and cytokines. BMSCs have been widely explored as a useful modality in disorders caused by insufficient angiogenesis such as chronic wounds [12, 15]. According to the research of Wu Y et al. BMSCs can promote wound healing by increasing local angiogenesis in wounds [7]. BMSCs are involved in all stages of angiogenesis, not only in the early steps such as proliferation or migration of EC but also in the later phases which involve blood vessel maturation [10]. Lei Shen et al. suggested that BMSCs stimulated by neurotrophin-3 promoted HUVEC proliferation and migration, and significantly accelerated diabetic foot wound healing [4]. In this study, we also observed the accelerating wound healing in diabetic mice induced by BMSCs. Moreover, BMSCs could promote the proliferation and migration of HUVEC.

It has been testified that miRNAs regulate the key elements of tissue repair such as stem cell biology, hypoxia-response, inflammation and angiogenesis [10, 16]. Dysregulation of the miR system might result in compromised wound healing through perturb the function of target genes. To detect whether miRNAs involved in the function of BMSCs on wound healing in diabetic mice, we examined the expression of miR-155, miR-21, miR-126, miR-29b, miR-143 and miR-196a in wound tissues. It has been shown that only the mRNA level of miR-155 was different between BMSCs group and control. We further found that overexpression of miR-155 reversed the effect of BMSCs on HUVECs proliferation and migration and worsened the wound healing in diabetic mice treated with BMSCs.

Recent data suggest that miR-155 has distinct expression profiles and plays a crucial role in a large number of pathological and physiological

processes, such as hematopoiesis, inflammation, immunity, cardiovascular diseases and cancer [17]. In the proliferation phase of wound healing, miR-155 inhibit the expression of keratinocyte growth factor (KGF) in fibroblasts [18]. Coen van Solingen et al. reported that the absence of miR-155 has beneficial effects in the wound healing process [19]. For the important role of miR-155 in inflammation, miR-155 is considered a tentative target to reduce the enhanced inflammation observed in diabetic foot patients [20].

In this study, we also discovered the dysregulation of Sirt1 in wound tissues treated by BMSCs. In addition, miR-155 could regulate the expression of Sirt1 in HUVECs. Sirt1 knockdown also reversed the effect of BMSCs on HUVECs proliferation and migration. Mounting evidence indicated that Sirt activators could accelerate the healing process [21]. It has been shown that agonists of Sirt1 have negative effect on matrix metalloproteinases (MMPs) transcription in the skin [22]. MMPs could break down extracellular matrix components such as collagen, leading to poor extracellular matrix formation, which is essential for wound healing. MMPs, in particular MMP-8 and MMP-9, play a key role in diabetic wound healing [23].

In conclusion, our study provides evidence that BMSCs accelerates wound healing in diabetic mice. BMSCs in wound tissue results in significantly increased Sirt1 expression via down-regulating miR-155. Furthermore, overexpression of miR-155 or Sirt1 knockdown reversed the effect of BMSCs on HUVECs proliferation and migration. These findings demonstrated that application of stem cells in tissue engineering for treating diabetic foot ischemic ulcers is a promising approach and miR-155 played important roles in the wound healing of diabetic foot by BMSCs.

Disclosure of conflict of interest

None.

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