EPO-activated MSC promote healing ulcers by improving microenvironment

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Abstract

Background: Mesenchymal stem cell (MSC) transplantation is an effective treatment therapy for ischemic ulcers. However, in high-glucose microenvironment, the original inflammation-inhibiting function of MSCs leads to turns into secreting large amounts of inflammatory mediators, such as tumor necrosis factor(TNF- α) e.g., which decreases their capacity and becomes poor quality stem cells over inflammation cells for diabetic foot ulcers repair in the healing of diabetic foot ulcers. Erythropoietin (EPO) is an anti-inflammatory, pro-angiogenic cytokine. It is unclear whether EPO-activated MSCs with biomaterials can promote the effective healing of diabetic foot ulcers.

Methods: Cultivated MSCs in MSC-L, MSC-H, EPO-G, Akt-G and mTOR-G, then separated the supernatant-conditioned medium of these groups to stimulate HUVECs on proliferation and migration experiments;A new type of biomaterial planted with the EPO-activated MSCs was applied to the diabetic foot ulcers of the C57 mice.

Results: *In vitro* experiments showed that EPO could stimulate MSCs to secrete VEGF in high-glucose microenvironment. More importantly, EPO could reduce the damage to MSCs by high-glucose microenvironment, promote their proliferation and migration functions, and inhibit the high-glucose-induced MSCs from secreting the inflammatory mediator TNF- α . *In vivo* experiments showed greater angiogenesis in EPO-MSC group than in control group, ulcer healing in EPO-MSC group was significantly better than in control group, and MSCs partially differentiated into endothelial cells. EPO-activated MSCs could inhibit the monocyte invasion of localized diabetic foot ulcers.

Conclusions: Our results indicate that EPO-activated MSCs can promote the effective

healing of diabetic foot ulcers. The mechanism is that EPO can change stem cells from excessive inflammation into general inflammation and improved diabetic foot ulcers inflammatory microenvironment.

Key words: Diabetic foot ulcers; Mesenchymal stem cells; Erythropoietin; Anti-inflammatory; Angiogenesis

1. Introduction

It is anticipated that the worldwide prevalence of diabetes in adults will reach 5.4% by 2025, and the total number of diabetic adults will reach 300 million [1]. Among these, 1.0%–4.1% will experience diabetic foot ulcers to differing degrees, and the rates of lower limb amputation will be 0.21%–1.37% [2].

Endothelial cells and mesenchymal stem cells are important in treating diabetic foot ulcers [3,4]. MSCs play the role of promoting angiogenesis and inhibiting inflammation in the skin wound as it heals. MSCs promote angiogenesis mainly through two ways: first, they secrete VEGF, which promotes the proliferation and migration of endothelial cells through paracrine pathways [5,6]; second, MSCs can differentiate into endothelial cells directly, thereby promoting revascularization in ulcerous tissue [7,8]. However, the current treatment results showed that MSC therapy to treat diabetic foot ulcers did not demonstrate the expected positive healing results that have been observed in other types of skin wounds [9]. This could be related to the high-glucose, hypoxic microenvironment of diabetic foot ulcers, which results in MSC dysfunction, reduced paracrine function and differentiation, and angiogenesis disorders [10]. It is especially

important to note that high-glucose and anoxic microenvironment prevent MSCs from originally inhibiting tissue inflammation to secreting large amounts of inflammatory mediators such as TNF- α , which leads to enhances local inflammation and results healing disorders [11].

EPO is a red blood cell regulating growth factor which is mainly produced in the body by the kidneys. It can stimulate the proliferation and differentiation of erythroid progenitor cells. In particular, EPO's function is to protect the kidneys. Therefore, it is widely used in clinical treatment of a variety of anemia, such as neonatal and aplastic [12]. However, recent studies have found that the erythropoietin receptor (EPOR) is also widely distributed in various systems other than the kidneys, such as the brain. Therefore, EPO serves such functions as neuroprotection [13, 14] antioxidation, anti-inflammation [15], anti-apoptosis [15, 16] and so on. EPOR is also distributed in MSCs, but it is unclear whether EPO-activated MSCs can promote the healing of ischemic ulcers, as is the mechanism by which they might do so. Therefore, we used EPO as a supplement to MSC therapy. Our aim was to reduce the negative stimulation of the MSCs from the high-glucose, hypoxic microenvironments, improve MSC function, and reduce the MSCs' secretions of inflammatory mediators. Thereby, we can change poor quality MSCs to high quality MSCs for the treatment of diabetic foot ulcers.

Based on the above, we planted EPO-activated MSCs on biomaterials, deposited the biomaterials on the diabetic foot ulcers of C57 mice, and studied the therapeutic effect.

2. Materials and Methods

2.1. Cell culture

We prepared a α -MEM medium(Hyclone Laboratories, USA) containing 10% fetal bovine serum, L-glutamine (2 mM)(Hyclone Laboratories, USA), penicillin (100U/ml)(Sangon.Com, China) and streptomycin (100 mg/ml)(Sangon.Com, China) as the basic MSC medium. Separately, 5.6 mM glucose was added to the basic MSC medium to form low-glucose microenvironment (LG) as MSC-L to simulate the normal blood glucose environment of body; 25 mM glucose was added to the basic MSC medium to form high-glucose microenvironment (HG) as MSC-H to simulate the diabetic blood glucose environment of body [17]; we also added EPO (100IU/ml)(Sigma, USA) to the MSC-H as the EPO group (EPO-G) [18]. The Akt inhibitor Triciribine (5 μ M)(Kangchen.com, China) and mTOR inhibitor Rapamycin (5 μ M)(Sigma, USA) were also added to the EPO-G, respectively, as the Akt (Akt-G) and mTOR blocking groups (mTOR-G) [19,20]. When contrasted the results among these groups, the MSC-L also as the control group in low-glucose microenvironment related to the MSC-H, EPO-G, Akt-G and mTOR-G, and the MSC-H as the control group in high-glucose microenvironment related to the EPO-G, Akt-G and mTOR-G. We prepared an RPMI-1640 (Hyclone Laboratories, USA) medium containing 10% fetal bovine serum(Gibco, USA), penicillin (100U/ml) and streptomycin (100 mg/ml) as the basic HUVEC medium. The MSCs and HUVECs were cultivated by basic MSC medium and basic HUVEC medium, and placed in a 37°C, 5% CO₂ incubator. We observed the cell growth with an inverted microscope and used the logarithmic phase cells in the

experiment.

2.3. MSC migration assays

We chose MSCs (GFP-transfected human bone marrow mesenchymal stem cells (Cyagen Biosciences (Guangzhou, China))) that were in the logarithmic phase. We then digested them with trypsin, prepared a cell suspension in a α -MEM basal medium, and counted the cells with an inverted confocal microscope. Then, we adjusted the cell density to $10^5/\text{ml}$ before planting the MSCs into 24-well plates. 600 μl MSC-H, EPO-G, Akt-G and mTOR-G were separately added to the 24-well plates. A 100 μl MSC suspension (1×10^4 cells) was added to the 25 mm 8.0 μm Transwell chamber. We routinely cultured the cells in the incubator for 6 hours at 37 °C, 5% CO₂. Then we fixed them with 90% ethanol after removing the chamber, stained them with 0.5% crystal violet (Sigma, USA) staining solution and observed and photographed the cells, counting and calculating the average MSC numbers at four randomly selected low magnifications. Selected randomly four sampling points of four control groups (MSC-H), EPO-G, Akt-G and mTOR-G at low magnification, counted the number of cells in each group sampling points of the average value.

2.4. Preparation of the MSC supernatant

We prepared MSCs that were in the logarithmic phase and planted them into 6-well plates. Then, the cells were incubated with MSC-L, MSC-H, EPO-G, Akt-G and mTOR-G for 48 hours, respectively. Then, a supernatant-conditioned medium (CM) was filtrated with a 0.22 μm filter needle and -70 °C preserved, and these were labeled

MSC-CM (LG), MSC-CM (HG), EPO-CM, Akt-CM, and mTOR-CM, respectively [21].

When contrasted the results among these groups, the MSC-CM (LG) as the control group in low-glucose microenvironment related to the MSC-CM (HG), EPO-CM, Akt-CM and mTOR-CM, and the MSC-CM (HG) as the control group in high-glucose microenvironment related to the EPO-CM, Akt-CM and mTOR-CM.

2.5. HUVEC migration assay

We chose HUVECs (human umbilical vein endothelial cells (Cyagen Biosciences (Guangzhou, China))) that were in the logarithmic growth phase. The cells were planted into 6-well plates and added to a 2 ml basal HUVEC medium at a dilution ratio of 1:5 for the MSC-CM (LG), MSC-CM (HG), EPO-CM, Akt-CM, and mTOR-CM media, respectively. The scratch test was conducted after the cells adhered to the walls. Then, we washed the plates by PBS and added them to an RPMI-1640 medium containing 0.1% serum. The scratches were observed and photographed with an Olympus BX50 microscope (Japan) at 0 and 24 hours. Lastly, we measured the scar areas using IPP software (Media Cybernetics) [22].

We chose HUVECs that were in the logarithmic phase. We then digested them with trypsin, prepared cell suspensions in a RPMI-1640 medium, and counted the cells with an inverted confocal microscope. Then, we adjusted the cell density to $10^5/\text{ml}$ before planting them into 24-well plates. We used 600 μl basal HUVEC medium at a dilution ratio of 1:5 for the MSC-CM (LG), MSC-CM (HG), EPO-CM, Akt-CM, and mTOR-CM media, respectively. A 100 μl HUVEC suspension (1×10^4 cells) was added

to the 25 mm 8.0µm Transwell chamber. We routinely cultured the cells in the incubator for 6 hours at 37 °C, 5% CO₂. Then, we fixed the cells with 90% ethanol after removing the chamber and stained them with 0.5% crystal violet staining solution. We finally observed and photographed the cells, counting and calculating the average cell numbers at four randomly selected low magnifications [23].

2.6. HUVEC proliferation assays

We chose HUVECs that were in the logarithmic phase. Then, we digested them with trypsin, prepared a cell suspension using a basal HUVEC medium, and counted the cells with an inverted confocal microscope. The cells were planted into 96-well plates using 100 µl basal HUVEC medium at a 1:5 dilution ratio for the MSC-CM (LG), MSC-CM (HG), EPO-CM, Akt-CM, and mTOR-CM media, respectively; cultured them for 48 hours after the addition of a 20 µl MTT(Sigma, USA) solution; and incubated them for 4 hours before the culture was terminated. 150 µl DMSO (Sigma, USA) was added to each well. After 10 minutes of shocking, the crystal was entirely dissolved. The light absorption value of each well was measured using a microplate reader (Molecular Devices), and we recorded the standard curve.

2.7. Detection of cytokines

We extracted the MSC-CM (LG), MSC-CM (HG), EPO-CM, Akt-CM, and mTOR-CM and detected the levels of TNF- α and VEGF by enzyme-linked immunosorbent sandwich assay (Elisa) (RD, USA). We detected the cytokines in strict accordance with

the Elisa kit instructions.

2.8. *Animal experiments*

All experiments performed with animals were reviewed approved by the Animal Ethics Committee of the Third Military Medical University (Chongqing, China). The nanofiber network of biomaterial was made by a mixture of 80% polylactic, 10% protein collagen and 10% silk) through electrospinning technology. Male C57BL/6J mice (5–8 weeks) (from the Third Military Medical University Experimental Animal Center) were used in the experiments. These mice were intraperitoneally injected with streptozotocin (35 mg/kg)(Sigma, USA) for 5 days uninterrupted. Subsequently, the mice with fasting blood glucose levels over 16.7 mmol/L were selected as the diabetic mice [24]. The EPO-activated MSCs (EPO-MSC group) and non-EPO-activated MSCs (MSC group) (2×10^4 cells) were planted on the biomaterials [19]. We ligated the proximal femoral artery of each mouse's lower limb, leading to lower limb ischemia and resulting in full-thickness 5 mm diameter dorsal skin wounds in each thigh; we then constructed models of diabetic foot ulcers [25]. The ulcers were separately planted with the EPO-MSC biomaterials, MSC biomaterials, blank biomaterials and the control group (no biomaterial). After surgery, the mice were given adequate food and water and were raised separately. Beginning on the date of the operation, we photographed the wounds at 0, 3, 7 and 10 days and analyzed the wound areas with IPP software. We cut the skin wound areas at 0, 3, 7 and 10 days based on the observation time, fixed them in 4% paraformaldehyde, produced 9 μ m frozen sections, and photographed these. There after

the sections at 7 and 10 days were stained by hematoxylin (HE) and the sections at 7 days were labeled as CD31 antibodies stained (Dako Cytomation. Denmark). While we cut the skin wound areas at 2 and 4 days and the sections were labeled as CD68 antibodies stained (Dako Cytomation. Denmark). Additionally, we quantitatively calculated the new blood vessel density of the local tissue ulcerations [24] in the former sections and the monocyte density of the local tissue ulcerations in the other ones.

2.9. Statistics analysis

Each experiment was replicated three times. The data were analyzed using SPSS software (version 18.0), and a confidence level of $P < 0.05$ was considered significant.

3. Results

3.1. EPO promotes MSCs migration in high-glucose microenvironment

We investigated the effects of EPO on MSCs by detecting MSC migration speeds in high-glucose conditions. We used Transwell chamber experiments to detect the effects of EPO on MSCs migration. In high-glucose microenvironment, the cell migration ability of the EPO-G was much greater (up to 261%, $\#p < 0.01$) than that of the control group (MSC-H). There were no significant differences between the mTOR-G and the EPO-G, while there was significantly less cell migration in the Akt-G (down to 60.3%, $*p < 0.01$) than that was in the EPO-G (Fig. 1A). From these results, we can conclude that in high-glucose microenvironment, EPO can mitigate the adverse effects of high glucose on MSCs by promoting MSCs migration directly through Akt pathways.

3.2. *EPO promoted MSCs secreting VEGF and inhibited MSCs secreting TNF- α in high-glucose microenvironment*

VEGF is considered to be a key factor in promoting the proliferation of HUVECs. Therefore, we used an Elisa assay to detect the vascular endothelial growth factor levels in each group. The results showed that in high-glucose microenvironment the VEGF content of the EPO-CM group was higher (up to 132%, $^{\#1}p<0.01$) than that of the control group; the VEGF content of the mTOR-CM group was considerably lower (down to 35%, $^{\#2}p<0.01$) than that of the EPO-CM group (Fig. 1B). This experimental result showed that in high-glucose microenvironment, EPO can promote HUVECs proliferation by promoting MSCs secretion of VEGF. This process is accomplished through the mTOR pathway.

We detected the impact of TNF- α expression in high-glucose microenvironment after the EPO stimulation of MSCs by Elisa assay. In high-glucose microenvironment, significantly more TNF- α was produced by MSCs than that was produced in the low-glucose microenvironment, which indicated that high-glucose microenvironment can stimulate MSCs to secrete a large number of inflammatory mediators, resulting in excessive local tissue inflammation and leading to ulcer wound healing difficulties. In high-glucose microenvironment, the EPO-CM led to a 37% ($^{\#}p<0.01$) decrease in TNF- α levels compared with the control group (Fig. 1C), suggesting that EPO can impede MSCs from generating TNF- α in high-glucose microenvironment. However, the supernatant TNF- α levels were lower in the Akt-CM and mTOR-CM groups than they were in the EPO-CM group, which showed that EPO decreased the MSCs' production of

inflammatory mediators in high-glucose microenvironment by neither Akt nor mTOR pathways but possibly through other mechanisms.

3.3. *EPO-CM promoted HUVECs proliferation*

We detected an effect of EPO-CM on HUVECs proliferation using the MTT assay. The OD values of MSC-CM without any reagent in the high-glucose microenvironment were much lower than those in the low-glucose microenvironment. In the high-glucose microenvironment, the OD values of the EPO-CM group were significantly higher (up to 181%, $\#^1p<0.01$) than those of the control group. The OD values of the Akt-CM group were lower (down to 63%, $\#^2p<0.01$) than those of the EPO-CM group; meanwhile, the OD values of the mTOR-CM group were also lower (down to 53%, $\#^3p<0.01$) than those of the EPO-CM group (Fig. 2A). This suggests that in high-glucose microenvironment, EPO-CM can significantly promote HUVECs proliferation. However, this effect can be blocked by both triciribine and rapamycin, which indicates that EPO-CM can promote HUVECs proliferation through mTOR and Akt pathways.

3.4. *EPO-CM can promote the formation of vascularization by HUVECs*

We detected whether EPO-CM could stimulate HUVECs to generate blood vessels using the scratch test and Transwell chamber experiment [21, 23]. Without any reagent, the scratch closure rate of HUVECs in the high-glucose microenvironment was much lower than that in the low-glucose microenvironment. In high-glucose microenvironment, the scratch closure rate of the EPO-CM group was higher (up to 217%, $\#^1p<0.01$) than that

of the control group. The closure rate of the mTOR-CM group almost have no difference with the EPO-CM group (Fig. 2B). In the Transwell chamber experiment, without any reagent there was considerably fewer cell migration in the high-glucose microenvironment than that in the low-glucose microenvironment. In high-glucose microenvironment, the cell migration in the EPO-CM group exceeded that in the control group (up to 225%, $^1p < 0.01$), while in the Akt-CM group, the cell migration rate was fewer (down to 57%, $^2p < 0.01$) than that in the EPO-CM group, in the mTOR-CM group, the cell migration rate was fewer (down to 71%, $^3p < 0.01$) than that in the EPO-CM group (Fig. 2C). These results indicated that a high-glucose microenvironment can lead to HUVECs' diminished ability to form blood vessels whereas EPO-CM can mitigate the adverse effect of high glucose on HUVECs and improve their ability to form blood vessels. Nevertheless, this effect can be blocked by Akt pathway, which can decrease HUVECs' ability to form blood vessels.

3.5. EPO-MSC can promote angiogenesis in diabetic foot ulcers and accelerate diabetic foot wound healing

To probe the healing effects of EPO-activated MSCs on diabetic foot ulcers, we overlaid biomaterials planted with EPO-MSCs, MSCs and blank biomaterials on C57 mice with diabetic foot ulcers. We also set a control group (ulcers without biomaterial). We observed the wound healing at 0, 3, 7, and 10 days. The results showed that the best group for wound healing was EPO-MSC group, the second one was MSC group, the third one was blank biomaterial group, and the last one was control group (Fig. 3),

indicating that EPO played a active role in promoting the healing of diabetic foot ulcers. For local tissue in diabetic feet, the greatest obstacle to healing is angiogenesis. To explore whether EPO-activated MSCs could promote angiogenesis, we observed the HE staining sections. The staining after 7 days indicated that the blood vessel density in the EPO-MSC group was significantly higher (up to 320%, $\#p<0.01$) than that in the MSC group (Fig. 4C), after 10 days the blood vessel density in the EPO-MSC group was still higher (up to 214.3%, $\#p<0.01$) than that in the MSC group (Fig. 4F). Our results also found that compared with the MSC group, in EPO-MSC group the density of HUVECs increased 89% ($\#p<0.01$) (Fig. 4J) and the density of MSCs increased 110% ($\#p<0.01$) (Fig. 4N), besides the distribution of MSC increased along the HUVECs in EPO-MSC group (Fig. 4I), which indicated EPO can enhance MSCs differentiate into HUVECs in local diabetic foot tissue, and the EPO most likely stimulated MSCs to improve the HUVECs' paracrine secretion, which can induce peripheral vascular regeneration.

3.6. EPO-MSC can inhibit local tissue invasion of monocytes in diabetic foot ulcers

One of the important reasons for the difficulty in ulcer healing is the excessive inflammation in localized diabetic foot areas. To explore whether EPO-activated MSCs could partially inhibit this excessive inflammation, we took sections stained by CD68 to observe the results. The tissue sections taken at 2 days showed that the monocyte density in the EPO-MSC group was markedly lower (down to 46.7%, $\#p<0.01$) than that in the MSC group (Fig. 5E), and at 4 days the monocyte density in the EPO-MSC group was still markedly lower (down to 43.5%, $\#p<0.01$) than that in the MSC group (Fig. 5F).

The results show that EPO-activated MSCs can inhibit the monocyte invasion of localized diabetic foot ulcers.

4. Discussion

The difficulties in healing of diabetic foot ulcers are local microenvironment inflammation and angiogenesis disorders [3, 26, 27]. MSC therapy is traditionally thought to be an effective treatment for diabetic foot ulcers. MSCs can differentiate into HUVECs in vitro and can also secrete VEGF to promote local tissue ulceration angiogenesis [5, 6], in the same time MSCs can also inhibit the healing process of skin inflammation. However, the function and differentiation capacity of VEGF are affected. MSC therapeutic effect is weakened owing to the high-glucose hypoxic conditions in the local microenvironment and a high degree of MSC apoptosis [9]. Further impeding healing, high-glucose stimulation releases inflammatory cytokines that inhibit MSCs from secreting sufficient amounts of inflammatory mediators. This will gradually increase local ulceration and impede healing. Local injury site revascularization is considered to be a prerequisite for regenerating tissue [28]. VEGF has appeared to be more closely related to cytokine VEGF [29]. One of MSCs' key features in treating diabetic foot ulcers is their ability to enhance the secretion of the growth factors, such as VEGF, that can enhance HUVECs' formation of vascularization. However, the results of animal experiments showed that under high-glucose microenvironment, there was less vascular regeneration of skin ulcers than there was in low-glucose microenvironment [9, 10]. Our research revealed that EPO-activated MSCs secreted more VEGF than

non-EPO-activated MSCs did under high-glucose microenvironment. However, this effect could be impeded by Akt inhibitors. Thus, in high-glucose microenvironment, EPO-enhanced HUVEC vascular function may be mainly achieved by VEGF paracrine secretion. Section staining showed that the density of HUVECs in the local tissue of the EPO-MSC group was far greater than that for the control group. Moreover, the density of HUVECs that were directly differentiated from the MSCs exceeded that of the control group. This indicated that EPO also has the direct function of promoting MSC differentiation into HUVECs as well as the function of VEGF paracrine secretion [30]. EPO enhanced MSCs' differentiation and regeneration capacities and their ability to secrete angiogenesis factors. This process works as a synergistic catalyst for the partial revascularization of diabetic foot ulcers.

Inflammation is an essential factor in skin wound healing [26] and could be one contributing factor to diabetic foot ulcer progression. In low-glucose microenvironment, MSCs can inhibit local inflammation and effectively promote wound healing by partially limiting the release of inflammatory cytokines. However, in high-glucose microenvironment, MSCs released many inflammatory cytokines (such as tumor necrosis factor (TNF- α)) in diabetic foot ulcer wound microenvironment, an effect that would have been limited by local inflammation in a low-glucose microenvironment [11]. TNF- α is one of the most important factors that can induce an inflammatory response. It is also considered the beginning of dynamic inflammation. TNF- α is primarily secreted by monocytes, macrophages and T cells. It begins the inflammation response and magnifies it as it develops, and it can cause the secretion of the inflammatory cytokines

IL-8, IL-11, IL-12, and IFN- γ as well as other lipids such as interleukin leukotrienes and prostaglandins. TNF- α can also further amplify the inflammatory reaction, causing excessive inflammation [31, 32]. TNF- α is mainly synthesized in the heart, liver, lungs, and kidneys, which distribute most of the EPOR. Our experimental results showed that in a high-glucose microenvironment, EPO can inhibit MSCs' release of TNF- α , stimulating MSCs in high-glucose microenvironment to better repair diabetic foot ulcers. Besides, monocyte is a natural important and protective composition in inflammatory response, which is but also a negative factor to delay the wound healing of diabetic foot ulcers. After stimulated by EPO, the density of monocyte in EPO-MSC group decreased obviously. It suggests that EPO may stimulate MSCs paracrine secretion and indirectly inhibit the monocyte invasion of localized diabetic foot ulcers.

5. Conclusions

In summary, our results indicate that EPO-activated MSCs can promote the effective healing of diabetic foot ulcers. The mechanism is that EPO can change stem cells from excessive inflammation into general inflammation and thus improved diabetic foot ulcers inflammatory microenvironment. Currently, diabetic ulcers is very difficult to cure, whether it is cell transplantation or biological agents therapy, neither of them can promote the healing of ulcers effectively, our results suggest that in experimental animals, the combination of cell transplantation and biological agents provides a new ideas to diabetic ulcers; second, EPO is more of systemic drugs, and topical application is of rare, our experiment also provides that the use of EPO in topical application is feasible.

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Disclousure

The authors declare no competing financial interests.

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Figure legends

Fig 1. Effect of EPO on MSCs migration in high-glucose microenvironment, and the concentration of VEGF and TNF- α in the MSC-CM. The migration of MSCs determined by Transwell chamber experiment. Concentration of VEGF and TNF- α in the MSC-CM determined by ELISA assay. (A) Results of Transwell chamber experiment of MSCs in high-glucose medium. # $p<0.01$ control group (MSC-H) vs. EPO-G; * $p<0.01$ EPO-G vs. Akt-G. (B) After stimulated by EPO for 24h, VEGF concentration in the supernatant. #1 $p<0.01$ EPO-CM group vs. control group (HG); #2 $p<0.01$ EPO-CM group vs. mTOR-CM group (HG). (C) After stimulated by EPO for 24h, TNF- α concentration in the supernatant. # $p<0.01$ EPO-CM group vs. control group (HG). (D-G) The cell migration of control group (MSC-H), EPO-G, Akt-G and mTOR-G (orig. mag. $\times 100$).

All values are means $\pm$ SEM.

Fig 2. MSC-CM stimulated by EPO promotes proliferation and migration of HUVECs. Cell proliferation determined by MTT assay and cell migration determined by cell scratch and Transwell chamber experiment. (A) Results of cell proliferation, absorbance was calculated at a wavelength of 492 nm. #1 $p < 0.01$ EPO-CM group vs. control group (HG); #2 $p < 0.01$ EPO-CM group vs. Akt-CM group (HG); #3 $p < 0.01$ EPO-CM group vs. mTOR-CM group (HG). (B) Results of cell scratch, #1 $p < 0.01$ EPO-CM group vs. control group (HG); #2 $p < 0.01$ EPO-CM group vs. Akt-CM group (HG). (C) Results of Transwell chamber experiment, #1 $p < 0.01$ EPO-CM group vs. control group (HG); #2 $p < 0.01$ EPO-CM group vs. Akt-CM group (HG); #3 $p < 0.01$ EPO-CM group vs. mTOR-CM group (HG). (D-H) respectively were the cell scratch results of control group (LG), control group (HG), EPO-CM group (HG), Akt-CM group (HG) and mTOR-CM group (HG) (orig. mag. $\times 40$); (I-M) respectively were the Transwell chamber experiment results of control group (LG), control group (HG), EPO-CM group (HG), Akt-CM group (HG) and mTOR-CM group (HG) (orig. mag. $\times 100$). All values are means $\pm$ SEM.

Fig 3. Evaluation of the effect of modified-biomaterials planted with MSCs on healing diabetic wound. (A) EPO-MSC group accelerated wound healing faster than MSC group, on day 0, 3, 7 and 10. $p < 0.01$ $n = 10$ per group. Material group accelerated wound healing faster than control group, but was slower than EPO-MSC group and MSC group, on day

0, 3, 7 and 10. $p < 0.01$ $n = 10$ per group. (B) The biomaterial imaged by a digital camera. (C) Images of diabetic foot ulcers healing in each group on day 0, 3, 7 and 10. (D) The biomaterial imaged by SEM. All values are means $\pm$ SEM.

Fig 4. EPO promoted angiogenesis and enhanced endothelial differentiation of MSCs in diabetic foot ulcers. (A, B, D, E) Wounds sections were stained by HE (orig. mag. $\times 200$). (A) Sections of MSC group on day 7; (B) Sections of EPO-MSC group on day 7; (C) The blood vessel density in sections obtained on day 7. # $p < 0.01$ MSC group vs. EPO-MSC group. (D) Sections of MSC group on day 10; (E) Sections of EPO-MSC group on day 10; (F) The blood vessel density in sections obtained on day 10. # $p < 0.01$ MSC group vs. EPO-MSC group. Took sections on day 7, (G, K) MSCs were transfected with GFP (green fluorescence) and (H, L) HUVECs were stained with CD31 (red fluorescence), then merged images (I, M) of MSCs and HUVECs (orig. mag. $\times 400$). (G, H, I) EPO-MSC group, the merged image shows the distribution of MSC increased along the HUVECs; (K, L, M) MSC group. (J) The density of HUVECs, # $p < 0.01$ MSC group vs. EPO-MSC group; (N) The density of MSCs, # $p < 0.01$ MSC group vs. EPO-MSC group. All values are means $\pm$ SEM.

Fig 5. EPO-activated MSCs inhibited the monocyte invasion of localized diabetic foot ulcers. Took sections of EPO-MSC group and MSC group on day 2 and 4. (A-D) Sections were stained with CD68 (red fluorescence) to observe distribution of monocyte

in local tissues of diabetic foot ulcers (orig. mag. $\times 400$). (A) The distribution of monocyte in MSC group on day 2; (B) The distribution of monocyte in EPO-MSC group on day 2; (C) The distribution of monocyte in MSC group on day 4; (D) The distribution of monocyte in EPO-MSC group on day 4; (E) The monocyte density on day 2, # $p < 0.01$ MSC group vs. EPO-MSC group; (F) The monocyte density on day 4, # $p < 0.01$ MSC group vs. EPO-MSC group. All values are means $\pm$ SEM.

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