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Hyperbaric oxygen potentiates diabetic wound healing by promoting fibroblast cell proliferation and endothelial cell angiogenesis

Running title: Hyperbaric oxygen promotes diabetic wound healing

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Abstract

Background: Diabetic foot ulcer (DFU), one of the diabetic complications, brings high burden to diabetic patients. Hyperbaric oxygen therapy (HBOT) has been proven to be an effective clinical method for the treatment of DFU. However, the mechanisms still to be elucidated.

Methods: Diabetic foot mice model was established, and treated with hyperbaric oxygen. Haematoxylin & Eosin (H&E) staining and Masson's trichrome staining were used for the analysis of wound healing. Human skin fibroblast (HSF) and Human Umbilical Vein Endothelial Cell (HUVECS) were exposed to high glucose and hyperbaric oxygen for studying the mechanism of hyperbaric oxygen promoted wound healing *in vitro*. Wound healing assay, Reactive Oxygen Species (ROS) assay, cell proliferation assay and tube formation assay were used for the analysis of wound healing. Quantitative-Polymerase Chain Reaction (Q-PCR), Western blotting and Enzyme-Linked Immunosorbent Assay (ELISA) were used for the analysis of gene expression.

Results: HBOT facilitated wound healing in DFU mice model, and promoted the expression of HIF-1α, NF-κB, VEGFA, SDF-1, VEGFR2 and CXCR4. Hyperbaric oxygen promoted the proliferation, migration and ROS production, as well as the expression of SDF-1 and VEGFA in HSF. HBOT stimulated the proliferation, migration and tube formation, as well as the expression of CXCR4 and VEGFR2 in HUVECS. **Conclusion:** Hyperbaric oxygen potentiates angiogenesis and diabetic wound healing by activating HIF-1α signaling, so as to promote the expression of VEGF/SDF-1 in HSF and the expression of VEGFR/CXCR4 in HUVECS, ultimately to promote the proliferation of HSF and the angiogenesis of HUVECS.

Keywords: Diabetic foot ulcer; Hyperbaric oxygen therapy; VEGF/VEGFR; SDF-1/CXCR4; HIF-1α

Introduction

Diabetes mellitus (DM) is a major global disease, and is caused by a disorder of glucose metabolism [1]. Due to the high epidemic of diabetes in the world, it has become a global concern for public health [2]. Various diabetic complications bring higher burden to patients [3]. One of the most common complications is diabetic foot ulcers (DFU) [4]. The symptoms of DFU include a disintegration of the foot dermal tissues, non-healing ulcers on the skin of diabetic foot, failure of wound healing, and ischemic pain in rest [5]. About 15% of the diabetic patients suffer from DFU, and more than 15% of the DFU patients have to be operated a subsequent amputation, which may result in a relative high rate of mortality [6].

In the past decades, studies of the pathophysiology of DFU demonstrated that, the impairment of angiogenesis in diabetic patients plays a pivotal role in the development of DFU [7, 8]. Angiogenesis is one of the most important steps in the process of wound healing [9-13], therefore the reduction of angiogenesis caused by diabetes facilitates the development of diabetic skin ulceration, especially DFU [14]. The interplay of fibroblasts and endothelial cells is of great importance in angiogenesis. Generally, fibroblasts are considered to play a key role in the regulation of endothelial cells mediated angiogenesis [15]. Fibroblasts produce different kinds of regulatory factors, such as Vascular Endothelial Growth Factor (VEGF [16] and Platelet-Derived Growth Factor (PDGF) [17], to stimulate the proliferation, migration and finally tube formation of endothelial cells. Importantly, investigations showed that fibroblasts promote endothelial cells mediated sprouting and lumen formation. An early study indicated that the *in vitro* co-cultivation of fibroblasts with endothelial cells promoted tube formation [18]. Later studies confirmed that fibroblasts stimulated the migration, proliferation, alignment, tube formation of endothelial cells by producing VEGF and other growth factors [19]. These studies demonstrated that the interactions of fibroblasts and endothelial cells are

crucial for angiogenesis. For diabetic patients, there is an impairment of angiogenesis and the resultant defect of wound healing [20]. More studies indicated that the elevated glucose level in diabetic patients is the major cause for the impairment of angiogenesis and delay of wound healing [21]. The *in vivo* study revealed that endothelial cells exposed to high glucose concentration resulted in an integrity loss and increased susceptibility to apoptosis, detachment, and circulation into the bloodstream, thus will be disorder in angiogenesis [22]. Taken together, these changes of molecular and cellular state lead to the development of DFU.

Stromal cell-derived growth factor-1 (SDF-1 or C-X-C Motif Chemokine Ligand 12 (CXCL12) is a chemotactic cytokine and has high level of expression in injured tissues [23]. It was found that SDF-1 can act to its receptor C-X-C chemokine receptor type 4 (CXCR4) to recruit endothelial progenitor cells from the bone marrow, and induce angiogenesis independent of VEGF.[24]. Previous reports demonstrated that the inhibition of SDF-1 impairs wound healing significantly, by decreasing angiogenesis and increasing inflammation in diabetic mice [25]. This study suggests that SDF-1 is a major component for angiogenesis and wound healing.

VEGF is one of the most important factors for the regulation of angiogenesis [26]. There are six variant types of VEGF have discovered, from type A to F [27]. Among them, VEGF-A is the most common one in the regulation of angiogenesis [28]. VEGF regulates endothelial cells in different stages of angiogenesis. In the inflammation stage, VEGF elevates the permeability of vessels and affects the expression of Selectin and intercellular adhesion molecule of endothelial cells, ultimately facilitates the recruitment of leucocytes to the injured part [29]. In the proliferation stage, VEGF strongly stimulates proliferation of endothelial cells [30]. Additionally, VEGF was found to regulate cell migration of endothelial cells [31]. In the remodeling stage, VEGF induces the assembly of endothelial cells to facilitate lumen formation [32].

Hyperbaric oxygen therapy (HBOT) has been proved to be an effective treatment for DFU [33]. The clinical treatment of DFU with HBOT has a history for more than 20 years [34]. HBOT can improve wound healing by stimulating angiogenesis, and lower the amputation rate [35]. Several studies revealed that HBOT improves DFU by reducing tissue hypoxia [36] and reducing inflammation [37]; however, the molecular and cellular mechanism of HBOT is still to be elucidated. In the current study, we investigated the molecular mechanism of HBOT for treating DFU. We found that HBOT activated HIF-1α and nuclea factor-κB (NF-κB) signaling, so as to stimulate the expression of SDF-1 and VEGF in fibroblasts and modulate cell proliferation and migration of endothelial cells to facilitate angiogenesis and wound healing.

Materials and Methods

Diabetic animal model, diabetic foot animal model and HBOT

To generate this mouse model, a glucose transporter specific inhibitor Streptozocin was administrated to the C57BL/6J mice for 5 times with a dose of 45mg/Kg. Body weight and blood glucose level were tested every two days. The mouse with blood glucose level tested as not lower than 16.7 mM for continuous 3 days can be considered as a successful diabetic mouse model. Based on the successful establishment of diabetic mouse model, a diabetic wound animal model was built to mimic DFU by a skin resection (5 x 5 mm^2) on the limb. The diabetic wound mice were sub-divided to two groups, one for non-HBOT, and the other for HBOT, with a treatment of hyperbaric oxygen therapy 90 min/day (A progressive increase in pressure for 15 min, followed by 60 min of continuous exposure to 100% oxygen at 2 ATA, then the pressure was slowly reduced over a 15-min period) in an NG90-ⅢB medical hyperbaric oxygen chamber (Ningbo Hyperbaric Oxygen Chamber Plant, Ningbo, China) for 30 days. The wound area was detected and calculated by a digital photography and image processing system (Analyze, version 6.0; AnalyzeDirect, Lenexa, KS). The experimental procedure was approved by the Central South University Institutional Animal Care and Use Committee

H&E staining

After 30 days of treatment by HBOT, C57BL/6J mice with or without HBOT were sacrificed. Next, the wound skin tissues were collected and fixed in 4% paraformaldehyde for 24h, embedded in paraffin and sectioned at 5 μm thickness. For H&E staining, the sections were stained with hematoxylin and eosin following standard procedures.

Cell culturing and hyperbaric oxygen treatment

Human skin fibroblast (HSF) was obtained from American Type Culture Collection. HSF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) High Glucose Cell culture medium supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1% Sodium-pyruvate (Sigma-Aldrich) and 1% Gentamycin solution (Sigma-Aldrich). Human umbilical vein endothelial cells (HUVECS) (Lonza, Walkerville, MD) were cultured in endothelial cell basal medium bullet kits (Lonza) containing 20% fetal bovine serum (FBS; Gibco, Aukland, New Zealand) supplemented with L-glutamine and antibiotics on 100 mm plates coated with type I rat tail collagen (Millipore/Upstate, Temecula, CA) at 10 μg/cm^2. To mimic diabetic condition, HSF or/and HUVECS were treated with high glucose, with 25 mM glucose, for 2 days then cells were transferred to a compression chamber (Drass Galeazzi, Italy) and exposed to 100% oxygen at a pressure of 2.5 ATA for 90 minutes per day. After the treatment for 3 days, cells were collected for assays.

Cell co-cultivation and Transwell permeability assays

To test the migration of HUVECs, HUVECs and HSF were seeded at a density of 3.3 × 10^4 cells/cm^2 on collagen-coated (10 μg/cm^2) Transwell polycarbonate filters (pore size = 0.4 μm, exposed area = 1 cm^2; Costar, Brumath, France). HSF was seed in the upper chamber, and HUVECS was seed in the lower chamber. For hyperbaric oxygen treatment, the co-cultivated cells were transferred to a compression chamber (Drass Galeazzi, Italy) and exposed to 100% oxygen at a pressure of 2.5 atmosphere absolute (ATA) for 90 minutes per day. After co-cultivation of the two cells for indicated time, the HUVECS cells were collected for assays. For transwell permeability assay, HUVECS cells were treated with high glucose for 2 days, then were treated with hyperbaric oxygen for 1, 2 and 3 days. The migrated HUVECS stained by crystal violet staining.

Wound healing assays to detect cell migration

For the wound healing assay, HSF cells were seeded into 6-well cell culture plates and cultured to ~100% confluence. After 6 hours of starvation, an artificial, homogeneous wound was created by scratching the monolayer with a sterile 200 μL pipette tip. Images of cells migrating into the wound were captured after 24 hours using a microscope.

Reactive Oxygen Species (ROS) assay HSF cells were treated with high glucose (HG, 25 mg/mL). For HBOT experiments, cells were treated with HG for 2 days, and then cells were treated with hyperbaric oxygen for 3 days. The duration of HBOT to the cells is 90 min for each day. Then ROS was measured, and results were presented. Reactive oxygen species were measured by a ROS assay kit (Thermo Fisher Scientific) and were performed following the manufacture's instructions.

Cell proliferation assay

To evaluate the effects of HBOT for cell proliferation, HSF cells were firstly treated with high glucose for 2 days, then were treated with hyperbaric oxygen for 5 days. Cell Counting Kit 8 (Sigma-Aldrich) was used to detect the number of viable cells and determine the cell proliferation of HSF. The assay was done according to the manufacturer instructions.

Tube formation assay

Matrigel (BD Biosciences, Bedford, MA, USA) was added to 12-well plates and incubated for 1 hour at 37°C. HUVECS in low serum medium (M199 containing 5% FBS) were seeded on the top of the polymerized Matrigel. Tube formation was imaged under a light microscope. The effect of

tube formation was calculated by measuring the length of the capillary-like network and number of tubules.

RNA extraction and Q-PCR

Cells were harvested at indicated time points, and total RNA was extracted by Trizol reagent (Invitrogen) according to manufacturer's instructions. 2-5 μg of RNA was used for reversed transcription, with 200 U Superscript II (Invitrogen) synthetic oligo dT. The resultant cDNA was used for Q-PCR, with the SYBR Green Q-PCR mix (KaTaRa), according to manufacturer's instructions. Q-PCR was carried out in an ABI 7500 thermocycler with fluorescence detection (Applied Biosystems). Primers are as followed: VEGFA forward: 5'-CTGCCGTCCGATTGAGACC-3'; VEGFA reverse: 5'-CCCCTCCTTGTACCACTGTC-3'; SDF-1 forward: 5'-TGCATCAGTGACGGTAAACCA-3'; SDF-1 reverse: 5'-CACAGTT-TGGAGTGTTGAGGAT-3'; GAPDH forward: 5'-AGGTCGGTGTGAACGGATTTG-3'; GAPDH reverse:5'-GGGGTCGTTGATGGCAACA-3'; VEGFR2 forward: 5'-GGCCCAAT-AATCAGAGTGGCA-3'; VEGFR2 reverse: 5'-CCAGTGTCATTTCCGATCACTTT-3'; CXCR4 forward: 5'-ACTACACCGAGGAAATGGGCT-3'; CXCR4 reverse: 5'-CCCACAATGCCAGTTAAGAAGA-3'; HIF-1α forward: 5'-TCTCGGCGAAGCAAA-GAGTC-3'; HIF-1α reverse: 5'-AGCCATCTAGGGCTTTCAGATAA-3'; NF-κB RELA or p65 forward: 5'-TGCGATTCCGCTATAAATGCG-3'; NF-κB RELA or p65 reverse: 5'-ACAAGTTCATGTGGATGAGGC-3'. The gene expression of VEGFA, SDF-1; HIF-1α and NF-κB were analyzed in the cultivated HSF cells and the tissues of mice animal model. The gene expression of VEGFR2 and CXCR4 were analyzed in the cultivated HUVECS cells and the tissues of mice animal model.

Western blotting

Total proteins of the cells were extracted by Golden lysis buffer (Tris-HCl, PH 8.0, 400 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM Na pyrophosphate, 1% Triton X-100, 10% glycerol), with supplement of protease inhibitors (Roche, Indianapolis, IN). The concentration of total protein was measured by a BCA kit (Pierce, Rockford, IL). 10 mg of protein was loaded onto 10% SDS-PAGE gel and was transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were then incubated with primary and secondary antibodies. Protein signals were detected via ECL method. The primary antibodies used in this study include anti-VEGFA (1:1000, Abcam), anti-SDF-1(1:1000, Abcam), anti-beta-actin (1:1000, Abcam), anti-VEGFR2 (1:1000, Abcam), anti-CXCR4 (1:1000, Abcam), anti-HIF-1α (1:1000, Abcam), anti-NF-κB (1:1000, Abcam). All the primary antibodies were incubated overnight at 4°C. The HRP-conjugated secondary antibody (1:5000, Sigma-Aldrich) was incubated for 2h at RT. The protein level of VEGFA, SDF-1; HIF-1α and NF-κB were analyzed in the cultivated HSF cells and the tissues of mice animal model. The protein level of VEGFR2 and CXCR4 were analyzed in the cultivated HUVECS cells and the tissues of mice animal model.

ELISA

In the co-cultivation system, the level of secreted form of SDF-1 and VEGFA were evaluated by ELISA. The SDF-1 and VEGFA ELISA kits (ThermoFisher) were used for the detection, guided by the instructions from the manufacturer. Briefly, 50 μL of medium after the cultivation for indicated times were collected and added to the coating plates, and 100 μL of biotin-conjugated labeling antibody was added to the plates. After incubation for 45 min at room temperature, the solutions in

the plates were removed and plates were cleaned by washing buffer for 4 times. Next, HRP-conjugated Streptavidin were added to the plate and incubated for 45 min. After removal of the solutions in the plates, 4 times wash by washing buffer were performed. HRP substrate TMB was added to the plates, and after 15 min incubation at 37 °C, ELISA signals were measured at 450 nm.

SiRNA and transfection

HIF-1α siRNA was customized ordered from GenePharma (Shanghai, China). SiRNA was transfected by Lipofectamine ™ 3000 according to the instruction of manufacturer.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) values. Comparisons between more than two groups were analyzed by one-way ANOVA followed by Tukey post hoc tests. Comparisons between two groups were made with two-tailed t-tests. Statistical analysis was performed using SPSS 10.0 for Windows. $P < 0.05$ was considered statistically significant.

Results

1. Hyperbaric oxygen therapy promoted the recovery of diabetic wound by stimulating angiogenesis in vivo

For the first step of exploration, the DFU mouse model was established by injected with Streptozocin, and a skin resection operation on the limb. Next, DFU mice were treated with or without hyperbaric oxygen. As shown by the pictures of Fig.1A, after 30 days treatment with HBOT, the wound area on the dorsum of foot was found to be notably smaller, and pyogenesis was not found, compared to the non-HBOT group (mice treated with Streptozocin but not HBOT). These results suggested that HBOT treated mice presented significantly better wound healing, characterized by a much smaller remaining wound area, a smooth recovered skin, and no remaining scar, compared to the non-HBOT group. The quantitative measurement of remaining wound area in DFU animal model was shown in Fig.1B. Results indicated the remaining wound area (mm^2) from 0 to 30 days after operation, suggested that HBOT group presented significantly smaller diabetic wound area than the non-HBOT group, from 19 to 30 days. These results demonstrated that HBOT facilitated wound healing in DFU animal model. Next, the expression of HBOT and angiogenesis associated genes, including NF-κB，HIF-1α，VEGFA，VEGFR2，SDF-1 and CXCR4 in DFU mice tissues (both HBOT and non-HBOT group) were measured by Q-PCR and Western blotting. As shown in Fig.1C and 1D, the expression of these genes was all found to be stimulated by HBOT, suggesting that NF-κB and HIF-1α signaling were activated, and HBOT induced recovery of DFU may be associated with VEGF/VEGFR and SDF-1/CXCR4 mechanism.

2. Hyperbaric oxygen stimulated the proliferation, migration and ROS production of fibroblasts

Due to the fact that fibroblasts are essential for wound healing, in the next step we investigated the effect of hyperbaric oxygen for the activity of fibroblasts, including proliferation, migration and ROS production. Fibroblast HSF was cultured with high glucose medium. For the assays, results of CCK8 assay revealed that, fibroblasts treated with hyperbaric oxygen for 90 min each day, after cultivation for 4 and 5 days, presented significantly higher proliferation rate, compared to the normal oxygen group (Fig.2A), suggesting that the proliferation of fibroblasts was stimulated by hyperbaric oxygen treatment. As shown in Fig.2B, our results indicated that hyperbaric oxygen treatment led to increased level of reactive oxygen species (ROS) in a time-dependent manner, suggesting that hyperbaric oxygen promoted the production of ROS in fibroblasts. As shown in Fig.2C, wound healing assay was used to test the migration of fibroblasts. Results indicated that, migration was inhibited by high glucose treatment, and stimulated by hyperbaric oxygen treatment.

3. Hyperbaric oxygen activated NF-κB/HIF-1α/VEGF/SDF-1 pathway in HSF

It is well accepted that NF-κB/HIF-1α cell signaling and the two factors VEGF/SDF-1 are critical for the regulation of endothelial cell mediated angiogenesis. Thus, we tried to examine whether hyperbaric oxygen affects this regulatory pathway in HSF. As shown in Fig.3A, the expression of NF-κB was up-regulated by the treatment of hyperbaric oxygen in a time-dependent manner, in the high glucose cultivation state, as revealed by Q-PCR results. Similarly, the expression of HIF-1α was stimulated as well by hyperbaric oxygen in a time-dependent manner (Fig.3B). Importantly, the expression of VEGFA and SDF-1 were also stimulated by hyperbaric oxygen, as shown in Fig.3C and 3D. Next, the protein level of NF-κB, HIF-1α, VEGFA and SDF-1 were evaluated by Western blotting (Fig.3E). Results of Western blotting are consistent with Q-PCR results, showing that the protein level of NF-κB, HIF-1α, VEGFA and SDF-1 were promoted by hyperbaric oxygen. Taken

together, we consider that hyperbaric oxygen activated NF-κB/HIF-1α/VEGF/SDF-1 pathway in HSF.

4. Hyperbaric oxygen simulated the tube formation and migration of endothelial cells when co-cultured with HSF.

To investigate the function of HBOT to endothelial cell during angiogenesis, a co-cultivation of fibroblasts (HSF) and endothelial cell (HUVECS) in a transwell system was performed. In this system, we can observe how HBOT affects the tube formation and migration of endothelial cell. As shown in Fig.4A, in the high glucose cultivation, the tube formation of HUVECS was found to be promoted by hyperbaric oxygen treatment in a time-dependent manner. In the condition of high glucose, the cell migration rate of endothelial cell HUVECS was found to be promoted by the treatment of hyperbaric oxygen, as shown by the migration assay results in Fig. 4B. Next, the mRNA level of CXCR4 and VEGFR2, which are the receptors of SDF-1 and VEGF, were examined by Q-PCR. Results indicated that the expression of CXCR4 and VEGFR2 in HUVECS were promoted by hyperbaric oxygen treatment in a time-dependent manner (Fig. 4C and 4D). Additionally, the time-dependent up-regulation of CXCR4 and VEGFR2 proteins were confirmed by Western blotting (Fig.4E). Additionally, the secreted form of SDF-1 and VEGF were found to be promoted, as indicated by the ELISA results (Fig.4F). Collectively, these results suggest that HBOT promoted the tube formation and migration of endothelial cells when co-cultivated with HSF.

5. Hyperbaric oxygen stimulated the migration and VEGF/SDF-1 expression of fibroblasts by activating HIF-1α

To investigate whether hyperbaric oxygen stimulates the migration of fibroblasts by activating HIF-1α, a knockdown of HIF-1α in fibroblast HSF was performed. As shown in Fig.5A, the gene silence of HIF-1α was confirmed by Q-PCR. Next, the mRNA level of VEGFA and SDF-1 were found to be decreased by the knockdown of HIF-1α, suggesting that HIF-1α regulates VEGF and SDF-1 expression in fibroblasts (Fig.5B and 5C). This suppression of VEGF and SDF-1 expression by the knockdown of HIF-1α was confirmed by Western blotting, as shown in Fig.5D. Moreover, we found that the migration of fibroblast was also suppressed by HIF-1α knockdown, as revealed by the wound healing assay of HSF (Fig.5E). These results suggested that hyperbaric induced expression of VEGFA and SDF-1, as well as cell migration of fibroblast are HIF-1α dependent.

6. Hyperbaric oxygen stimulated the tube formation and migration of HUVECS by activating HIF-1α

In this section of study, knockdown of HIF-1α was performed to study whether hyperbaric oxygen induces tube formation and migration of HUVECS by activating HIF-1α. After HIF-1α knockdown , HUVECS were co-cultivated with HSF. Next, tube formation was evaluated. As shown in Fig.6A, hyperbaric oxygen treatment promoted tube formation of HUVECS, however, this can be suppressed by the knockdown of HIF-1α. Moreover, as shown in Fig.6B, hyperbaric oxygen treatment promoted HUVECS migration, however, this was suppressed by HIF-1α knockdown. Additionally, the mRNA level of VEGFR2 and CXCR4 in HUVECS were found to be suppressed by HIF-1α knockdown (Fig.6C). As shown in Fig.6D, the suppression of VEGFR2 and CXCR4 protein level were confirmed by Western blotting. Additionally, as shown in Fig.6F, the level of the secreted form of SDF-1 and VEGF in the cultivated medium were confirmed to be reduced by the knockdown of HIF-1α. In summary, these results suggest that hyperbaric oxygen induces VEGFR2

and CXCR4, as well as HUVECS tube formation and migration through HIF-1α.

Discussion

Angiogenesis is an essential process in wound healing [38]. In diabetic patients, wound healing is delayed or impaired, resulting in the development of DFU. HBOT has been proved to be an effective treatment to improve wound healing and cure DFU [39]. Improvement of oxygen delivery to injured tissues by HBOT is considered as an important mechanism for HBOT induced wound healing. Studies revealed that HBOT can increase the amount of oxygen with 16 folds higher; this will meet the demand of oxygen for poorly perfused tissues [40]. Other studies focused on the molecular and cellular mechanism of HBOT induced angiogenesis. HBOT was found to stimulate endothelial nitric oxide synthase (eNOS) to initiate producing NO, thus triggers the activation and recruitment of endothelial progenitor cells [41]. In diabetic patients, HBOT reverses the inhibition of eNOS to facilitate angiogenesis and wound healing [42]. However, more molecular details are remained to be elucidated.

In the present study, based on the successful establishment of diabetic wound animal model, HBOT was proved to promote wound healing in diabetic mice, according to the measurement of the remaining wound area. These results are consistent with previous reports [43]. Additionally, we found that the protein level of NF-κB, HIF-1α, VEGFA, VEGFR2, SDF-1 and CXCR4 were all promoted by HBOT in DFU mice model, demonstrating that NF-κB/HIF-1α signaling were activated, and angiogenesis associated pathways VEGF/VEGFR and SDF-1/CXCR4 were activated. For the first time, our research results indicates that HBOT can activate NF-κB/HIF-1α signaling and VEGF/VEGFR and SDF-1/CXCR4 simultaneously *in vivo*.

Next, we attempted to investigate the effects of HBOT for fibroblasts and determine the role of these effects in the regulation of angiogenesis. We found that cell proliferation of fibroblasts can be stimulated by HBOT. This is consistent with previous investigates which indicated that the

proliferation of fibroblasts exerted positive effect for angiogenesis [44].

Besides, our results revealed that HBOT promoted the migration of fibroblasts hence may in turn facilitate angiogenesis. Additionally, HBOT was found to stimulate ROS production in our high glucose cell model. For the first time, our results revealed that HBOT regulates the proliferation and migration of fibroblasts to affect angiogenesis and wound healing. Importantly, evidence from Q-PCR and Western blotting indicated that HBOT stimulated fibroblasts to express VEGF, SDF-1, NF-κB and HIF-1α，which are critical factors and signaling components for angiogenesis. Previous studies have demonstrated that SDF-1 and VEGF are probably the two most important factors for stimulating vessel formation of endothelial cells [45]. Therefore, here we indicate that HBOT promotes the production of SDF-1 and VEGF in fibroblast, in turn to regulate the angiogenic activity of endothelial cells. The present study specifically demonstrates that HBOT stimulates fibroblasts mediated production of SDF-1 and VEGF to promote angiogenesis and probably diabetic wound healing, implying that the simultaneously stimulation of SDF-1 and VEGF is important for the angiogenesis and wound healing. This new insight into the regulatory mechanism of HBOT provides new information for the design of novel therapeutic agents for DFU. For example, a combination of factors including SDF-1 and VEGF can be used for the treatment of DFU, this may elicit better effect than the use of one factor singly.

Next, we focused on the study of the regulation of endothelial cells by HBOT. Endothelial cell HUVECS and fibroblast HSF were co-cultivated in a transwell system. In this study, tube formation of HUVECS was found to be stimulated by HBOT. Similarly, the migration of HUVECS was found to be stimulated by HBOT. Thus, we considered that HBOT promotes cell migration of endothelial cells. For deeper insights into the molecular change of HUVECS, examination of mRNA and protein of CXCR4 and VEGFR2 were performed. We identified that HBOT increased the

expression of CXCR4 and VEGFR2, which are receptors for SDF-1 and VEGF, respectively. The increased expression of receptors of the two major regulatory factors may facilitate the activity of HUVECS, includes migration and tube formation. It is well known that the expression of receptors can be stimulated by ligands, and a positive feed-back was formed [46]. The proposed SDF-1/CXCR4 and VEGF/VEGFR positive feed-back loop may be important for the promotion of the subsequent vessel formation. Our systematic study pointed out that HBOT stimulated the production of both SDF-1 and VEGF in fibroblasts, as well as the expression of receptors CXCR4 and VEGFR2, in turn to activate endothelial cells for migration and tube formation. The promotion of gene expression of ligands (SDF-1 and VEGF) and their respective receptors (VEGFR2 and CXCR4) in fibroblast and endothelial cell simultaneously suggests that the cooperation and balance of the two cells are important in the regulation of tube formation.

Our further studies investigated the signaling pathways that mediate HBOT induced SDF-1 and VEGF expression in fibroblasts. Previous reports proved that HIF-1α is a main regulator for the expression of SDF-1 and VEGF expression. We observed that the knockdown of HIF-1α suppressed the expression of VEGFA and SDF-1. This finding is consistent with previous studies which demonstrated that HIF-1α regulates the expression of SDF-1 and VEGF in various cell types, including T cells, macrophages, etc. [47]. Additionally, similar mechanisms have been found in the regulation of angiogenesis in rheumatoid arthritis synovium [48] and tumorigenesis [49]. Probably the molecular pathway that HIF-1α mediated regulation of SDF-1/VEGF expression is an essential and conserved pathway in angiogenesis process. Moreover, we found that the knockdown of HIF-1α in HUVECS inhibited tube formation and migration, as well as the production of VEGFR and CXCR4, demonstrated that the activation of HIF-1α is critical for the angiogenic activity of endothelial cells.

To our best of knowledge, the HBOT induced activation of HIF-1α is a new mechanism to explain

how HBOT facilitates angiogenesis and diabetic wound healing. Additionally, HIF-1α can be considered as a critical switch for HBOT mediated cure of wound healing, thus, HIF-1α is a potential effective therapeutic target for DFU. The promotion of HIF-1α signaling may benefit both fibroblast and endothelial cell simultaneously, thus can facilitate angiogenesis and wound healing in two ways. In addition to HBOT, the development of new therapeutic agents by stimulating HIF-1α may be a promising way for the treatment of DFU.

Collectively, the present study investigated the cellular and molecular mechanism of HBOT induced diabetic wound healing. We found that HBOT enhances diabetic wound healing by affecting both fibroblasts and endothelial cells. HBOT promotes the proliferation of fibroblasts; and induces the production of angiogenesis regulator SDF-1 and VEGF, which subsequently stimulate the expression of their respective receptors CXCR4 and VEGFR in endothelial cells; and stimulate the migration and vessel formation of endothelial cells. Furthermore, we identified that HBOT affects fibroblasts through the activation of HIF-1α signaling pathway. Our findings provide new mechanism to explain how HBOT improves angiogenesis and wound healing in diabetic patients. This new mechanism may facilitate the future development of new therapeutic agents or clinical methods for the treatment of diabetic wound healing.

Hightlights

1. HBOT facilitated wound healing in DFU;
2. HBOT promotes angiogenic activities endothelial cells
3. HBOT activates HIF-1α signaling;
4. HBOT promoted the expression of VEGF/SDF-1 in fibroblast and the expression of VEGFR/CXCR4 in endothelial cells

Abbreviations

DM: Diabetes mellitus

DFU : diabetic foot ulcers

VEGF: vascular endothelial growth factor

SDF-1: stromal cell-derived growth factor-1

HSF: human skin fibroblast;

DMEM : Dulbecco's modified Eagle's medium;

FBS: fetal bovine serum;

HUVECS : Human umbilical vein endothelial cells;

HG: high glucose;

SD :standard deviation;

eNOS: endothelial nitric oxide synthase;

H&E: haematoxylin & Eosin

ROS: reoxygen species

Q-PCR: quantitative-polymerase chain reaction

ELISA: enzyme-linked immunosorbent assay

DFU: diabetic foot ulcer

HBOT: hyperbaric oxygen therapy

HIF-1α: hypoxia-inducible factor-1α

NF-κB: nuclea factor-κB

VEGFA: vascular endothelial growth factor A

SDF-1: stromal cell derived factor 1

VEGFR2: vascular endothelial growth factor receptor 2

CXCR4: C-X-C chemokine receptor type 4

PDGF: platelet-derived growth factor

CXCL12: C-X-C motif chemokine ligand 12

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Ethics Approval

The experimental procedure was approved by the Central South University Institutional Animal Care and Use Committee.

Conflicts

The authors declare that they have no conflicts of interests.

Authors' Contributions

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study design: Xu Huang, Bimei Jiang;

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clinical studies: Le Guo, Xu Huang, Xv Cui;

experimental studies: Mitao Huang, Xiaoyuan Huang;

data acquisition: Xu Huang, Pengfei Liang;

data analysis: Xu Huang, Pengfei Liang;

statistical analysis: Mitao Huang, Xv Cui

manuscript preparation: Xu Huang, Le Guo

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Figure legends

Fig.1 Hyperbaric oxygen therapy promoted wound healing in DFU mice.

Representative pictures of DFU mice (**A**). The quantitative measurement of remaining wound area (mm^2) in non-HBOT and HBOT diabetic wound mice model from 0 to 30 days after operation. (n=3, t-test, *p<0.05) (**B**). The expression of NF-κB，HIF-1α，VEGFA，VEGFR2，SDF-1 and CXCR4 in DFU mice tissues were measured by Q-PCR in non-HBOT and HBOT diabetic mice model (**C**). The protein level of NF-κB，HIF-1α，VEGFA，VEGFR2，SDF-1 and CXCR4 in DFU mice tissues were measured by Western blotting in non-HBOT and HBOT diabetic mice model (**D**).

Fig.2 Hyperbaric oxygen stimulated the proliferation, ROS production and migration of fibroblasts

A To evaluate the effects of HBOT for cell proliferation of HSF cells, CCK8 assay (**A**) and ROS measurement (**B**) were performed. (n=3, t-test, *p<0.05). Cell migration of HSF was measured by wound healing assay (**C**).

Fig.3 Hyperbaric oxygen NF-κB/HIF-1α/VEGF/SDF-1 pathway in HSF

The mRNA level of NF-κB (**A**), HIF-1α (**B**), VEGFA (**C**) and SDF-1 (**D**) were measured by Q-PCR. The protein level of NF-κB, HIF-1α, VEGF and SDF-1 were measured by Western blotting (**E**).

Fig.4 Hyperbaric oxygen simulated tube formation, migration and VEGFR2/CXCR4 expression of endothelial cells when co-cultured with HSF.

Tube formation (**A**) and cell migration (**B**) were performed to test the angiogenic activities of HUVECS. mRNA level of VEGFR2 (C) and CXCR4 (D) were evaluated by Q-PCR. Additionally, protein level of VEGFR2 and CXCR4 in HUVECS were measured by Western blotting (**E**). The secreted form of SDF-1 and VEGFA in cultivated medium were measured by ELISA (**F**).

Fig.5 Hyperbaric oxygen stimulated migration of fibroblast by activating HIF-1α pathway.

In HSF, HIF-1α was knockdown by siRNA. The mRNA level of HIF-1α (**A**), VEGFA (**B**) and SDF-1(**C**) were measured by Q-PCR. The protein levels of VEGFA and SDF-1 were measured by Western blotting (**D**). Cell migration of HSF was measured by wound healing assay (**E**).

Fig.6 Knockdown of HIF-1α suppressed tube formation, migration and the expression of VEGFR2/CXCR4 in HUVECS

Tube formation of HUVECS was measured by tube formation assay (**A**), cell migration of HUVECS were measured by transwell assay (**B**). The mRNA level of VEGFR2 (**C**) and CXCR4 (**D**) were measured by Q-PCR. The protein levels of VEGFR2 and CXCR4 were measured by Western blotting (**E**). The secreted form of SDF-1 and VEGFA in cultivated medium were measured by ELISA (**F**).

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Hyperbaric oxygen potentiates diabetic wound healing by promoting fibroblast cell proliferation and endothelial cell angiogenesis

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Declarations

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Highlights:

1. HBOT facilitated wound healing in DFU;
2. HBOT promotes angiogenic activities endothelial cells
3. HBOT activates HIF-1α signaling;
4. HBOT promoted the expression of VEGF/SDF-1 in fibroblast and the expression of VEGFR/CXCR4 in endothelial cells;

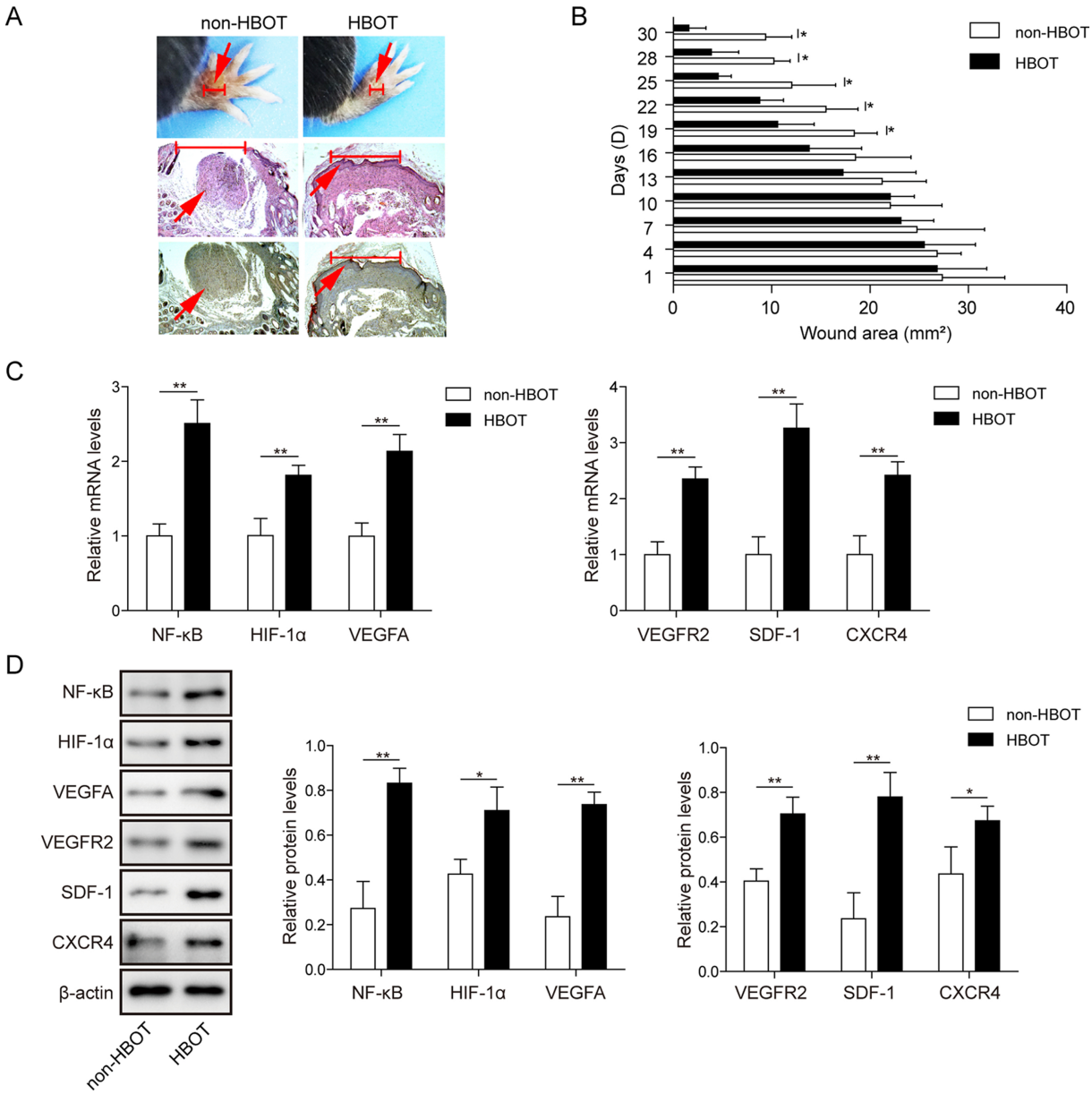
A
non-HBOT
HBOT
B
non-HBOT
HBOT
Days (D)
30
28
25
22
19
16
13
10
7
4
1
0
10
20
30
40
Wound area (mm²)
C
non-HBOT
HBOT
Relative mRNA levels
NF-κB
HIF-1α
VEGFA
VEGFR2
SDF-1
CXCR4
D
NF-κB
HIF-1α
VEGFA
VEGFR2
SDF-1
CXCR4
β-actin
non-HBOT
HBOT
Relative protein levels

Figure 1

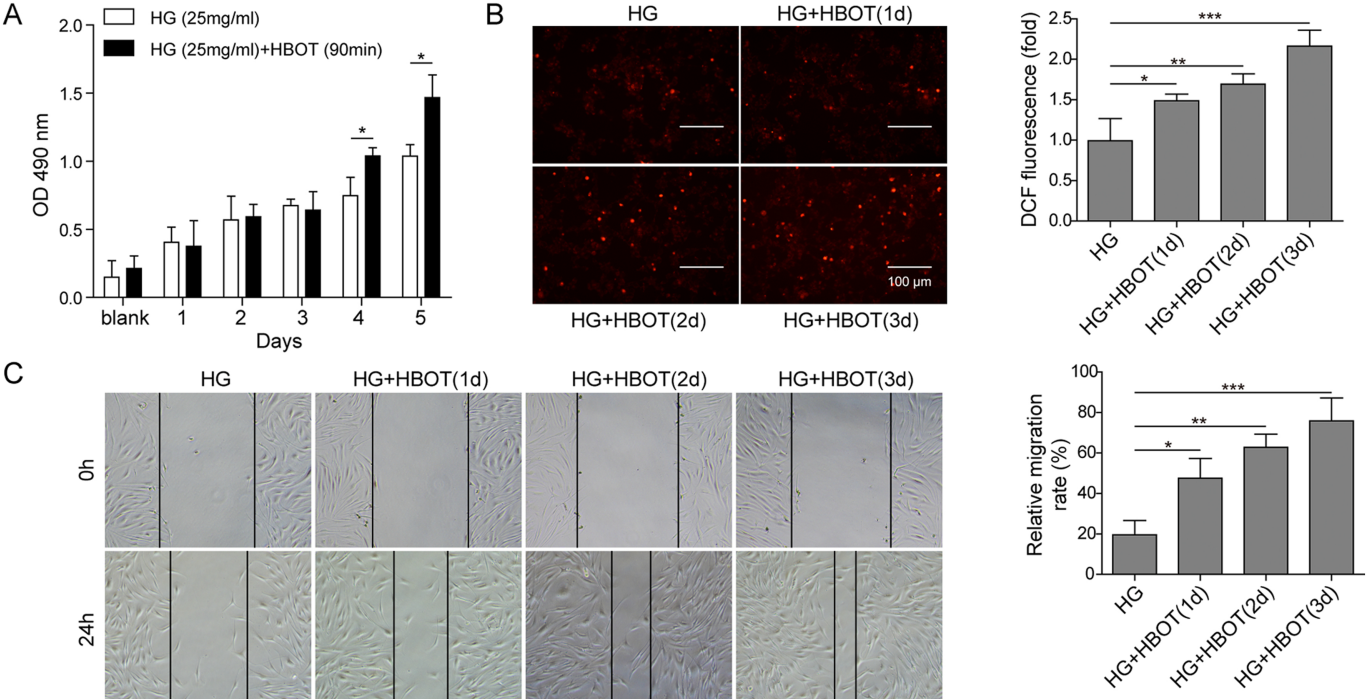
A
HG (25mg/ml)
HG (25mg/ml)+HBOT (90min)
OD 490 nm
2.0
1.5
1.0
0.5
0.0
blank
1
2
3
4
5
Days
*
*
B
HG
HG+HBOT(1d)
HG+HBOT(2d)
HG+HBOT(3d)
100 μm
DCF fluorescence (fold)
2.5
2.0
1.5
1.0
0.5
0.0
***
**
*
HG
HG+HBOT(1d)
HG+HBOT(2d)
HG+HBOT(3d)
C
HG
HG+HBOT(1d)
HG+HBOT(2d)
HG+HBOT(3d)
0h
24h
Relative migration rate (%)
100
80
60
40
20
0
***
**
*
HG
HG+HBOT(1d)
HG+HBOT(2d)
HG+HBOT(3d)

Figure 2

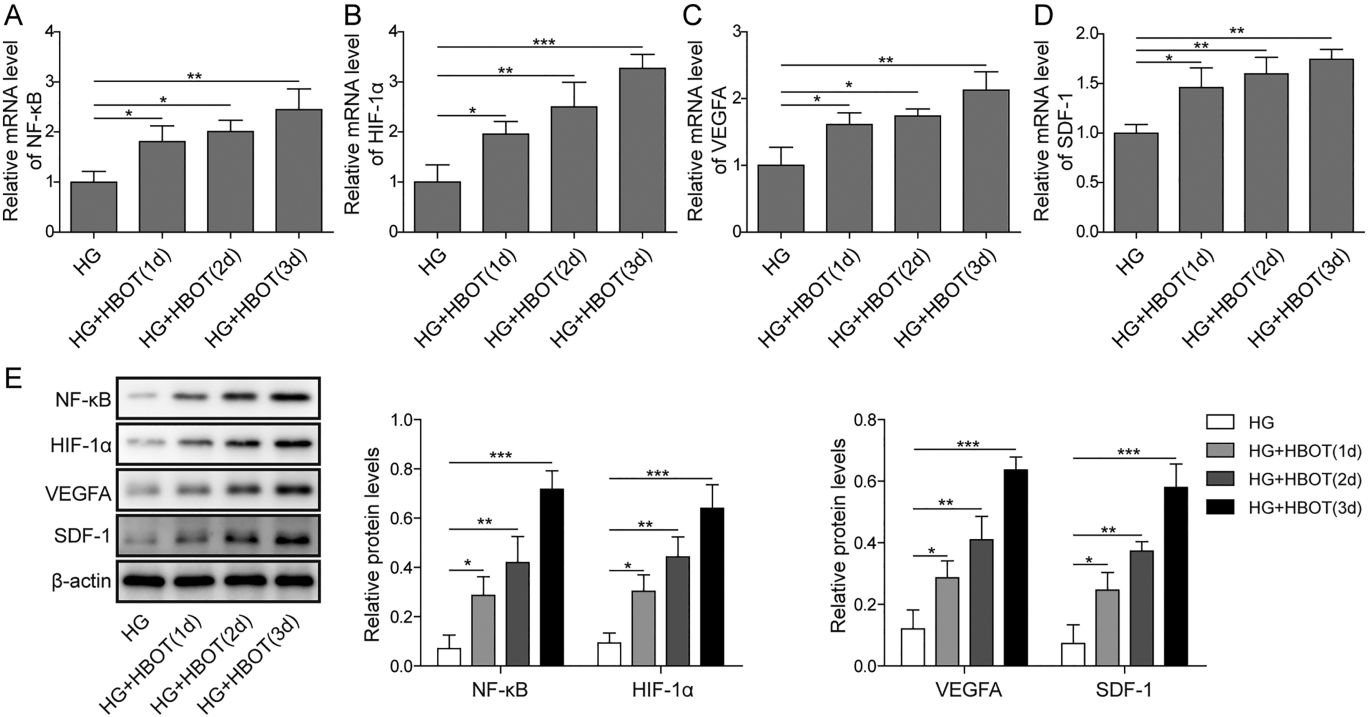
A
Relative mRNA level of NF-κB
B
Relative mRNA level of HIF-1α
C
Relative mRNA level of VEGFA
D
Relative mRNA level of SDF-1
HG
HG+HBOT(1d)
HG+HBOT(2d)
HG+HBOT(3d)
E
NF-κB
HIF-1α
VEGFA
SDF-1
β-actin
Relative protein levels
NF-κB
HIF-1α
VEGFA
SDF-1

Figure 3

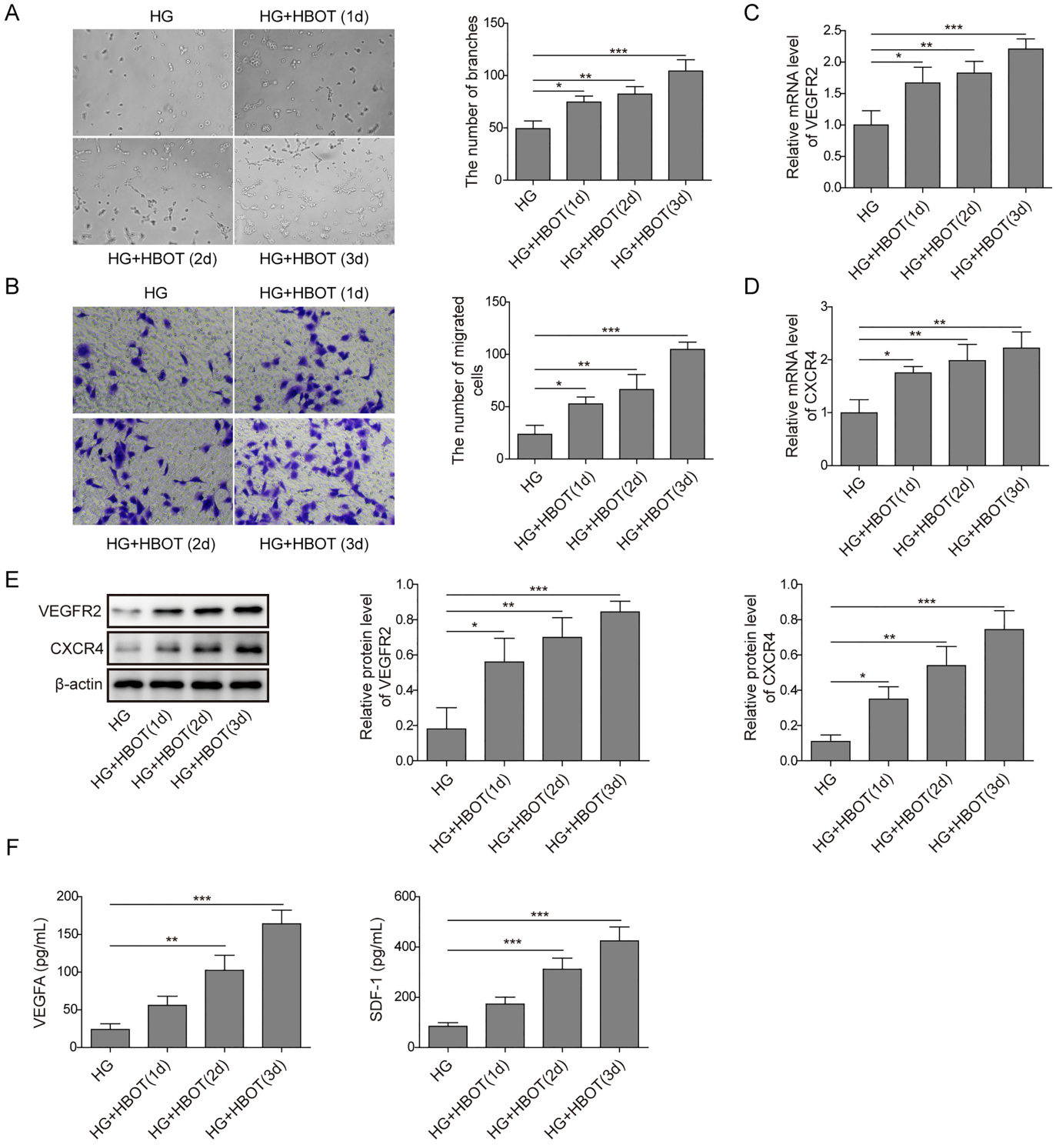
A
HG
HG+HBOT (1d)
HG+HBOT (2d)
HG+HBOT (3d)
The number of branches
B
The number of migrated cells
C
Relative mRNA level of VEGFR2
D
Relative mRNA level of CXCR4
E
VEGFR2
CXCR4
β-actin
Relative protein level of VEGFR2
Relative protein level of CXCR4
F
VEGFA (pg/mL)
SDF-1 (pg/mL)
HG
HG+HBOT(1d)
HG+HBOT(2d)
HG+HBOT(3d)

Figure 4

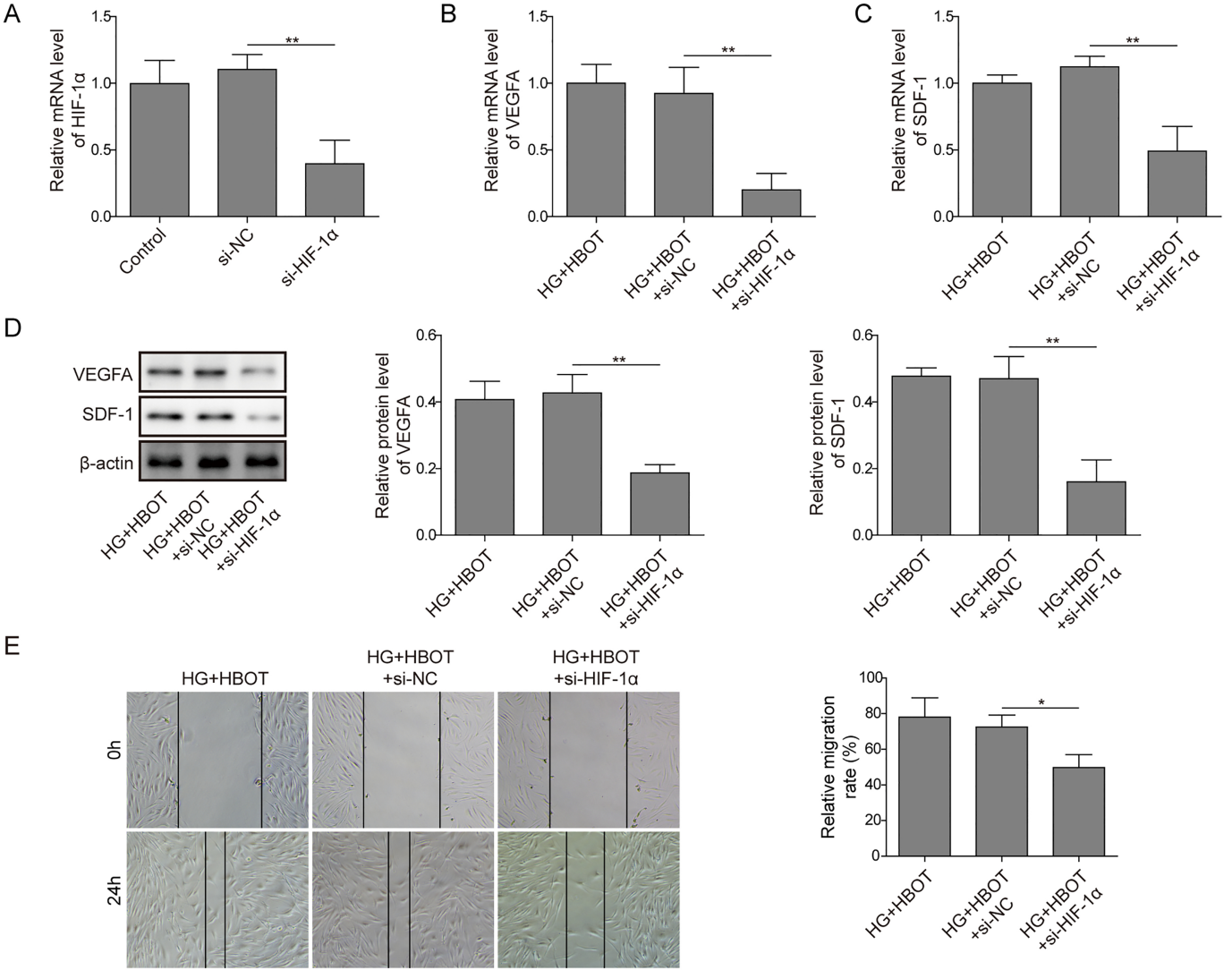
A
Relative mRNA level of HIF-1α
1.5
1.0
0.5
0.0
**
Control
si-NC
si-HIF-1α
B
Relative mRNA level of VEGFA
1.5
1.0
0.5
0.0
**
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
C
Relative mRNA level of SDF-1
1.5
1.0
0.5
0.0
**
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
D
VEGFA
SDF-1
β-actin
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
Relative protein level of VEGFA
0.6
0.4
0.2
0.0
**
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
Relative protein level of SDF-1
0.6
0.4
0.2
0.0
**
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
E
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
0h
24h
Relative migration rate (%)
100
80
60
40
20
0
*
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α

Figure 5

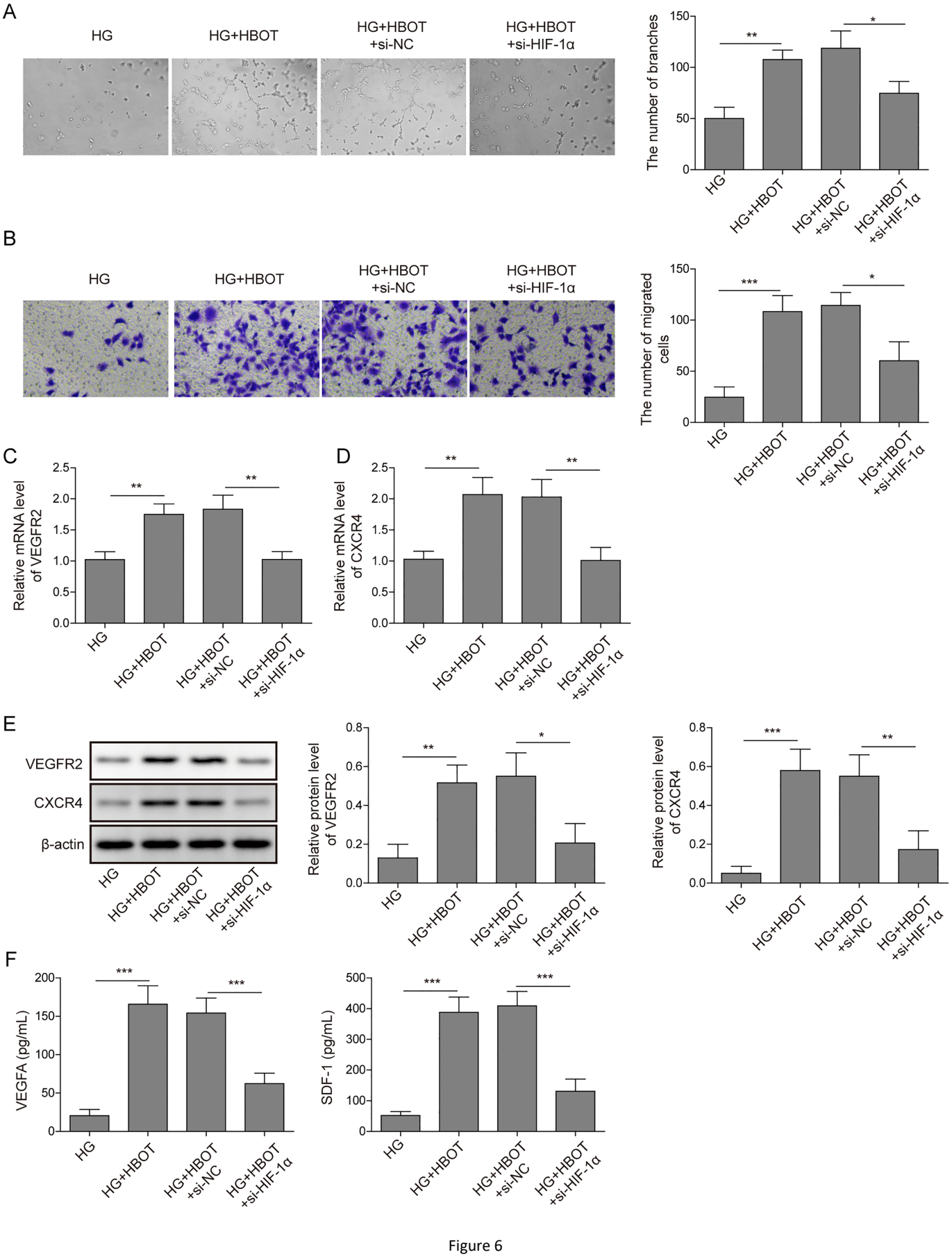
A
HG
HG+HBOT
HG+HBOT +si-NC
HG+HBOT +si-HIF-1α
The number of branches
B
The number of migrated cells
C
Relative mRNA level of VEGFR2
D
Relative mRNA level of CXCR4
E
VEGFR2
CXCR4
β-actin
Relative protein level of VEGFR2
Relative protein level of CXCR4
F
VEGFA (pg/mL)
SDF-1 (pg/mL)

Figure 6