



## Salidroside-Pretreated Mesenchymal Stem Cells Enhance Diabetic Wound Healing by Promoting Paracrine Function and Survival of Mesenchymal Stem Cells Under Hyperglycemia

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### ABSTRACT

Systemic abnormalities cause several complications in diabetes patients. Impaired wound healing is a serious complication that leads to severe foot ulcer and amputation. Mesenchymal stem cells (MSCs) have been considered a promising strategy for promoting wound healing due to their paracrine function. However, their poor survival after transplantation limits their therapeutic effect and applications. Salidroside, a glucopyranoside, has been reported to exert cytoprotective effects. Our previous study revealed that salidroside could promote the paracrine function of skeletal muscle cells. However, whether salidroside could improve MSCs survival under hyperglycemic condition and, subsequently, promote wound healing in diabetic model mice remains unknown. Here, we found that salidroside pretreatment effectively reversed the hyperglycemia-induced suppression of the expression of crucial wound healing factors in MSCs, such as heme oxygenase-1 (HO-1), fibroblast growth factor 2 (FGF2), and hepatocyte growth factor (HGF). Salidroside pretreatment also suppressed the hyperglycemia-induced intracellular reactive oxygen species (ROS) levels in MSCs, thereby lowering the apoptosis rate and enhancing MSCs survival rate. Furthermore, salidroside improved the MSCs migration potential that was impaired under hyperglycemia. *in vivo* experiments revealed that salidroside pretreatment prior to transplantation significantly enhanced the effect of MSCs in promoting wound closure in diabetic mice. Collectively, our results suggest that pretreatment with salidroside could be an effective strategy to enhance the survival rate and the therapeutic effect of MSCs. Thus, our article suggested a novel, potential MSC-based strategy for diabetic wound healing. *STEM CELLS TRANSLATIONAL MEDICINE* 2019;8:404–414

### SIGNIFICANCE STATEMENT

Mesenchymal stem cells (MSCs) transplantation is a potential therapeutic strategy for wound healing as they could secrete various cytoprotective and growth factors; however, high blood glucose, as well as the oxidative environment in wound site significantly affects their paracrine function, survival and migration potential. It was found that salidroside, a small molecule drug, could significantly increase MSCs paracrine function, as well as their survival, antioxidant defense and migration potential, most likely by restoring the expression levels of HO-1, FGF2, and HGF that decreased under hyperglycemia. Finally, it was showed that salidroside-pretreated MSCs could promote wound closure and re-epithelialization in diabetic model mice.

### INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, which induces oxidative stress and the generation of reactive oxygen species (ROS). Hyperglycemia also causes systematic disruption of various physiological functions, including angiopathy,

neuropathy, and impaired wound healing. Chronic wound healing, which affects 15% of all diabetic patients, is indeed a serious complication of diabetes with high risk of amputation [1, 2]. Diabetic wounds are characterized with diminished cell recruitment, impaired collagen matrix formation and lack of growth factors [2, 3]. Previous studies revealed that

hyperglycemia negatively regulates the expression of fibroblast growth factor 2 (FGF2) and hepatocyte growth factor (HGF) [4, 5], which in turn suppresses cell migration and causes retardation in re-epithelialization subsequently leading to impaired wound healing process [5, 6].

Mesenchymal stem cells (MSCs) are known to be beneficial for diabetic wound healing [7], particularly due to their paracrine function [8]. Owing to their ability to secrete growth factors and cytokines that modulate skin wound healing [9], transplantation of MSCs could elevate the amounts of growth factors in the injury site, leading to improved wound healing through increased rapid epithelialization, as well as enhanced granulation tissue formation and angiogenesis [9, 10]. However, hyperglycemia severely affects the survival of transplanted MSCs, as it enhances ROS generation which in turn induces apoptosis [11, 12]. Furthermore, hyperglycemia also disrupts MSCs migration potential, inhibits their homing to the injury site and thus limits their ability in successful MSC-based therapy [12]. Hence, promoting cell survival and migration potential of the MSCs are supposed to be the key factors for successful MSC-based therapeutic strategy for diabetic wound healing.

Salidroside (2-[4-hydroxyphenyl]ethyl beta-D-glucopyranoside) has been previously reported to exhibit antioxidant and antiapoptotic functions, as well as promotion of high altitude adaptation potential [13–15]. Previous studies have shown that salidroside reduces hyperglycemia-induced intracellular ROS generation in endothelial cells by inducing heme oxygenase-1 (HO-1) expression [16], and thus exerts cytoprotective activity by modulating oxidative stress-induced apoptosis [17]. Furthermore, our recent studies revealed that salidroside promotes the paracrine function of the skeletal muscle cells and enhances neovascularization in both nondiabetic and diabetic hind limb ischemic mice [18, 19]. However, whether salidroside could promote MSCs survival under hyperglycemia and subsequently enhance diabetic wound healing remains unknown.

In this article, we investigated the effect of salidroside pretreatment on the therapeutic effect of MSC-based therapy for diabetic wound healing. Salidroside significantly restored the paracrine function of the MSCs that were impaired under hyperglycemia by enhancing the expression of HO-1, which is crucial for promoting cell survival, as well as FGF2 and HGF, which are critical for MSCs migration and skin re-epithelialization. Concomitantly, salidroside robustly decreased the hyperglycemia-induced intracellular ROS levels and the apoptosis rate of MSCs, thereby enhancing the survival and migration potential of MSCs under hyperglycemia. Subsequently, we have demonstrated that salidroside pretreatment increased the therapeutic effect of MSCs on diabetic wound healing. Together, our results elucidated a novel, potential MSC-based therapeutic strategy for diabetic wound healing.

## MATERIALS AND METHODS

### Cell Culture

Mouse MSCs were purchased from Jenniobio (Guangzhou, China). Cells were maintained in Dulbecco's modified Eagle's medium basic (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Biological Industries,

Beit Haemek, Israel). Cells have been tested periodically for mycoplasma contamination using Mycoplasma Detection Kit-QuickTest (Biotoool, Houston, TX). MSCs from 3 to 9 passages were used for the experiments.

For hyperglycemia experiment, cells were cultured without serum under the presence of 25 mM glucose for 12 hours prior to treatment with salidroside or phosphate buffered solution (PBS). Cells cultured under 5.5 mM glucose were used as normoglycemia control. All experiments were performed under hypoxia by incubating the cells in a hypoxia chamber (Mitsubishi GAS Chemical, Tokyo, Japan; oxygen concentration: 0.1%) as previously described [18, 19].

For salidroside pretreatment, salidroside (purity  $\geq 98\%$ , Tauto Biotech, Shanghai, China, final concentration: 100  $\mu\text{g}/\text{ml}$ ) was added to cell culture, and then the cells were cultured further for 24 hours. Cells were then washed and cultured for another 24 hours under hypoxic and normoglycemic or hypoglycemic conditions. For control, cells were treated with same amount of PBS.

For experiment with HO-1 inhibitor, cells were cultured with medium containing zinc protoporphyrin IX (ZnPP, dissolved in dimethyl sulfoxide (DMSO), final concentration: 10  $\mu\text{M}$ ; APExBIO, Houston, TX) for 1 hour. For control, cells were treated with same amount of DMSO.

### Plasmids and Constructs

The shRNA control vector and shRNA expression vectors against murine FGF2 (NM\_008006) were constructed as described previously [18]. For shRNA expression vector against murine HGF (NM\_010427), specific target sites were predicted and vectors were constructed in the way described previously [20]. The sequences of specific target sites were: GCA AGA ACG GCG GCT TCT T (shFGF2-1), GCG AGA AGA GCG ACC CAC A (shFGF2-2), GAA GGA GAT ACT ACA CCT A (shHGF-1) and GGC TGG GGC TAC ACT GGA T (shHGF-2).

### Animal Experiment

For the *in vivo* diabetic wound healing study, C57BL/6 mice (male; body weight: 18–24 g; 8 weeks old) were purchased from the Third Military Medical University (Chongqing, China; permit number SYXK-PLA-20120031). Animal studies were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University, and were carried out in the Third Military Medical University according to the guidelines approved by the Animal Care and Use Committee of the Third Military Medical University. All efforts to minimize suffering were made. For inducing diabetic condition, mice were fed with high fat diet (20% kcal protein, 20% kcal carbohydrate, and 60% kcal fat) for 3 weeks prior to intraperitoneal injection of streptozotocin (Sigma–Aldrich, St. Louis, MO; 40 mg/kg per day dissolved in sodium citrate buffer) for 5 consecutive days. One week after the last injection, the mice were fasted overnight, and then blood glucose level was assessed using an Accu-Check Integra (Roche Diagnostics, Shanghai, China) by tail vein puncture blood sampling. Mice with blood glucose  $\geq 16.7$  mmol/l were considered to be diabetic and used for preparing diabetic wound model mice.

For establishing diabetic wound healing model, the diabetic mice were anesthetized by an intraperitoneal injection of ketamin/xylazine (80 mg/kg body weight). After the hair was depilated, a full thickness skin wound (6 mm in diameter) was

created on the dorsal section. One day after wounding,  $8 \times 10^5$  MSCs pretreated with PBS or salidroside in 100  $\mu$ l PBS were transplanted into the sites surrounding the wound. Wound areas were measured on day 0, 3, 7, and 14 after wounding. The rate of wound closure was calculated as described previously according to the following equation: wound closure rate (%) = [(original wound area – open area on final day)/original wound area]  $\times$  100% [21].

### Scratch Assay

MSCs were seeded in 6-well plates at the density of  $2 \times 10^5$  cells per well, and serum starved for 12 hours prior to treatment with PBS or salidroside (final concentration: 100  $\mu$ g/ml). After washing and changing the medium to culture medium without salidroside, cells were treated with cyclohexamine (purity  $\geq$ 95%, final concentration: 2.5 mg/ml, Cayman Chemicals, Ann Arbor, MI), then a scratch wound was created using a micropipette tip. Cells were then incubated under hypoxia for the indicated times.

### Transwell Migration Assay

Cells were cultured under hyperglycemia and serum starved for 12 hours prior to treatment with salidroside (final concentration: 100  $\mu$ g/ml). After washing, the cells were reseeded ( $7 \times 10^3$  cells per chamber) in the upper chamber of a transwell plate (Corning, NY), and cultured under hypoxia for 24 hours. Culture medium with glucose was placed in the lower chamber. Cells migrated to lower chamber were stained with crystal violet (Beyotime, China). Images were taken with Olympus IX71 (Japan). For control, cells were cultured under normoglycemia and serum starved for 12 hours prior to treatment with PBS. Transwell migration assay was then performed as described above, except that PBS was added into the culture medium placed in the lower chamber instead of glucose. For experiments using shRNA expression vectors against FGF2 and HGF, MSCs were seeded in 6-wells plate ( $1.5 \times 10^5$  cells per well) and cultured in medium with glucose. shRNA expression vectors were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol, and 24 hours later, cells were selected using puromycin to eliminate untransfected cells. Thirty-six hours later, cells were reseeded ( $7 \times 10^3$  cells per chamber) in the upper chamber of a transwell plate and cultured under hypoxia for 24 hours. Culture medium with glucose was placed in the lower chamber.

### Phalloidin Staining

Cells were seeded in a 35-mm glass bottom cell culture dish at a density of  $2 \times 10^4$  cells per well, serum starved for 12 hours prior to treatment with PBS or salidroside (final concentration: 100  $\mu$ g/ml), washed, and incubated under hypoxia for 24 hours. Cells were then fixed with 4% paraformaldehyde and permeabilized for 5 minutes with PBS containing 0.1% Triton X-100. After blocking with 1% bovine serum albumin for 1 hour, the samples were incubated at room temperature for 30 minutes with phalloidin, and images were taken with Microsystems-TCS SP5 (Leica, Heidelberg, Germany). The quantification of F-actin formed from G-actin polymerization was performed by fractal dimension analysis using ImageJ software.

### RNA Extraction and Quantitative RT-PCR Analysis

Total RNA from cells was extracted with Trizol (Invitrogen Life Technologies) according to the manufacturer's instruction, then 1  $\mu$ g of the total RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Dalian, China). The mRNA expression levels were assessed by quantitative reverse-transcribed polymerase chain reaction (qRT-PCR), which was performed using SYBR Premix Ex Taq (Takara Bio). The sequences of the primer pairs used for qRT-PCR were shown in Supporting Information Table S1.  $\beta$ -Actin was used to normalize sample amplifications. Quantification data was shown as relative to control.

### Western Blotting

Western blotting was performed as previously described [22]. For cell culture experiment, cells were collected and lysed with RIPA lysis buffer; while for animal experiments, the tissues were isolated and immediately homogenized with RIPA lysis buffer. Protease inhibitor and phosphatase inhibitor cocktail (complete cocktail; Roche Applied Science) were added to the lysis buffer to inhibit protein degradation. Equal amounts of the sample proteins were electrophoresed on sodium dodecyl sulfate polyacrylamide gel prior being transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The antibodies used are listed in Supporting Information Table S2, and the signals were measured using SuperSignal West Femto Maximum Sensitivity Substrate detection system (Thermo Scientific, Waltham, MA).  $\beta$ -Actin was used as a loading control. The quantitative analysis was performed using Quantity One (Thermo Scientific), and the results were shown as relative to the expression level in the controls, which were assumed as 1.

### ROS Measurement

Intracellular ROS level was assessed using the peroxide-sensitive fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime). Cells were cultured as describe above and subsequently exposed to 10 mol/l DCFH-DA for 30 minutes at 37°C. Fluorescence intensity was observed using DMI6000B (Leica).

### Cell Counting Assay

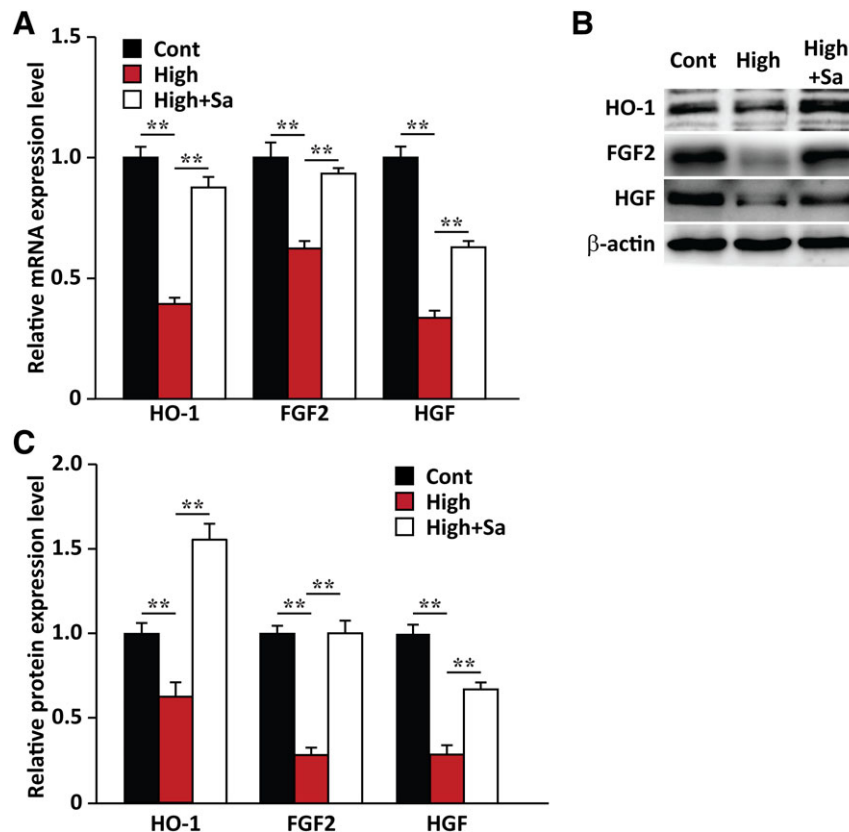
Cells treated with PBS or salidroside were reseeded in a 96-well cell culture dish. Cell numbers were counted at indicated time points using colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Madison, WI).

### Apoptosis Analysis

Cells were cultured as describe above in medium containing H<sub>2</sub>O<sub>2</sub> (final concentration: 100  $\mu$ M). Then the number of apoptotic cells were analyzed using Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen Biotech, Jiangsu, China) according to the manufacturer's instruction. Briefly, the cells were trypsinized and resuspended in a binding buffer containing Annexin V-FITC and PI at room temperature for 10 minutes before being analyzed using FACS Calibur (BD Biosciences).

### Hematoxylin and Eosin Staining

Mice were sacrificed at 14 days after wounding. The skin tissues from the wounds with a 3 mm margin were carefully



**Figure 1.** Salidroside restores the expression of cell survival and cell migration factors in mesenchymal stem cells (MSCs) under hyperglycemia. (A–C): Expression levels of heme oxygenase-1, fibroblast growth factor 2, and hepatocyte growth factor in MSCs treated with salidroside and cultured under hyperglycemia: (A) mRNA expression levels, as analyzed by quantitative reverse-transcribed polymerase chain reaction (qPCR); (B) protein expression levels, as examined by western blotting; and (C) quantification of the protein expression levels. All experiments were done under hypoxia. Cells cultured under normoglycemia and treated with phosphate buffered solution were used as control.  $\beta$ -Actin was used for normalization in qPCR, and as a loading control in western blotting. Quantification data were shown as relative to that of control, and expressed as mean  $\pm$  SD ( $n = 3$ ). \*\*,  $p < .01$ ; Cont: normoglycemia; high: hyperglycemia; Sa: salidroside.

removed and fixed with 4% paraformaldehyde for overnight prior to being embedded in paraffin. Then the tissues were sectioned at 5  $\mu$ m thickness using a cryostat. Sections were dewaxed using xylene and rehydrated before being stained with Hematoxylin and Eosin (Beyotime). Images were taken with Olympus IX71 (Japan).

### Statistical Analysis

Statistical analysis was performed using Student's *t* test. A value of \* $p < .05$  was considered statistically significant, while a value of \*\* $p < .01$  was considered highly significant. All values of the experimental results were presented as mean  $\pm$  SD. For animal experiments, statistical analysis was performed using one-way ANOVA.

## RESULTS

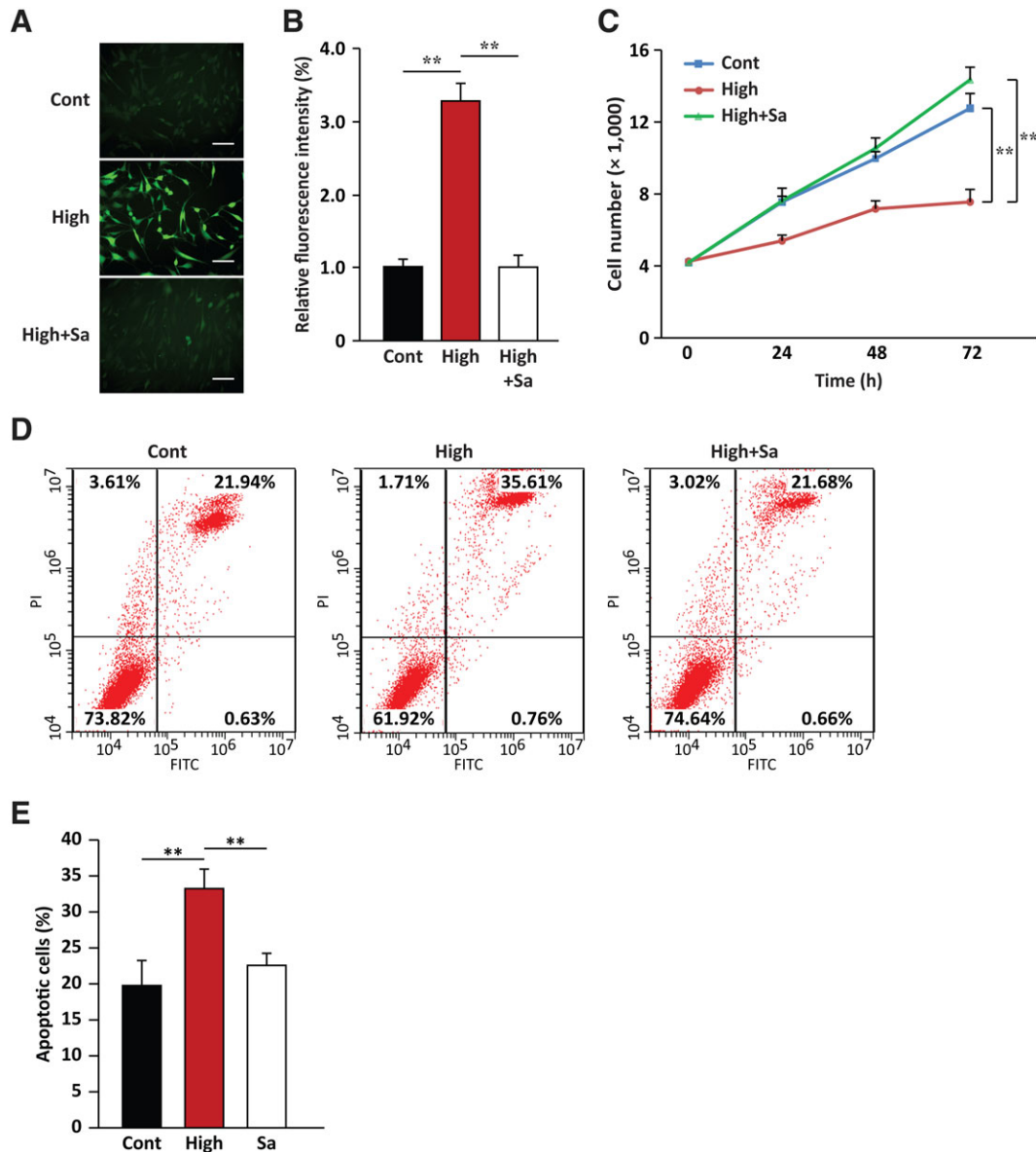
### Salidroside Promotes the Paracrine Function of MSCs

Diabetic condition results in systematic impairments leading to several complications. As shown in Supporting Information Figure S1, wound healing in diabetic model mice was significantly slower than that in nondiabetic mice. Wounds in nondiabetic mice healed almost completely at day 14 without significant scarring, while in diabetic mice, only approximately

60% of wound closure was observed. MSCs are considered an effective therapeutic strategy due to their paracrine function; however, hyperglycemia severely damages this function. Our previous reports have shown that salidroside could enhance and restore the hyperglycemia-induced suppression of paracrine function of the skeletal muscle cells [18]. In the present article, we found that while hyperglycemia disrupted the expression of HO-1, FGF2, and HGF, salidroside restored both the mRNA (Fig. 1A) and the protein (Fig. 1B, 1C) expression levels of these genes. FGF2 and HGF have been reported to promote cell migration potential, while HO-1 is a crucial factor that promotes cell survival rate under oxidative stress. Collectively, these results demonstrate that salidroside could enhance the paracrine function of MSCs impaired by hyperglycemia.

### Salidroside Protects MSCs from Apoptosis

Previous reports have shown that for a successful MSC-based therapy, the survival of the post-transplanted MSCs is the most critical factor [23]. However, hyperglycemia due to diabetes induces oxidative stress that damages cells and suppresses their survival. HO-1 has been known as a cell survival factor, and lack of HO-1 results in the aberrant antioxidant defense, leading to high apoptosis rate. Thus, we questioned whether salidroside could suppress the generation of ROS under hyperglycemia. Our



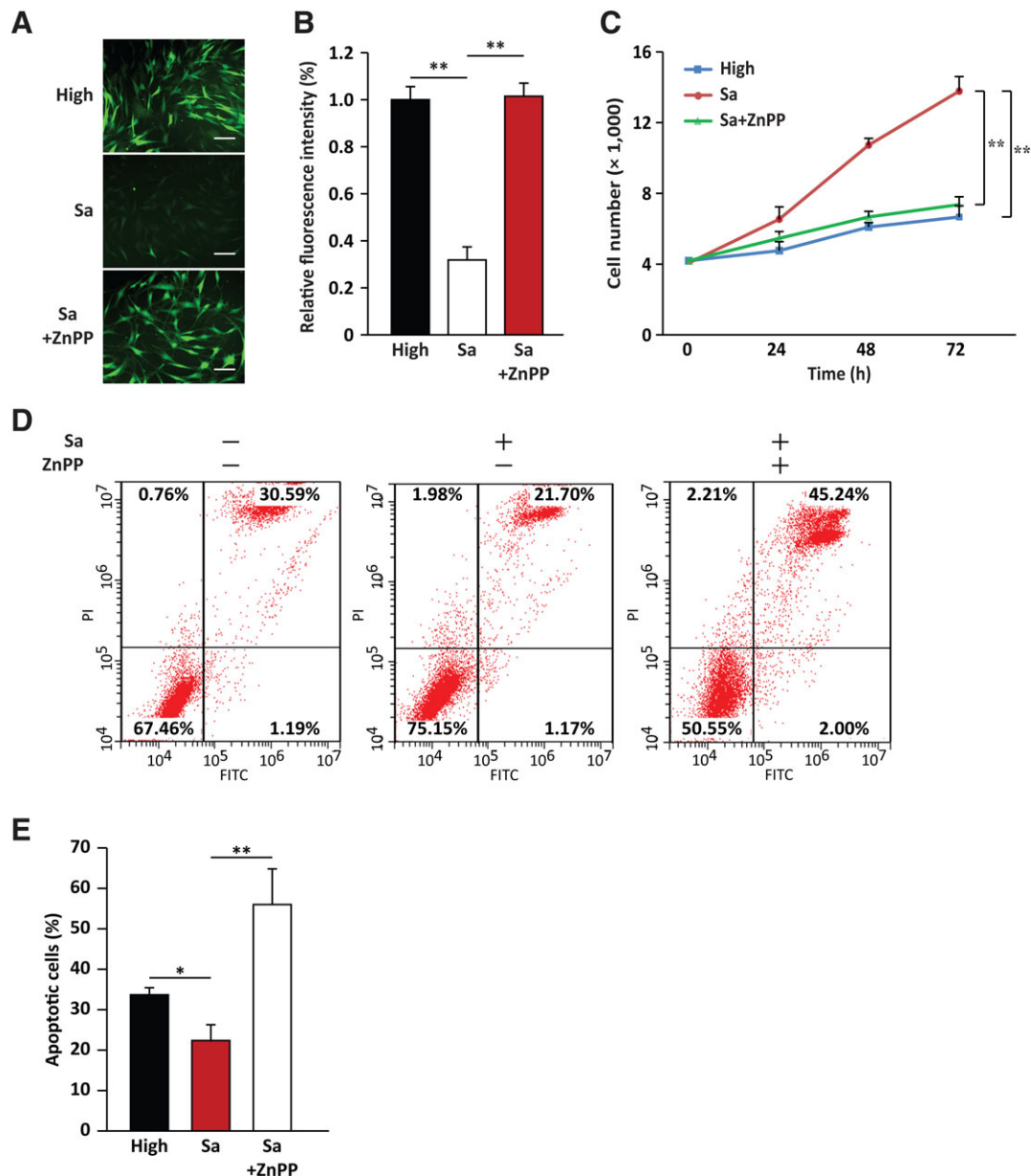
**Figure 2.** Salidroside enhances mesenchymal stem cells (MSCs) survival under hyperglycemia. **(A, B):** Intracellular reactive oxygen species (ROS) level in MSCs treated with salidroside and cultured under hyperglycemia, as determined using DCFH-DA: **(A)** representative images (scale bars: 200  $\mu$ m); and **(B)** quantification of relative fluorescence intensity. **(C):** Total cell number of MSCs treated with salidroside and cultured under hyperglycemia at the indicated time points. **(D, E):** The percentage of apoptotic cells in MSCs treated with salidroside and cultured under hyperglycemia were analyzed using Annexin V-FITC/PI staining and FACS analysis: **(D)** representative images of apoptotic cells; and **(E)** average of total apoptotic cells from 3 independent experiments. All experiments were done under hypoxia. Quantification data were expressed as mean  $\pm$  SD ( $n = 5$  for ROS quantification;  $n = 3$  for others). \*\*,  $p < .01$ ; Cont: normoglycemia; high: hyperglycemia; Sa: salidroside.

DCFH-DA staining results demonstrated that salidroside pretreatment significantly reduced the intracellular ROS levels that were increased by hyperglycemia (Fig. 2A, 2B). Concomitantly, we found that salidroside evidently restored the total number of cells suppressed by hyperglycemia (Fig. 2C).

Next, we assessed whether salidroside pretreatment could improve the antioxidant defense of MSCs. We analyzed the effect of salidroside pretreatment on the apoptosis rate of MSCs under hyperglycemia. As shown in Figure 2D, 2E, salidroside significantly suppressed the percentage of apoptotic cells that was increased under hyperglycemia. Together, these results demonstrated that salidroside could repress ROS

generation, enhance antioxidant defense, and subsequently promote MSCs survival under hyperglycemia.

We next investigated whether salidroside promoted the survival rate and antioxidant defense of MSCs by regulating HO-1 expression. Addition of ZnPP, an HO-1 inhibitor, significantly induced MSCs apoptosis (Supporting Information Fig. S2). We then analyzed the apoptosis rate of MSCs treated with both salidroside and ZnPP under hyperglycemia. Our results clearly showed that ZnPP addition increased the intracellular ROS level suppressed by salidroside treatment under hyperglycemia (Fig. 3A, 3B). The total number of cells which were restored by salidroside pretreatment, were suppressed upon treatment with



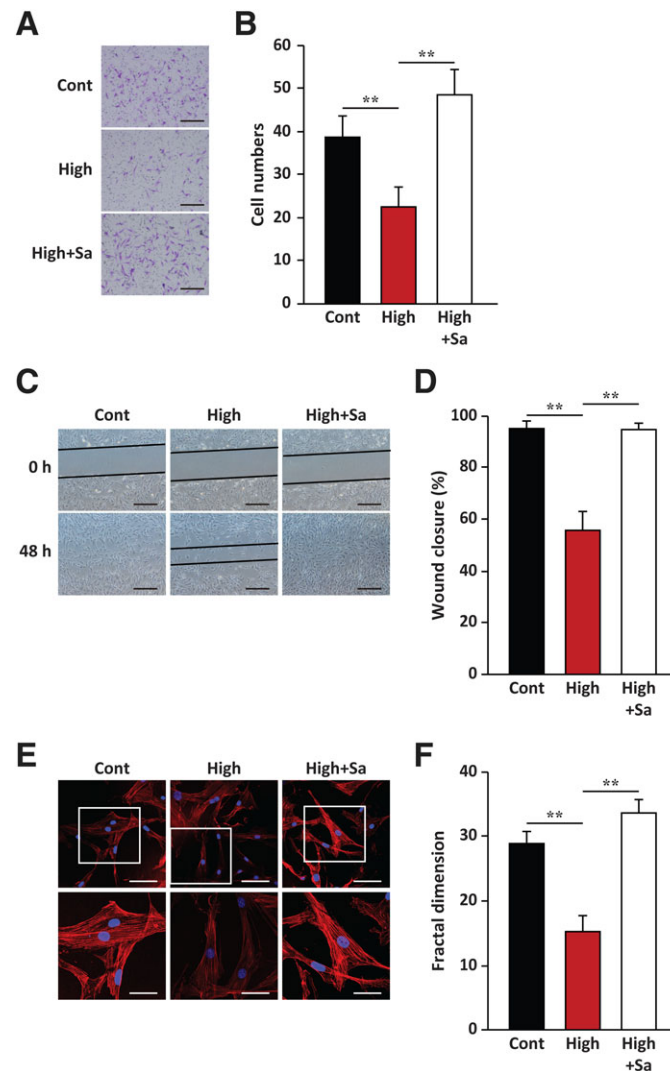
**Figure 3.** Heme oxygenase-1 is essential for salidroside-induced mesenchymal stem cells (MSCs) survival under hyperglycemia. **(A, B):** Intracellular ROS level in MSCs treated with salidroside and ZnPP cultured under hyperglycemia, as determined using DCFH-DA: **(A)** representative images (scale bars: 200  $\mu$ m); and **(B)** quantification of relative fluorescence intensity. **(C):** Total cell number of MSCs treated with salidroside and ZnPP cultured under hyperglycemia at the indicated time points. **(D, E):** The percentage of total apoptotic cells in MSCs treated with salidroside and ZnPP cultured under hyperglycemia, as analyzed using Annexin V-FITC/PI staining and FACS analysis. All experiments were done under hypoxia. Quantification data were expressed as mean  $\pm$  SD ( $n = 5$  for ROS quantification,  $n = 3$  for others). \*,  $p < .05$ ; \*\*,  $p < .01$ ; high: hyperglycemia; Sa: salidroside; ZnPP: zinc protoporphyrin IX.

ZnPP (Fig. 3C). Furthermore, the percentage of apoptotic cells increased in the cells treated with ZnPP (Fig. 3D, 3E). Together, these results demonstrated that HO-1 inhibition nullified the effect of salidroside on the antioxidant defense and survival of MSCs under hyperglycemia, suggesting that salidroside promotes the antioxidant defense of MSCs under hyperglycemia by, at least partly, enhancing HO-1 expression.

### Salidroside Improves MSCs Migration

For effective wound restoration, homing of MSCs to the wound site is essential, and thus migration potential is also a

determining factor for their therapeutic effect [9]. The migration potential of MSCs are suppressed under hyperglycemia mainly due to its suppressive effect on the expression and secretion of the various wound healing-related factors; and this is another causative reason for the decreased therapeutic effect of MSCs under hyperglycemia [12]. As described above, salidroside increased the mRNA expression levels of FGF2 and HGF, both of which are known to be essential factors for MSCs migration [24, 25]. Conform with this, our results also demonstrated that knocking down FGF2 (Supporting Information Fig. S3A–S3C) and HGF (Supporting Information Fig. 3D–3F)



**Figure 4.** Salidroside restores mesenchymal stem cells (MSCs) migration potential suppressed by hyperglycemia. **(A, B):** The migration potential of MSCs treated with salidroside or phosphate buffered solution (PBS) and cultured under hyperglycemia, as examined using transwell chamber assay: **(A)** representative images (scale bars: 200  $\mu$ m); and **(B)** quantification of migrated cells. **(C, D):** The migration potential of MSCs pretreated with salidroside or PBS and cultured under hyperglycemia, as examined using scratch assay: **(C)** representative images (scale bars: 200  $\mu$ m) and **(D)** percentage of wound closure. **(E, F):** Morphological changes of F-actin as examined by phalloidin staining: **(E)** representative images (scale bars: 100  $\mu$ m for upper panels and 50  $\mu$ m for lower panels, lower panels showed the enlarged images of the cropped part in the upper panels); and **(F)** quantification analysis of fractal dimension. All experiments were done under hypoxia. Quantification data were expressed as mean  $\pm$  SD ( $n = 6$ ). \*\*,  $p < .01$ ; Cont: normoglycemia; high: hyperglycemia; Sa: salidroside.

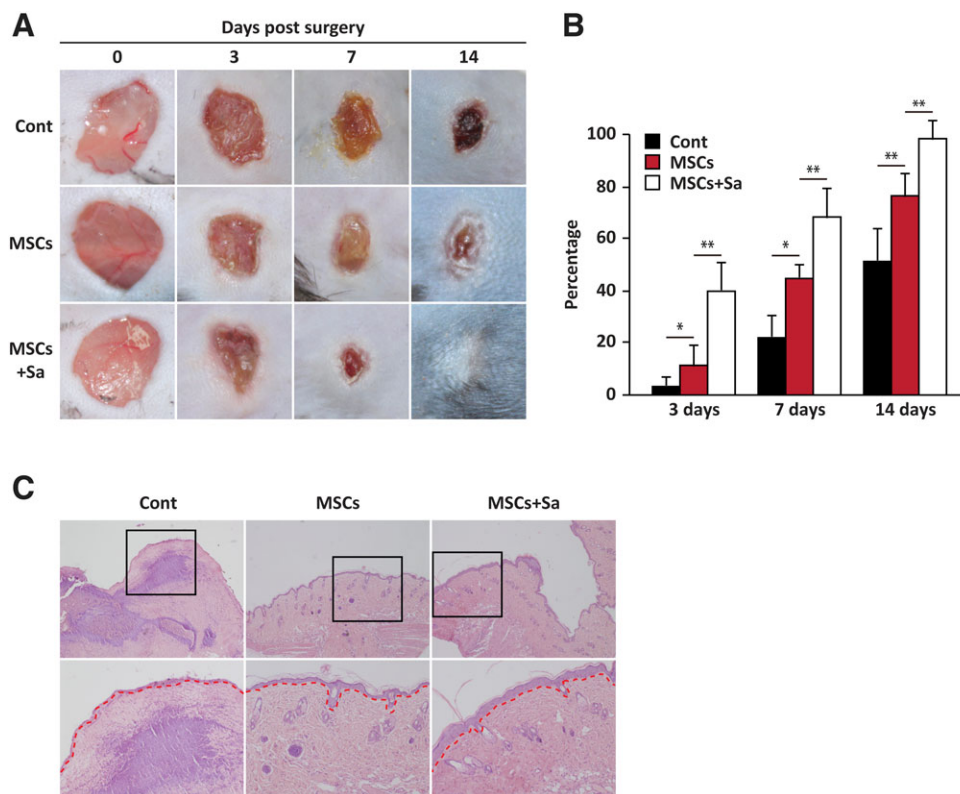
robustly suppressed the migration potential of the MSCs. Furthermore, we found that salidroside treatment also reincreased the expression levels of SDF-1 and CXCR4 suppressed by hyperglycemia (Supporting Information Fig. 4A, 4B). SDF-1/CXCR4 axis is crucial for MSCs migration potential, and FGF2 has been known to be able to induce CXCR4 expression [26, 27]. These results prompted the possibility that salidroside might also improve MSCs migration. Indeed, the results of transwell migration assay revealed that salidroside pretreatment restored the migration potential of MSCs that was substantially decreased under hyperglycemia (Fig. 4A, 4B). Data obtained from the scratch assay further confirmed this result (Fig. 4C, 4D).

Formation of F-actin by the polymerization of G-actin is critical for cell migration. Thus, we next examined the F-actin

polymerization of MSCs under hyperglycemia. We found that salidroside significantly enhanced F-actin polymerization in MSCs that was dramatically reduced under hyperglycemia (Fig. 4E, 4F). These results demonstrated that hyperglycemia decreases MSCs migration potential most probably by impairing F-actin polymerization; and that salidroside could restore the MSCs migration potential by reversing this impairment.

#### Salidroside-Pretreated MSCs Promote Wound Healing in Diabetic Mice

Our results have shown that salidroside could not only restore the paracrine function of MSCs but also their survival and migration potentials which were disrupted by hyperglycemia. To assess the therapeutic effect of salidroside-pretreated MSCs on diabetic wound healing, we established a mouse model of



**Figure 5.** Salidroside-pretreated mesenchymal stem cells (MSCs) promote wound healing in diabetic mice. **(A, B):** Wound closure rate of diabetic mice transplanted with salidroside-pretreated MSCs: **(A)** representative images taken at the indicated time points after wounding; **(B)** comparison of the wound closure rate at indicated time points after wounding. **(C):** H&E staining of the skin tissue surrounding wound site on day 14 after wounding. Quantification data were shown as relative to that control, and expressed as mean  $\pm$  SD ( $n = 6$ ). \*,  $p < .05$ ; \*\*,  $p < .01$ ; Cont: mice injected with phosphate buffered solution (PBS); MSCs: mice transplanted with PBS-pretreated MSCs; Sa: mice transplanted with salidroside-pretreated MSCs.

diabetic wound healing and transplanted MSCs pretreated with either salidroside or PBS. As shown in Figure 5A, 5B, while mice transplanted with PBS-pretreated MSCs showed an enhanced wound closure rate compared with the control group; pretreatment with salidroside further promoted the therapeutic effect of MSCs significantly. Moreover, hematoxylin and eosin staining results revealed an enhanced re-epithelialization at the wound site with transplantation of salidroside-pretreated MSCs (Fig. 5C). Furthermore, we found that the protein expression levels of HO-1, FGF2, and HGF were upregulated at the wound site in the mice transplanted with salidroside-pretreated MSCs (Fig. 6A, 6B).

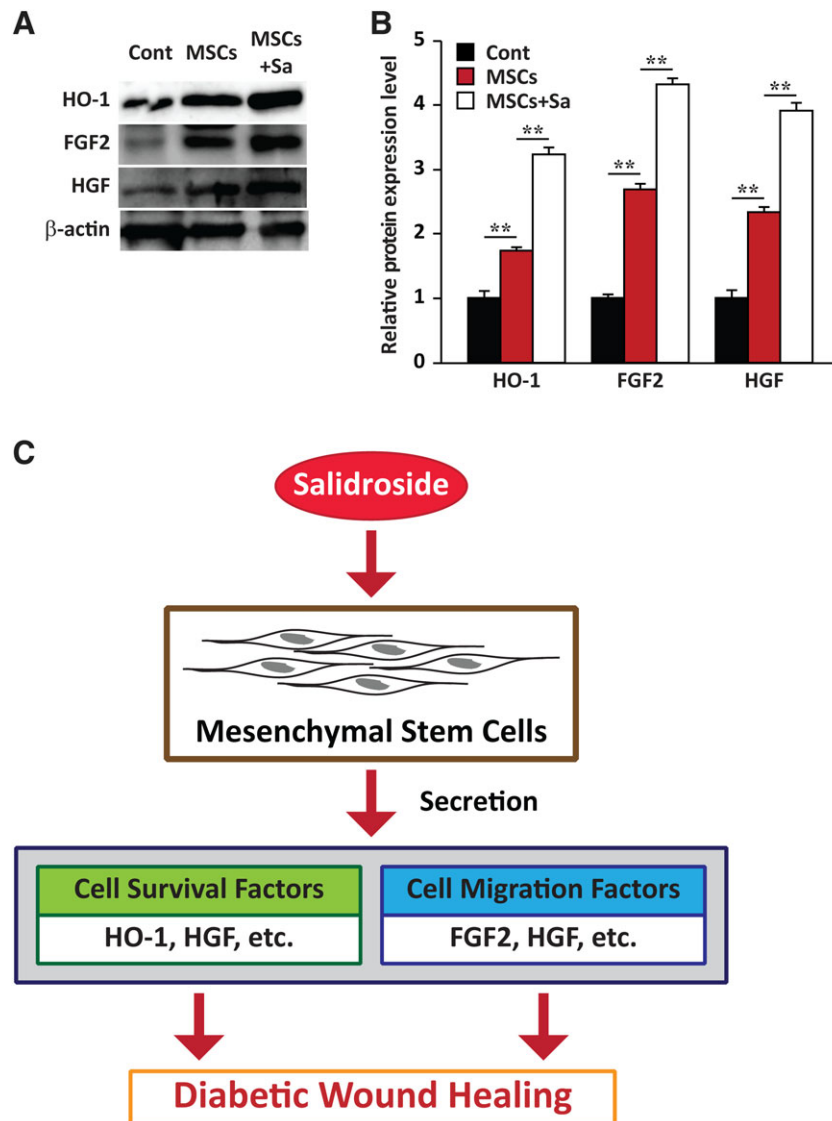
Collectively, our results demonstrate for the first time that salidroside enhances the paracrine function, survival rate, and migration potential of MSCs, thereby enhancing their therapeutic effect in diabetic wound healing (Fig. 6C).

## DISCUSSION

Hyperglycemia affects the expression of various factors, resulting in systematic impairment of physiological pathways and paracrine function, as well as the reduction of cell survival rate and migration potential. These abnormalities lead to several diabetic complications, such as angiopathy, neopathy, and nonhealing wound. Hyperglycemia severely disrupts

the survival rate and migration potential of MSCs most likely due to aberrant paracrine function. Our previous studies have shown the effect of salidroside in improving the paracrine function and migration potential of the skeletal muscle cells, both in normoglycemia and hyperglycemia [18, 19]. Salidroside exerts this function by inhibiting the expression level of prolyl-hydroxylase domain 3 (PHD3), which regulates a number of growth and angiogenic factors including HO-1, FGF2, and HGF; thus, treatment with salidroside provides benefit through the simultaneous induction of various factors [18, 19]. Previous report showed that HO-1, FGF2, and HGF are major components of the stem cells secretome, which is essential for wound healing [8]. Therefore, these facts prompt the possibility that salidroside might also be able to promote MSCs paracrine function, their survival and migration potential, and subsequently their therapeutic effect in diabetic wound healing. Indeed, our results have shown that salidroside pretreatment robustly induced the expression levels of cell survival factor HO-1, as well as FGF2 and HGF, which are crucial for cell survival and wound healing process under hyperglycemia. Wound healing is a complex process involving multiple factors. The ability of salidroside to simultaneously induce the expression and secretion of several factors yields higher benefits when compared with other therapeutic strategies for diabetic wound healing, such as administration of growth factor and genetic therapy.





**Figure 6.** Salidroside-pretreated mesenchymal stem cells (MSCs) promote the expression of cell survival and cell migration factors surrounding the wound site. **(A, B):** Protein expression levels of heme oxygenase-1, fibroblast growth factor 2, and hepatocyte growth factor from tissue surrounding the wound in diabetic mice transplanted with salidroside-pretreated MSCs: (A) protein expression levels, as examined by western blotting; and (B) quantification of protein expression levels. **(C):** Schematic diagram of the mechanism of salidroside-pretreated MSCs in promoting diabetic wound healing.  $\beta$ -Actin was used as a loading control in western blotting. Quantification data were shown as relative to that of control, and expressed as mean  $\pm$  SD ( $n = 3$ ). \*\*,  $p < .01$ ; Cont: mice injected with phosphate buffered solution (PBS); MSCs: mice transplanted with PBS-pretreated MSCs; Sa: mice transplanted with salidroside-pretreated MSCs.

Furthermore, compared with genetic manipulations, small molecules have a number of distinct advantages, especially from the safety and convenience aspects, and their effects can be fine-tuned by varying their concentrations and combinations [28, 29]. Thus, although whether other cell survival and cell migration factors are also involved in the effect of salidroside on MSC-based therapy needs to be investigated further, our results have nevertheless shown that salidroside pretreatment benefits MSC-based therapy.

MSCs are multipotent adult stem cells that have the potential for self-renewing and differentiating into multiple cell types [2]. MSCs secrete various cytokines and growth factors to support the functions of other cells, including their growth and migration [9]. Due to these characteristics, previous reports have described MSCs as a promising therapeutic

for diseases caused by tissue damage, including wound healing, ischemic heart failure, and hind limb ischemia [2, 7, 30]. MSCs participate in various steps of the wound healing process, including the enhancement of epidermal cell growth, angiogenesis, collagen deposition, anti-inflammation, and wound closure [9]. However, previous studies have shown that the therapeutic effect of MSCs is limited due to their poor survival after transplantation [30, 31]. Indeed, previous study showed that the survival rate of MSCs at 24 hours post-transplantation is relative low, and ROS make a great contribution of this low survival rate [32]. This problem is even more severe in diabetic wound healing, as hyperglycemia induces ROS generation, resulting in DNA damage and subsequently the activation of the apoptosis cascade. Thus, increasing the antioxidant defense potential of MSCs, thereby improving their

survival rate, is crucial for achieving a successful MSC-based therapy for diabetic wound healing. Increase of survived MSCs leads to the accumulation of secreted factors at the wound site, and thus is beneficial for the effect of MSC-based therapy. HO-1 is a critical factor for cell survival [33, 34]. Previous reports demonstrated that HO-1 upregulation could inhibit the apoptosis of MSCs after transplantation in treating acute liver failure, irreversible intestinal failure, and retina ischemia/reperfusion injury [35], while lack of HO-1 increased cellular damage caused by oxidative stress, leading to an enhanced apoptosis rate [36, 37]. Our results clearly demonstrated that salidroside restored the HO-1 expression in MSCs suppressed by hyperglycemia, and concomitantly, reduced hyperglycemia-induced intracellular ROS generation. Furthermore, salidroside enhanced the antioxidant defense of MSCs, and thereby, their survival under hyperglycemia.

Another key factor for the success of MSC-based therapy is potential of MSCs for homing to the injury site to exert their beneficial effects [38]. FGF2 is a crucial factor for the migration potential of cells, including MSCs migration, and it could indeed induce F-actin polymerization [8, 18, 24]. Our results have shown that both FGF2 expression and MSCs migration potential were severely decreased by hyperglycemia; while salidroside treatment grossly induced FGF2 expression, and in turn enhanced F-actin polymerization and cell migration. Conform with this, salidroside also induced the expression of SDF-1 and CXCR4. FGF2 could induce the expression of CXCR4, and SDF-1/CXCR4 axis plays important role in stem cell migration [26, 27]. Furthermore, FGF2-null mice show retardation in wound healing rate and re-epithelialization, as well as reduced collagen deposition at the wound site and thicker scabs, suggesting that FGF2 is crucial for re-epithelialization and wound repair [39, 40]. Concomitantly, our results showed that salidroside pretreatment promoted wound healing under hyperglycemia. Thus, the ability of salidroside to enhance FGF2 expression most plausibly is one of the factors underlying its enhancing effects on MSCs migration potential and wound healing rate.

Salidroside also significantly increased HGF expression in MSCs. HGF has been reported to enhance wound healing by promoting keratinocytes at the wound site [6, 8]. Furthermore, previous studies also showed that HGF treatment could also enhance MSCs migration [25], and that combination therapy using FGF2 and HGF could promote wound healing in a mouse model [6]. HGF could also exert antiapoptotic activity as it induces phosphorylation of c-Met, which binds and activates PI3K and further promotes cell survival [41, 42].

Collectively, our results show that salidroside enhances multiple characteristics of MSCs that are crucial for wound healing, thereby improving their paracrine effects and enabling the orchestrated, synergistic action of various cytokines and growth factors secreted by the MSCs. These factors in turn increase MSCs survival and migration potential under

hyperglycemia, and subsequently enhance diabetic wound healing. Furthermore, although further investigation is needed to show the involvement of other factors, our article has nevertheless shown that salidroside pretreated-MSCs is a novel therapeutic strategy for diabetic wound healing, and that HO-1, FGF2 and HGF are crucial for the therapeutic effect of salidroside pretreated-MSCs.

## CONCLUSION

Our data clearly demonstrate that salidroside pretreatment significantly improves the efficacy of MSC-based therapy for diabetic wound healing. Salidroside pretreatment reversed the hyperglycemia-induced suppression of MSCs paracrine function and the expression of HO-1, FGF2, and HGF. Thus, salidroside could not only protect MSCs from apoptosis and promote their survival under hyperglycemia, but also restore their migration potential, which was also impaired by hyperglycemia. These beneficial effects significantly improved the wound closure rate and re-epithelialization in diabetic mice transplanted with salidroside-pretreated MSCs. In conclusion, our findings demonstrate that transplantation of salidroside-pretreated MSCs into the injury site might be a promising novel therapeutic strategy for diabetic wound healing. These findings will also open-up the possibility of using this combinatorial therapy for other MSC-based treatments.

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## AUTHOR CONTRIBUTIONS

V.K.: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript; S.W.: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript; A.D.A.: most of the experiments; G.W.: analyzed part of the data; J.Z.: part of the animal experiment; O.M.: part of the cellular experiment; D.A.N.: part of the animal experiment.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

A patent has been filed with Chinese patent application No. 201810152377.7. The authors indicated no potential conflicts of interest.

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