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Research article

Hypoxic preconditioning promotes galvanotaxis of human dermal microvascular endothelial cells through NF-κB pathway

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ABSTRACT

Angiogenesis plays an important role in wound healing, especially in chronic wound. The directional migration of the human dermal microvascular endothelial cells (HDMECs) is the key regulation of angiogenesis. The wound healing can be regulated by numerous microenvironment factors including the electric fields, hypoxia and chemotaxis. During wound repair, the electric fields mediates the directional migration of cells and the hypoxia, which occurs immediately after injury, acts as an early stimulus to initiate the healing process. However, the mechanism of hypoxia and the endogenous electric fields coordinating to promote angiogenesis remain elusive. In this study, we observed the effect of hypoxia on the directional migration of HDMECs under electric fields. The galvanotaxis of HDMECs under the electric fields (200 mV/mm) was significantly improved, and the expression of VEGF/VEGFR2 was up-regulated after 4h of hypoxic preconditioning. In addition, the knockdown of VEGFR2 reversed the directivity of HDMECs promoted by hypoxia in the electric fields. Moreover, knockdown of VEGFR2 inhibited the migration directionality of HDMECs in the electric field after hypoxic preconditioning. Hypoxia decreased the activation of NF-κB in HDMECs. Activated NF-κB by fusicoccin decreased the expression of VEGFR2/VEGF and negatively regulated the migration direction of HDMECs in the electric fields. Enhancing the galvanotaxis response of cells might therefore be a clinically attractive approach to induce improved angiogenesis.

1. Introduction

Angiogenesis is an important part of wound healing. It is formed by the development of existing capillaries or posterior capillaries. Functional neovascularization can provide stable nutrients and oxygen source for the process of wound healing, which is very important for acute and chronic skin wound healing [1]. Angiogenesis depends on extracellular matrix conditions and directional migration of vascular endothelial cells [2].

Angiogenesis is affected by a variety of wound microenvironments, including hypoxia, endogenous bioelectrical fields and various chemokines [3]. Hypoxia is an important part of the wound microenvironment and is one of the main stimuli to initiate angiogenesis [4]. After the formation of the wound, due to the local blood circulation disorder and the increase of oxygen consumption of wound edge cells, the wound forms a local hypoxic microenvironment [5]. Studies have shown that the central oxygen partial pressure of the wound is the lowest (0–1.3%), and the closer to the wound edge, the oxygen partial pressure gradually increases (7.9%) [6, 7]. Furthermore, matrix metalloproteinase (MMP), fibroblast growth factor-2 (FGF-2) and vascular growth factor (VEGF) promote the migration and proliferation of vascular endothelial cells [8]. As early as the mid-19th century, it was found that DC electric fields would be generated at the moment of skin damage, with the wound center as the negative electrode and the wound edge as the positive electrode [9]. The electric field intensity was maintained at 42-200mv/mm until the re epithelialization of the wound was completed [10, 11]. The density and growth direction of neovascularization are significantly dependent on the electric field intensity. In this process, VEGF/VEGFR2 signaling pathway plays a crucial role effect. Regulating the expression of VEGFR2 can significantly affect the diameter and density of wound neovascularization [12].

However, it has not been reported whether the hypoxic microenvironment has an effect on the galvanotaxis migration of vascular endothelial cells in the process of wound healing. A large number of studies

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are carried out under normoxic conditions, ignoring the effect of hypoxic environment on angiogenesis in the process of wound healing. In our previous studies, we found that hypoxic preconditioning for 2–6 h can significantly enhance the direction and speed of epidermal cells in the electric field through oxidative stress [13].

P65 is the main subunit of NF-κB and play a significant role in NF-κB function [14]. NF-κB plays an important role in cellular inflammatory response and the immune response plays a key role [15]. In renal cell carcinoma cells, NF-κB is a target site for tumor treatment by inhibiting tumor angiogenesis. Hypoxia can significantly reduce the expression of NF-κB p65 in granulation tissue of refractory wounds [16]. In addition, studies have shown that pulsed electric fields can also change the activity of p65 [17], but the effect of the direct current electric fields on NF-κB is rarely reported. In our previous experiment, an electric fields can significantly improve the expression and nuclear displacement of p65 protein, providing a new direction for the study of synergistic regulation of wound healing microenvironment. Moreover, studies have shown that the activation of NF-κB pathway can induce the expression of inflammatory factors and chemokines to increase, which can lead to the decrease of VEGF/VEGFR2 expression [18].

Therefore, in this study, we used human dermal microvascular endothelial cells (HDMECs) to observe the migration of cells in the biological electric field after normoxia and hypoxia preconditioning [19]. Some results showed that hypoxic preconditioning could promote the expression of VEGF/VEGFR2 pathway and enhance the galvanotaxis of HDMECs in biological electric fields. The activate NF-κB can reduce the expression of VEGF/VEGFR2 pathway and reverse the galvanotaxis of HDMECs promoted by hypoxic preconditioning. The treatment of cells with NF-κB activity inhibitor can enhance the galvanotaxis of cells in biological electric fields. Our findings deeply reveal the mechanism of angiogenesis regulated by a variety of wound microenvironment factors, and provide a new theoretical basis for wound clinical treatment.

2. Materials and methods

2.1. Cell culture

Human dermal microvascular endothelial cells (HDMECs) were cultured in Endothelial Cell Medium (ECM), supplemented with fetal bovine serum (FBS, 5% as the final concentration) , Endothelial Cell Growth Supplement (ECGS, 1% as the final concentration) and P/S (Penicillin streptomycin) solution (1% as the final concentration, Scien Cell, USA). For some experiments, following a 1–2h recovery period after removal of the transfection mixture, the following inhibitors and activator

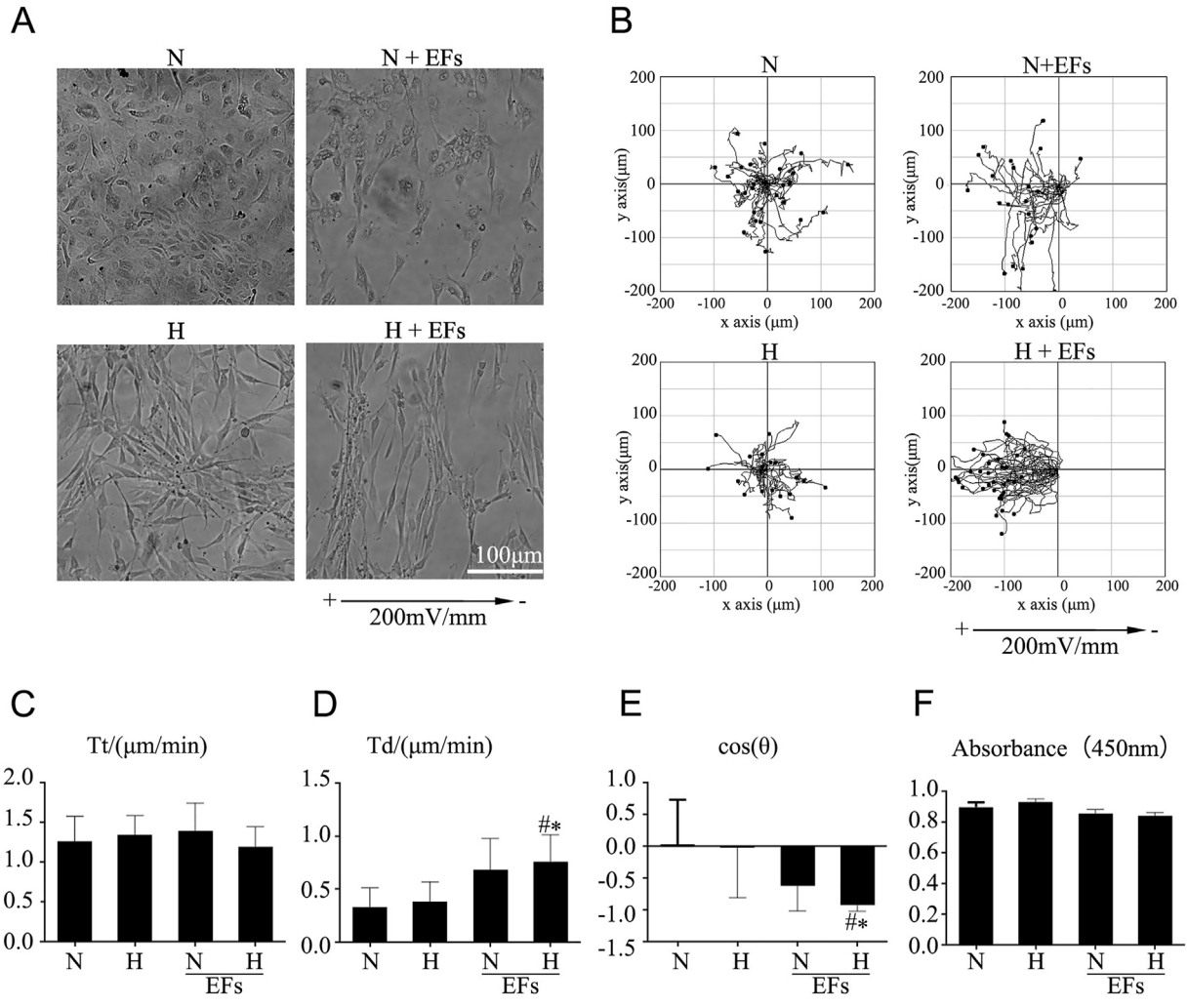

Figure 1. Effect of hypoxic preconditioning on the migration of HDMECs in the electric fields. (A) Effect of hypoxia preconditioning on the arrangement of HDMECs in the electric fields. (B) The migration trajectories of HDMECs guided by hypoxic preconditioning or not and EFs or not. #, $p < 0.05$ compared with H group. *, $p < 0.05$ compared with H group. (C–E) Quantitative analysis of trajectories by time (min) and direction (cos(θ)) of HDMECs migration. #, $p < 0.05$ compared with H group. *, $p < 0.05$ compared with N + EFs group. (F) The cellular activity of HDMECs guided by hypoxic preconditioning or not and EFs or not.

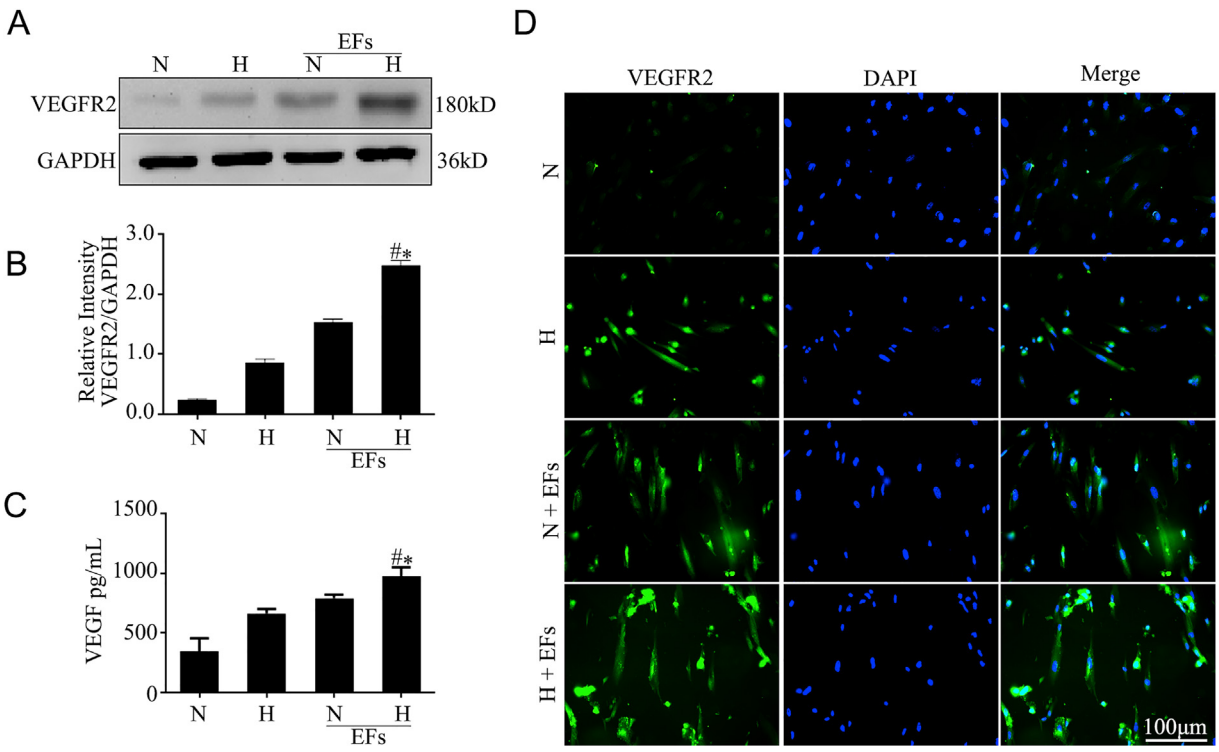

Figure 2. Effect of hypoxic preconditioning on VEGF/VEGFR2 pathway in HDMECs in the electric fields. (A and B)Hypoxic preconditioning could enhance the expression of VEGFR2 in HDMECs in the electric fields. (C) Hypoxic preconditioning can also promote VEGF secretion of HDMECs in electric fields. (D)The expression and distribution of VEGFR2 in cells under hypoxia preconditioning and the electric fields were observed by immunofluorescence. Bar = 100μm. #, $p < 0.05$ compared with H group. *, $p < 0.05$ compared with N + EFs group.

(Abcam, UK) were added: the NF-κB inhibitor PDTC (ab141406, 150 μM for 24h) and the NF-κB activator Fusicoccin (ab145208, 12.5mM for 48h) [20,21].

2.2. Hypoxia preconditioning and EFs stimulation

The hypoxia condition was to culture cells in the hypoxia chamber filled with 5% CO_2 and 2% O_2. The strength of EFs (200 mV/mm) was based on the relevant literature and our previous research. As mentioned earlier, EFs stimulation was used. In summary, EFs stimulated HDMECs by two silver electrodes immersed in Steinberg's solution, and two agar bridges were attached to the medium [22].

2.3. Cytotoxicity analysis by CCK-8

At the density of $2\times10^3/cm^2$ cells, the cells were seeded on the climbing piece, and the control group, hypoxic preconditioning group, electric field treatment group, and hypoxic preconditioning four superimposed electric field group were set. The EFs treatment was 200 mV/mm for 6 h, and the hypoxic preconditioning was treated with 2% oxygen concentration for 4 h. After treatment, trypsin was used to collect cells. The HDMECs were seeded into a 96-well plate at a density of 2×10^3 cells per well. Cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan) was used according to the manufacturer instructions. Briefly, 100 μL of the cell suspension was added to each well, and 10 μL of CCK-8 solution was added to each well, and the plate was incubated at 37 °C for 3h. The optical density (OD) value was measured at 450nm.

2.4. Small interfering RNA transfection

To knockdown VEGFR2 in HDMECs, a pool of siRNA for the VEGFR2 (ID:3791) gene and non-specific control siRNA (ID:3791) were purchased from GenePharma (Carlsbad, CA). Second passage HDEMC were transfected with small interfering RNA (siRNA) for VEGFR2 or negative control according to the manufacturer's protocol.

2.5. Imaging of single-cell motility

For the single cell migration assays, HDMECs were seeded into a modified plates at a density of $2\times10^3/cm^2$ in ECM medium. And based on previous studies, the electric fields strength (200 mV/mm) was chosen [23]. The ECM is connected to the DC power supply through a salt bridge and Ag/AgCl electrodes are immersed in the Steinberg's (ST) solution. After the cells were subjected to siRNA transfection or treated with the NF-κB inhibitor or activator, Zeiss imaging system (Carl Zeiss Meditec in Jenner, Germany) was used to conduct delayed imaging to monitor the movement of single cells, which excluded cell proliferation and death. The images were taken every 5 min for 6 h. The trajectories of the cells were subsequently obtained by tracing the positions of the cell nuclei at frame intervals of 10 min using NIH ImageJ software. Count the Trajectory (Tt) and Displacement (Td) of each cell. The trajectory speed (μm/min) of each cell was defined as the total length (μm) of the trajectories divided by time (min), which reflects cell motility. The cos(θ) represents the direction of cell migration, which is calculated according to the ratio of x-axis to Y-axis. The closer the cos(θ) is to +1 or -1, the more obvious the direction of cell movement. Randomly select the microscope field of vision, and count the movement state of no less than 50 cells.

2.6. Detection of VEGF by ELISA

VEGF release was quantified with ELISA as directed by the manufacturer. Briefly, HDMECs were grown in serum-free ECM medium, and the cells were hypoxic Preconditioning for 4 h, and then grown in ECM-complete medium in EFs for 6h. After incubation for the indicated times with EFs in medium, the cultures up supernatant was removed and evaluated for VEGF content using the corresponding ELISA kit.

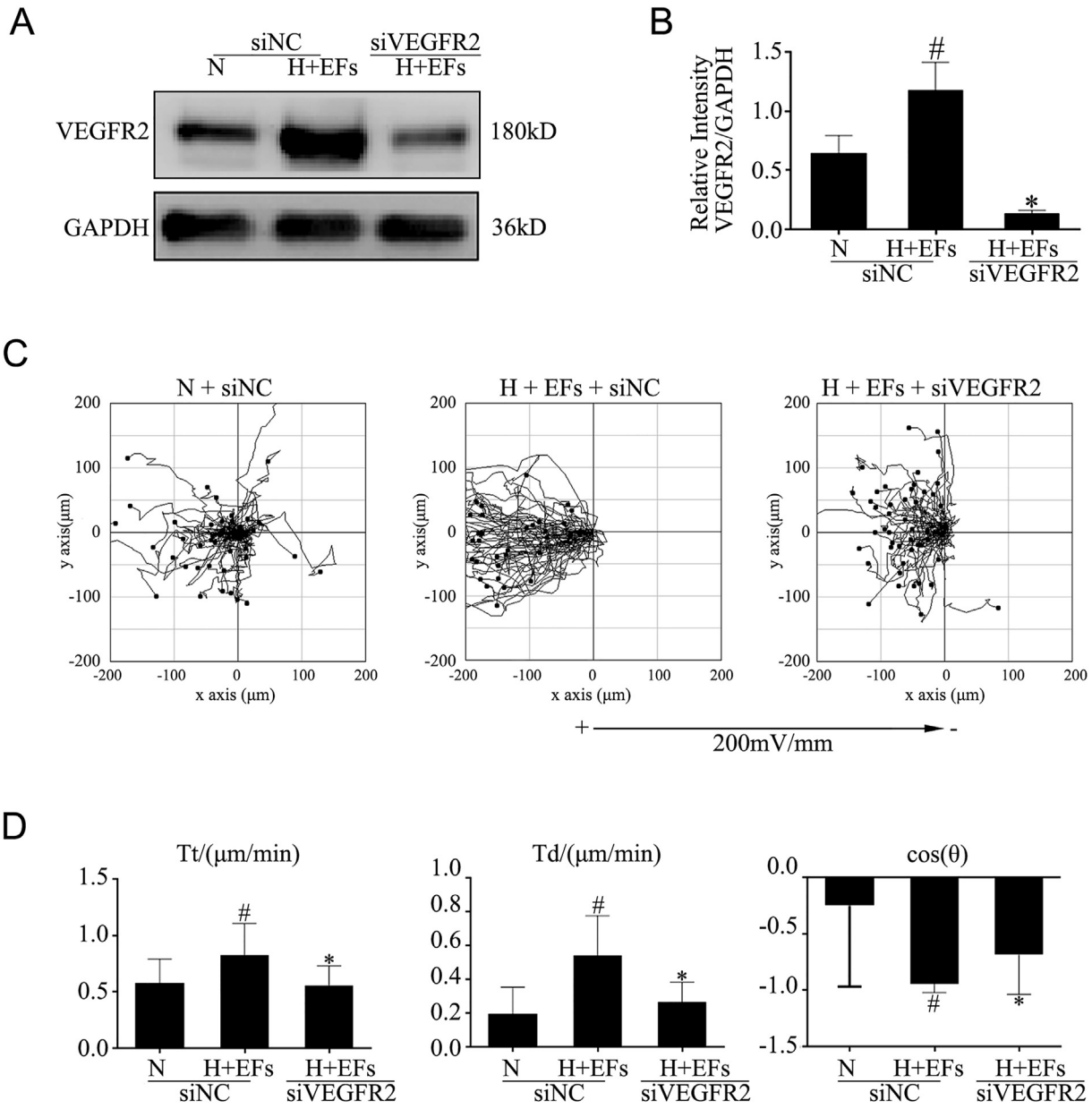

Figure 3. The effect of VEGFR2 on hypoxia-promoted HDMECs directional migration in the EFs. (A-B)The expression of VEGFR2 was determined by Western blot. The data was shown as the mean ± SEM (n = 3). #, $p < 0.05$ compared with N + siNC group. *, $p < 0.05$ compared with H + EFs + siNC group. (C) Cell migration was recorded by time-lapse microscopy at 1 frame per 5 min and was analyzed by Image J. (D) Quantitative analysis of cos(θ), Td/t (μm/min) and Tt/t (μm/min) of HDMECs migration. The data was shown as the mean ± SEM (n = 3). The effect of siRNA on the expression and distribution of VEGFR2 in HDMECs cells was observed by immunofluorescence. #, $p < 0.05$ compared with N + siNC group. *, $p < 0.05$ compared with N + EFs + siNC group.

2.7. NF-κb activity and nuclear transport analysis

Cell culture media were collected and NF-κB analyzed in the NF-κB activity activated nuclear transport test kit (sn368 Beyotime Shanghai). Cells stimulated by EFs were fixed with 4% paraformaldehyde and blocked with 5% goat serum albumin to diminish background signals. The cells were subsequently incubated with the primary antibody, corresponding fluorescence-labeled secondary antibody, and DAPI. The slides were imaged using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.8. Western blot

Protein extracts were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blocked with 5% non-fat milk or bovine serum albumin. The desired bands were incubated with the appropriate primary and secondary antibodies and were subsequently visualized. The antibodies used in this study included anti-VEGFReceptor2 antibody (AB39638, Abcam, USA), the anti-NF-κB p50/105 antibody (AB32360, Abcam, USA), GAPDH HRP-60004 (Proteintech, USA), Anti-NF-κB p65 (phospho S536) antibody (ab76302, Abcam, USA), Anti-NF-κB p65 antibody (ab32536, Abcam, USA) and MAPK (Erk1/2) Antibody (8690, CST, USA), and Phospho-MAPK Rabbit mAb (2325, CST, USA).

2.9. Statistical analysis

All experiments were repeated at least three times. The data were expressed as mean ± (SD). Students' t-test was used to evaluate the statistical significance among groups. $P < 0.05$ was considered statistical significance.

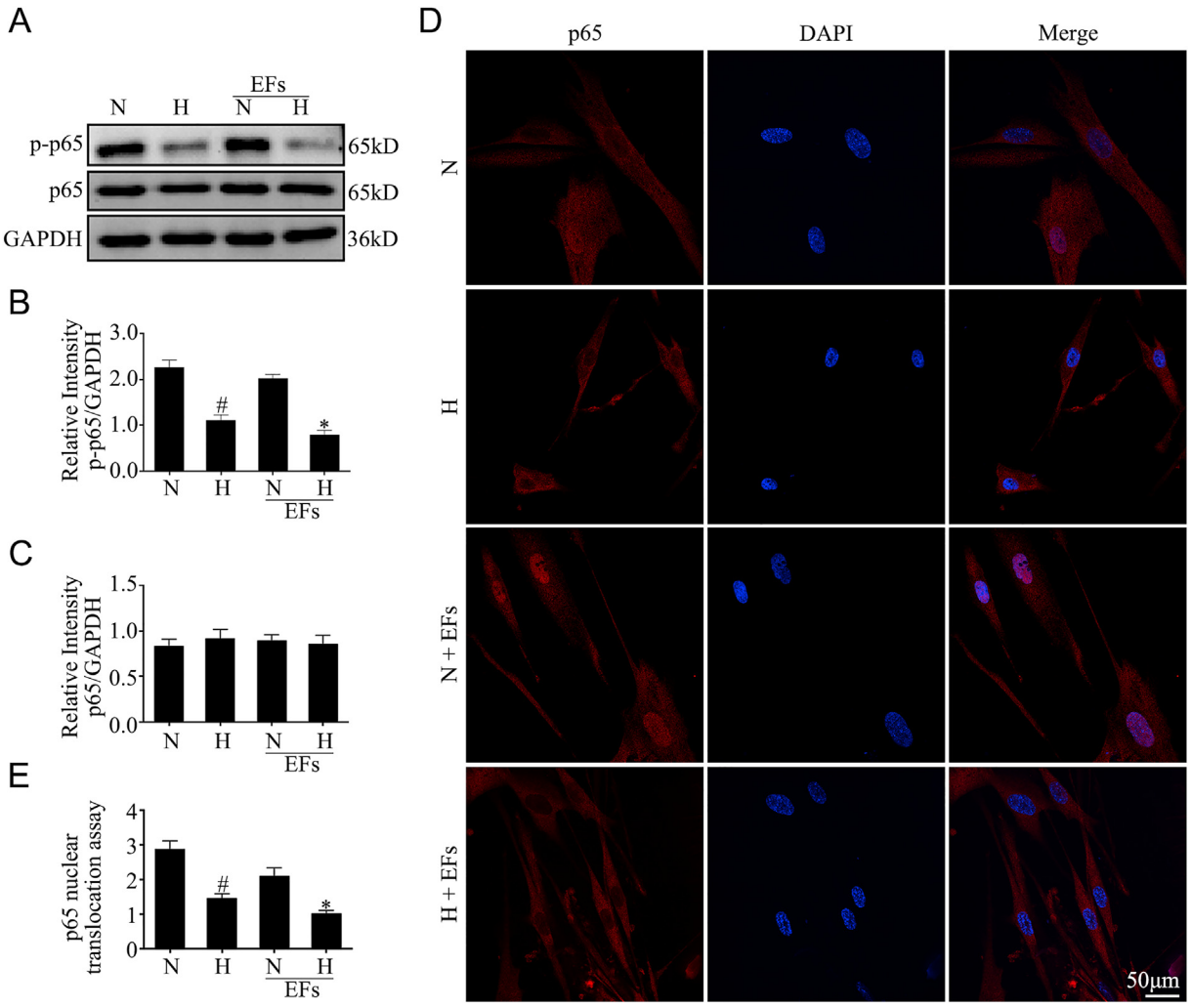

Figure 4. The role of NF-κB p65 activity in the effect of hypoxic preconditioning on cell galvanotaxis. (A) The levels of p-p65 and p65 in HDMECs was tested by Western blot. (B–C) The results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). #, $p < 0.05$ compared with N group. *, $p < 0.05$ compared with N + EFs group. (D) The activity detection kit was used to detect the p65 activity. (E) The results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). #, $p < 0.05$ compared with N group. *, $p < 0.05$ compared with N + EFs group.

3. Results

3.1. The galvanotaxis of HDMECs in the electric fields was enhanced by hypoxia

To study the effect of hypoxia on the migration of HDMECs in the electric fields, we observed the trajectory of HDMECs under an electric fields after hypoxic Preconditioning by time-lapse microscopy. As shown in Figure 1A, after the EFs stimulation, the arrangement of HDMECs changed significantly (200 mV/mm), while in the hypoxic preconditioning superimposed electric fields group, the arrangement of cells changed significantly (hypoxia 4h and EFs 6h). As shown in Figure 1 B, the cos(θ) of HDMECs in the EFs is closer to 1 after 4h of hypoxic Preconditioning than that in EFs only group (Figure 1E). Therefore, the galvanotaxis of HDMECs in the electric fields was stimulated by hypoxic Preconditioning. The displacement velocity of HDMECs in electric fields Td/t (μm/min) did not increase significantly due to hypoxic Preconditioning (Figure 1C and D). The cytotoxicity was detected by CCK-8. The results showed that hypoxic preconditioning and electric field had no obvious cytotoxicity to cells (Figure 1F).

3.2. VEGF/VEGFR2 pathway was enhanced by hypoxia in the electric fields

To verify whether VEGFR2 is involved in hypoxia promoting the improvement of galvanotaxis of HDMECs in the EFs, we treated HDMECs with hypoxia (4h), EFs (6h) and hypoxia superimposed EFs, respectively. We detected the expression of VEGFR2 by Western blotting, the secretion of VEGF by ELISA, and observed the distribution of VEGFR2 by immunofluorescence staining. Quantitative analysis showed that both hypoxia group and EFs group could significantly increase VEGFR2, while H+EFs increased more than other groups (Figure 2A and B). The same trend is shown in ELISA, that H+EFs can significantly further promote VEGF content than H and EFs (Figure 2C). Immunofluorescence staining further proves that hypoxia can promote the arrangement of HDMECs changed significantly in the EFs (Figure 2D).

In this study, hypoxia can also significantly promote VEGF/VEGFR2 alone, which is consistent with previous results. But we pay more attention to the role of hypoxia in EFs.

3.3. The galvanotaxis of HDMECs induced by hypoxia was down-regulated by the inhibition of VEGFR2

The above results showed that hypoxia could increase the expression of VEGFR2 in EFs. Reverse regulation of protein expression is a common method of studying target proteins. Therefore, we used interfering RNA (siRNA) to reduce the expression of VEGFR2. To verify whether VEGFR2 is involved in hypoxia-induced galvanotaxis of HDMECs, we used siRNA to treat HDMECs before hypoxia superimposed EFs. After 48h of treatment, the expression of VEGFR2 decreased by 76.8% (Figure 3A and B).

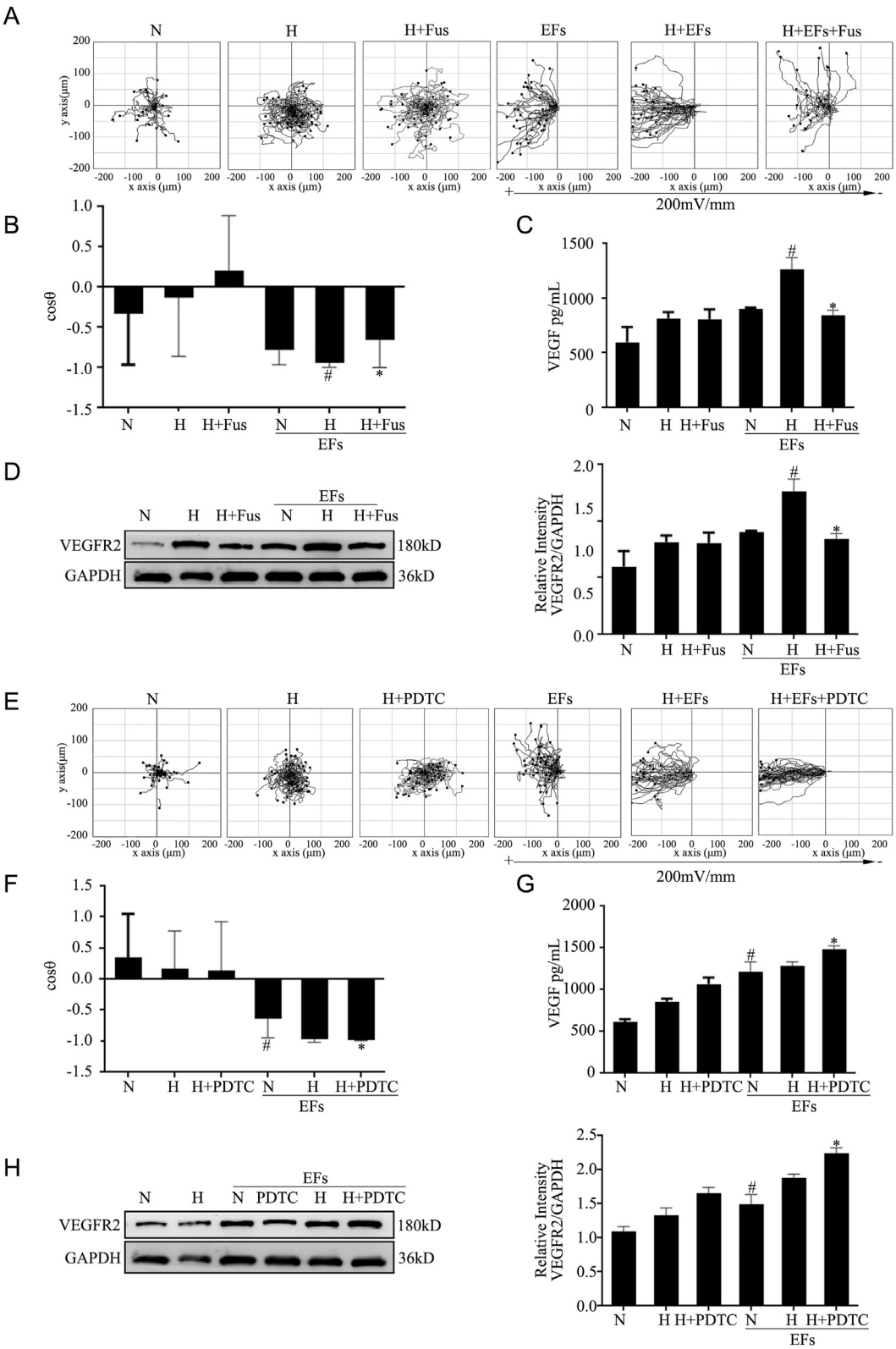

Figure 5. NF-κB is involved in cell galvanotaxis promoted by hypoxic preconditioning. (A–B) Cell migration was recorded by time-lapse microscopy at 1 frame per 5 min and was analyzed by Image J. Quantitative analysis of cos(θ) of HDMECs migration. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with H + EFs group. #, $p < 0.05$ compared with N + EFs group. (C) VEGF content in HDMECs was detected by ELISA. #, $p < 0.05$ compared with N + EFs group. *, $p < 0.05$ compared with H + EFs group. #, $p < 0.05$ compared with N + EFs group. (D) The levels of VEGFR2 in HDMECs were tested by Western blot. *, $p < 0.05$ compared with H + EFs group. #, $p < 0.05$ compared with N + EFs group. (E–F) Cell migration was recorded by time-lapse microscopy at 1 frame per 5 min and was analyzed by Image J. Quantitative analysis of cos(θ) of HDMECs migration. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with PDTC + EFs group. #, $p < 0.05$ compared with N + EFs group. (G) VEGF content in HDMECs was detected by ELISA. #, $p < 0.05$ compared with N + EFs group. *, $p < 0.05$ compared with H + EFs group. #, $p < 0.05$ compared with H + EFs group. (H) The levels of VEGFR2 in HDMECs were tested by Western blot. *, $p < 0.05$ compared with H + EFs group. #, $p < 0.05$ compared with N + EFs group.

The cell trajectory was observed by a delay microscope (Figure 3C). The results showed that knockdown of VEGFR2 made HDMECs directional (cos(θ)) decrease by 25.3%. The distribution of VEGFR2 in HDMECs was observed by immunofluorescence staining (Figure 3D).

3.4. NF-κb activity was decreased by the synergistic effect of hypoxia and electric fields

To study the correlation between NF-κB and VEGFR2 in HDMECs migration in hypoxia superimposed electric fields, the phosphorylated NF-κB p65 and NF-κB p65 were detected by Western blot (Figure 4A, B and C). And we detected the activity of NF-κB through the NF-κB activity detection kit (Figure 4D). Hypoxic preconditioning can reduce the NF-κB activity of HDMECs in the electric fields by 36.6%. The p65 nuclear translocation assay showed that the p65 activity of H+EFs group was 55.23% lower than that of EFs group (Figure 4E).

3.5. NF-κB signal is involved in the galvanotaxis of HDMECs induced by hypoxia

PTDC (a NF-κB activity inhibitor) has been used to prevent nuclear transfer and Fus (Fusicoccin, the NF-κB activator) to activated nuclear transfer, to study whether the change of VEGFR2 depends on the activity of NF-κB p65 and determine the relationship between NF-κB activity and HDMECs chemotaxis.

The motility (cos(θ)) of HDMECs was analyzed by a delay microscope (Figure 5A and E). The results showed that activating NF-κB p65 activity with Fus could reduce the expression of VEGFR2 and the secretion of VEGF (Figure 5C, D, G, H and Figure S2A, S2D), and reduce the direction of motion (cos(θ)) of HDMECs under the condition of hypoxia superimposed electric fields 24.3% (Figure 5B and F). Western blot verified the effectiveness of regulation (Figure 5D and H). The secretion of VEGF was studied by ELISA (Figure 5C and G). These results show that NF-κB p65 plays a key role in promoting the galvanotaxis of HDMECs in hypoxia (see Figure 6).

4. Discussion

Exogenous physiological intensity electric field has been proved by many studies to be able to promote a variety of physiological activities including wound healing and angiogenesis [24]. The process of wound healing is affected by directing epidermal cells to migrate to the wound center, transforming fibroblasts, inducing the release of various growth factors, activating and modifying various cell surface receptors, etc. The sensitivity of cells to electric fields is crucial in this process [25]. The healing speed of chronic wounds is much slower than that of acute wounds. Wound microenvironment is one of the most important factors that affect cells' perception of electric field stimulation, including oxygen partial pressure concentration, pH, inflammation, etc., which can affect the response ability of various cells in electric field. In the acute trauma microenvironment, hypoxia is one of the most potentially influential trauma microenvironments [26, 27, 28].

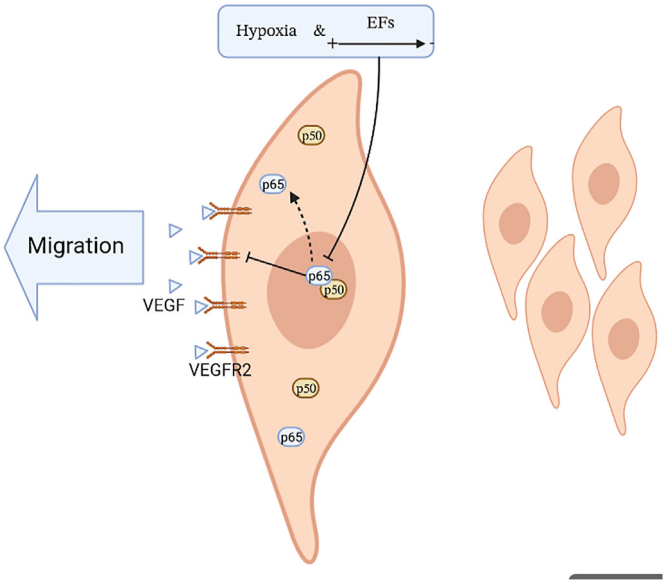

Figure 6. Schematic diagram of hypoxic preconditioning promoting galvanotaxis of HDMECs in the electric fields.

Hypoxia is caused by vascular injury during wound formation [29]. Hypoxia initiates the related angiogenesis and wound repair processes through multiple reactions such as oxidative stress, cascades, etc., and activates the corresponding cells to complete the re-epithelialization process [30, 31]. Our previous studies have shown that hypoxic preconditioning significantly enhances the motility of epidermal cells in electric fields. Hence, we hypothesized that hypoxic facilitation of cell galvanotaxis is a widespread phenomenon. As an important microenvironmental factor in wound formation, few studies currently focus on the combined effects of hypoxia with other factors, and the majority of studies individually investigate its role and mechanism [13]. However, the microenvironmental factors in the wound healing process are very complex, so exploring the synergistic effects of multiple microenvironmental factors is necessary to gain insights into the mechanism of wound healing [32].

In addition, the galvanotaxis of cells is very sensitive to many factors, such as cell matrix microenvironment, ion channel status of cells, and intercellular junctions. There are many molecular pathways initiated by hypoxia, such as oxidative stress. Furthermore, bioelectrical fields can also influence the regulation of cellular hypoxic microenvironment by modulating the level of oxidative stress, membrane potential, etc. [33]. Therefore, on the basis of this study, further, in-depth studies on the mutual effects between hypoxia and bioelectric fields under *in vivo* and *in vitro* conditions are required to gain insights into potential therapeutic targets in the wound healing process [34, 35].

In real wounds, both hypoxia and bio-electric fields play a continuous role throughout the re-epithelialization process. Therefore, it is equally important to simultaneously study the response of cells to bio-electric fields during hypoxic treatment. In the present study, due to the limitations of the cell culture model device, electric field stimulation could only be performed after hypoxic preconditioning. In future studies, we will try new physiological strength electric field stimulation devices for coupling studies of multiple microenvironmental factors.

Analysis of vessel formation is an important research indicator to study the process of angiogenesis [36]. However, HDMECs, which are the main cells involved in wound microangiogenesis, grew slowly in the *in vitro* culture environment. In the previous preliminary experiment, we found that HDMECs took a longer time to form the vascular ring (3–5 weeks continuous culture), which far exceeded the research cycle on bioelectric field in the acute wound healing process. The currently used electric field model has many problems for performing the ring formation experiments. Due to the above considerations, vascular ring formation experiments were not performed in this study.

In conclusion, angiogenesis is a complex physiological process involved in wound healing. Many factors can influence the complex process of wound healing, including electric field, chemical stimulation and other physiological factors. Therefore, exploring the effects and mechanisms of multi-factor interactions in wound healing can provide more ideas and prospects for wound healing treatment [37].

In conclusion, our study reveals that hypoxia can enhance the galvanotaxis of cells in the electric field, so that the electric field can play a more significant role in guiding the directional in cells migration. Angiogenesis in wound healing is an extremely complex physiological process, including electric field, chemical stimulation and other physiological factors. This process has brought many uncertain factors for

wound healing treatment. Therefore, exploring the effect and mechanism of multi factor interaction in wound healing can provide more ideas and prospects for wound healing treatment.

Declarations

Author contribution statement

Ze Zhang; Chao Wu; Jinrui Yang; Jie Liu; Yi Li; Luojia Liu; Meng Kong; Jiaping Zhang; Xupin Jiang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors have declared that no competing interest.

Additional information

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