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Original Article

Recombinant human erythropoietin promotes angiogenesis by activating SMAD3 and stimulating endothelial progenitor cells during wound healing

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Abstract: Angiogenesis is one of the essential steps in the wound healing process. Multiple growth factors and cytokines, including EPO, have been shown to accelerate wound healing by promoting angiogenesis. However, the exact mechanism remains unclear. In this study, we demonstrated that treatment of RhEPO significantly reduced wound closure time in animal model compared with control group. This effect is partly mediated by increased p-SMAD3 level in wound tissue. Furthermore, systemic administration of RhEPO was observed to be able to stimulate $CD133^+Flk^+$EPCs in peripheral blood. The concurrent increase in MVD in grafted PDAM suggested that the systematic administration of RhEPO can improve the success rate of PDAM implantation. Based on these data, we hypothesized that RhEPO accelerates wound healing by activating SMAD3 and stimulating EPCs in the peripheral blood.

Keywords: RhEPO, SMAD3, wound healing, angiogenesis, EPCs

Introduction

In recent years, poor wound healing has become a major clinical problem worldwide. In the United States alone, at least 6.5 million patients are affected by chronic wounds [1]. This figure continues to arise due to the aging population and a sharp rise in the incidence of diabetes and obesity around the world. Wound healing is a complicated process that involves inflammation, tissue formation and tissue remodeling. These occur concurrently and influence each other during tissue regeneration [2]. Besides, multiple growth factors and cytokines, including vascular endothelial growth factor (VEGF), basic fibroblast growth factors (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and platelet-derived growth factor (PDGF), play pivotal roles in wound healing by promoting cell proliferation, angiogenesis and extracellular matrix remodeling [2-4]. Though it has been demonstrated that administration of exogenous growth factor and/or cytokines to wound tissues can reduce wound closure time, there are still limited clinical applications of growth factors in treating chronic wounds. As growth factors are constantly exposed to proteases and hydrolysase in the wounded tissues, they can often be inactivated by enzymatic activity and rendered ineffective. In addition, the exogenous cytokine applied may interact with endogenous cytokines and disrupt the balance. Lastly, while growth factors promote wound healing, they may cause undesirable outcomes such as keloid formation and hypertrophic scars. Bearing these in mind, it is imperative to develop novel molecular therapy to improve the process of wound repair.

Erythropoietin (EPO) is a glycoprotein hormone and its role in the development and maturation of erythroid cells has been studied in detail for a long time [3, 5]. Only until very recent have several studies highlighted EPO's involvement in vasoproliferative processes and wound healing. In the study by Jaquet, et al., it was shown that *in vitro* treatment of human adult myocardial tissues with recombinant human erythropoietin (RhEPO) stimulated capillary growth up to 220% compared to untreated tissue [6]. In a rat model of diabete, topical EPO treatment sig-

nificantly reduced the wound closure time. Furthermore the EPO treated animals showed higher microvessel density (MVD), VEGF and hydroxyproline contents. Percentage of apoptotic cells in the wound tissues treated by EPO were also reduced [7]. These are consistent with the findings by Sorg H, *et al.* who showed that mice receiving EPO treatment exhibited accelerated wound epithelialization, reduced wound cellularity and enhanced maturation of newly formed microvascular networks [5]. Nevertheless, it remains unclear how EPO promotes angiogenesis and revascularization.

In this study, we demonstrated that treatment of mice with RhEPO significantly reduced wound closure time compared to the control group. The effect is partly mediated by increased p-SMAD3 level in the wound tissue. Moreover, we observed that systemic administration of RhEPO stimulated $CD133^{+}Flk^{+}$EPCs in peripheral blood. Most importantly, we demonstrated *in vivo* administration of RhEPO significantly increased MVD in grafted PDAM, suggesting that systemic administration can effectively improve PDAM implantation success rate. Based on these data, we hypothesized that RhEPO accelerates wound repair by activating Smad3 and stimulating EPCs in the peripheral blood. Our data revealed the underlying molecular mechanism of EPO in promoting would healing and provided evidences that systematic administration of RhEPO may serve as a novel approach for clinical application of RhEPO in wound healing.

Material and methods

Reagents and antibodies

Recombinant human erythropoietin (RhEPO) was purchased from Sinopharm. Co. Ltd (China). Antibodies in this study were purchased from eBioscience (CA), except for the antibody against phospho-SMAD3, which was purchased from Cell Signaling Technology (MA). Porcine Acellular Dermal Matrix (PADM) was obtained from Uni-Trump (China).

Western blot

Minced tissues were homogenized and lysed in lysis buffer (Cell Signaling, MA) which contained PMSF. Proteins resolved by SDS-PAGE were electro blotted to a nitrocellulose membrane (Amersham, Buckinghamshire) which was then incubated overnight at 4°C with blocking buffer (PBS containing 5% (w/v) skim milk and 0.05% (v/v) Tween-20). Primary and secondary antibody applications were performed in blocking buffer. The membrane was finally washed with PBS containing 0.05% (v/v) Tween-20 followed by analysis done using the Super signal Chemiluminescent kit (Pierce, IL) according to the manufacturer's instructions.

Wound healing assessment

The animals were handled in accordance with the guiding principles in the care and use of animals, approved by the council of Wannan Medical College. Thirty six-week old male BALB/c mice (20.00 ± 0.50 g) (Shanghai Laboratory Animal Center, China) were anesthetized with isoflorane (Santa Cruz Biotechnology, CA). Areas between the two scapula bones on the dorsum were shaved and a 2.5 cm diameter round full-thickness wound was created on the shaven skin. The day of surgery was defined as day 0. Equal numbers of animals were randomized into three treatment groups: 1 mL saline, 50 U/mL and 100 U/mL RhEPO. Animals were treated once a day for 7 consecutive days. Wound areas were measured daily using a calibrator. The percentage of healed area was calculated by the formula: 100 × (Wound area on day 1 - Wound area on day *x*) ÷ Wound area on day 1.

Endothelial progenitor cells (EPCs) analysis

A total of 108 six-week old male BALB/c mice (20.00 ± 0.50 g) were randomized into three treatment groups: saline, 1000 IU/kg and 3000 IU/kg RhEPO. Eight mice from each group were terminated on day 3, 7 and 14. The blood was collected into Vacutainer Blood Collection Tubes (BD Biosciences, CA) by cardiac puncture. Red blood cells (RBC) were lysed by RBC hypotonic lysis solution (Sigma, CA). The remaining cells were washed twice with staining buffer (5% (w/v) BSA, 2 mM EDTA, 2 mM NaN_3 in PBS) followed by incubation for 30 minutes on ice with anti-CD133 (FITC) and anti-Flk-1 (PE). Cells were washed once with staining buffer and resuspended in 300 μL staining buffer. The percentages of EPCs were analyzed on a FASCAN flow cytometry (BD Biosciences, CA). Data were analyzed by FlowJo (OR).

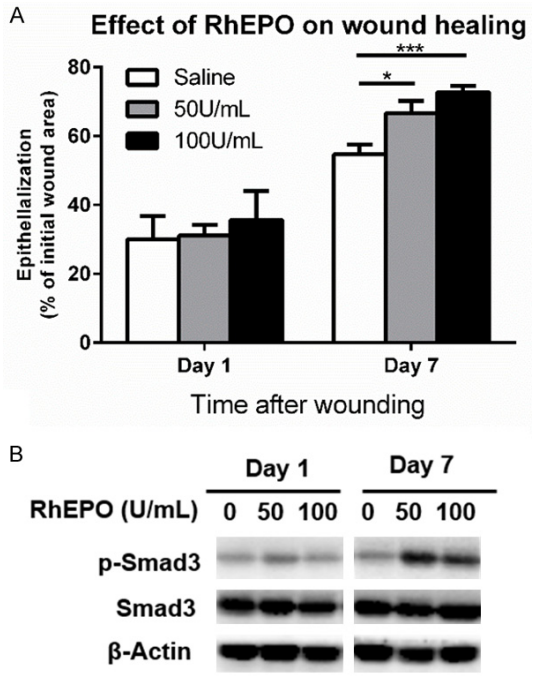

Figure 1. The Effect of RhEP on wound healing in mouse. A. Plantimetric analysis on wounded mice treated with saline, 50 U/mL RhEPO or 100 U/mL EPO daily for 7 days (n = 10 *per* group). Values are means se; $*P < 0.05$, $***P < 0.001$. B. The representative image on p-SMAD3 and total SMAD3 expression level in wound tissues harvest for mice treated with saline, 50 U/mL RhEPO or 100 U/mL EPO daily. β-actin was used as the loading control. Data were representative of three time experiments.

Microvessel density (MVD) analysis

A total of 144 six-week old male mice (20.00 ± 0.50 g) were randomized into two treatment groups: saline or 1000 IU/kg RhEPO that were treated for 7 consecutive days. On day 7, a 225 mm^2 of porcine acellular dermal matrix (PADM) was subcutaneously implanted between the two scapula bones on the dorsum. The PADM was harvested on day 3, 7, 14 and 21 after implantation and was subjected to immunohistochemical (IHC) analysis of CD31. Pictures of 5 fields per tissue were taken at 200 × magnification. The MVD was determined in a blinded manner by counting using Imagine_0.16.

Statistics

Data were expressed as means ± standard deviations (SD). ANOVA and independent samples t-test were used to calculate the significance among the groups (SPSS Inc., IL). P-value of < 0.05 was considered statistically significant.

Results

Human recombinant erythropoietin (RhEPO) treatment promotes wound healing

To evaluate the effect of RhEPO on wound healing, we established a wound-healing model in mice by creating a circular full thickness skin wound of 2.5 cm diameter between the two scapula bones on the dorsum. Then the mice were treated with 1 mL saline or 50 U/mL and 100 U/mL RhEPO for 7 days. Percentage of epithelialization in the wounding area was used as the measurement for wound healing. The degrees of epithelialization were similar among all three treatment groups at base line. After 7 days of treatment, the percentage of epithelialization in 50 U/mL RhEPO treatment group increased from 33.13% to 66.63% and in 100 U/mL treatment group the percentage increased from 35.66% to 72.73%. These increases are dose-dependent and significantly higher than that in the saline control group, which showed a mere increase from 30.03% to 54.71% (**Figure 1A**). The data suggest that RhEPO treatment promotes wound healing and are consistent with previous reports which showed that EPO administration enhanced wound repair in diabetic and ischemic wound model [6, 8].

Next, we investigated the wound healing mechanism mediated by RhEPO through measuring the phosphorylated SMAD3 (pSMAD3) level in wound tissue. Mice epithelium at the leading edge of the wound was harvested after treatment with saline, 50 U/mL and 100 U/mL RhEPO on day 1 and 7. The protein was extracted and separated using Western Blot. P-SMAD3 level increased substantially after 3 days of 100 U/mL RhEPO treatment compared to the saline group. The enhanced phosphorylation of Smad3 was slightly delayed in the lower dose treatment group (50 U/mL), but a similar amplitude of increase was observed after day 7 (**Figure 1B**).

RhEPO stimulates endothelial progenitor cells (EPCs)

Based on previous reports that SMAD3 is associated with stem cell renewal and stimulates vascular cell dedifferentiation [9], our study

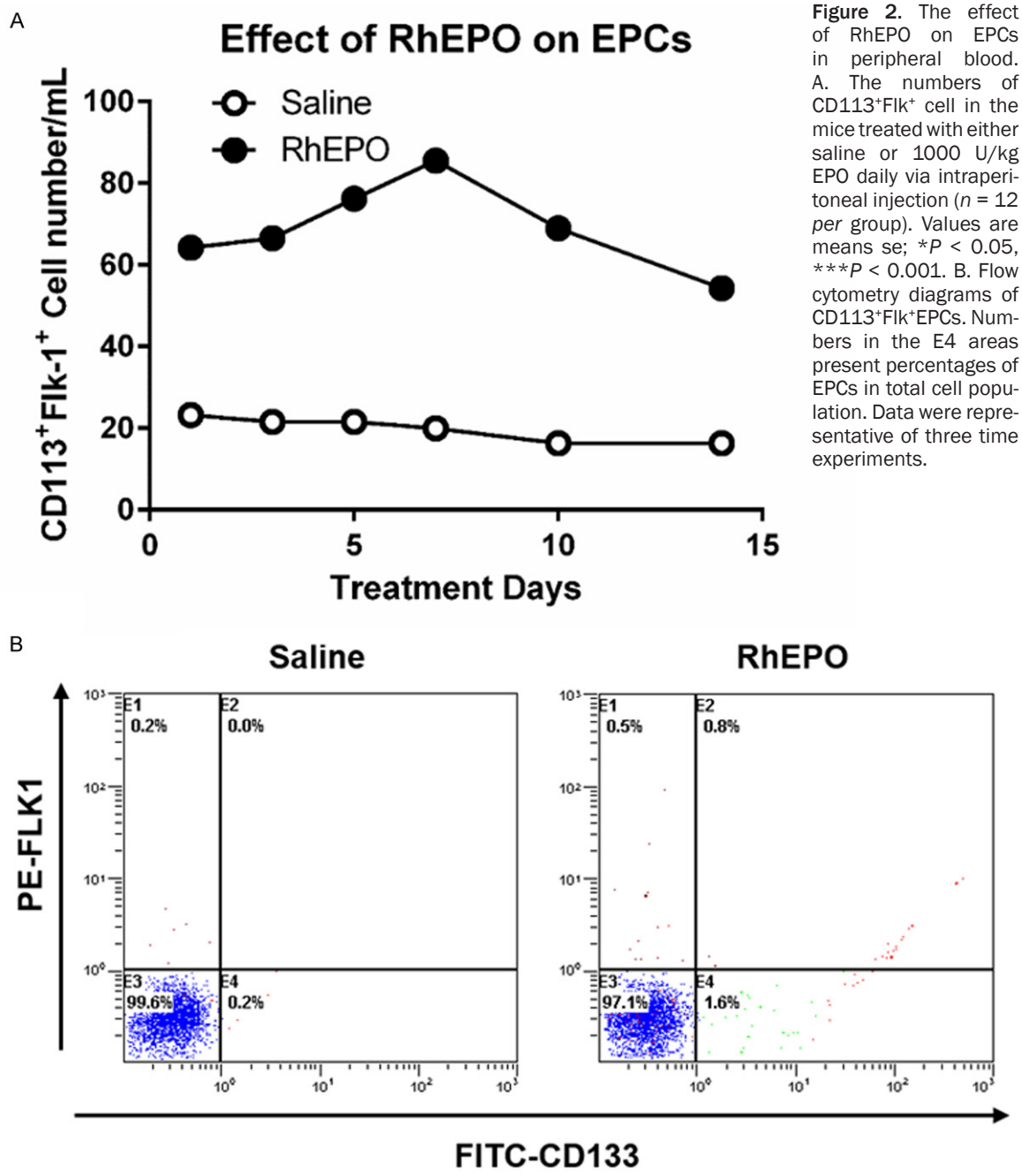

Figure 2. The effect of RhEPO on EPCs in peripheral blood. A. The numbers of $CD113^+Flk^+$ cell in the mice treated with either saline or 1000 U/kg EPO daily via intraperitoneal injection (n = 12 *per* group). Values are means se; $*P < 0.05$, $***P < 0.001$. B. Flow cytometry diagrams of $CD113^+Flk^+$EPCs. Numbers in the E4 areas present percentages of EPCs in total cell population. Data were representative of three time experiments.

also aims at validating the capability of RhEPO treatment in stimulating EPCs. Commonly expressed markers specific for EPC lineage include CD133, CD34 and Flk-1. We only included CD133 and FLK-1 to determine the presence of EPCs in this study because it has been found that a subset of EPCs are lack of CD34 expression and therefore inclusion of CD34 may confound the findings [10]. In this experiment, we treated the mice with RhEPO via intraperitoneal injection for 1, 3, 7, 10 and 14 days. The peripheral blood was collected via cardiac puncture and the EPCs was stained by anti-CD113 (FITC) and anti-Flk (PE) antibodies. The percentage of $CD113^+Flk\text{-}1^+$EPCs number remained unchanged in saline treatment group throughout the 14 days of treatment. After treatment with 300 IU/kg of RhEPO for 3 days, the EPCs number in mouse peripheral blood increased significantly from 71.42% to 95.25% on day 7 ($P < 0.05$). However, the proliferative effect started to diminish after day 7 (**Figure 2**).

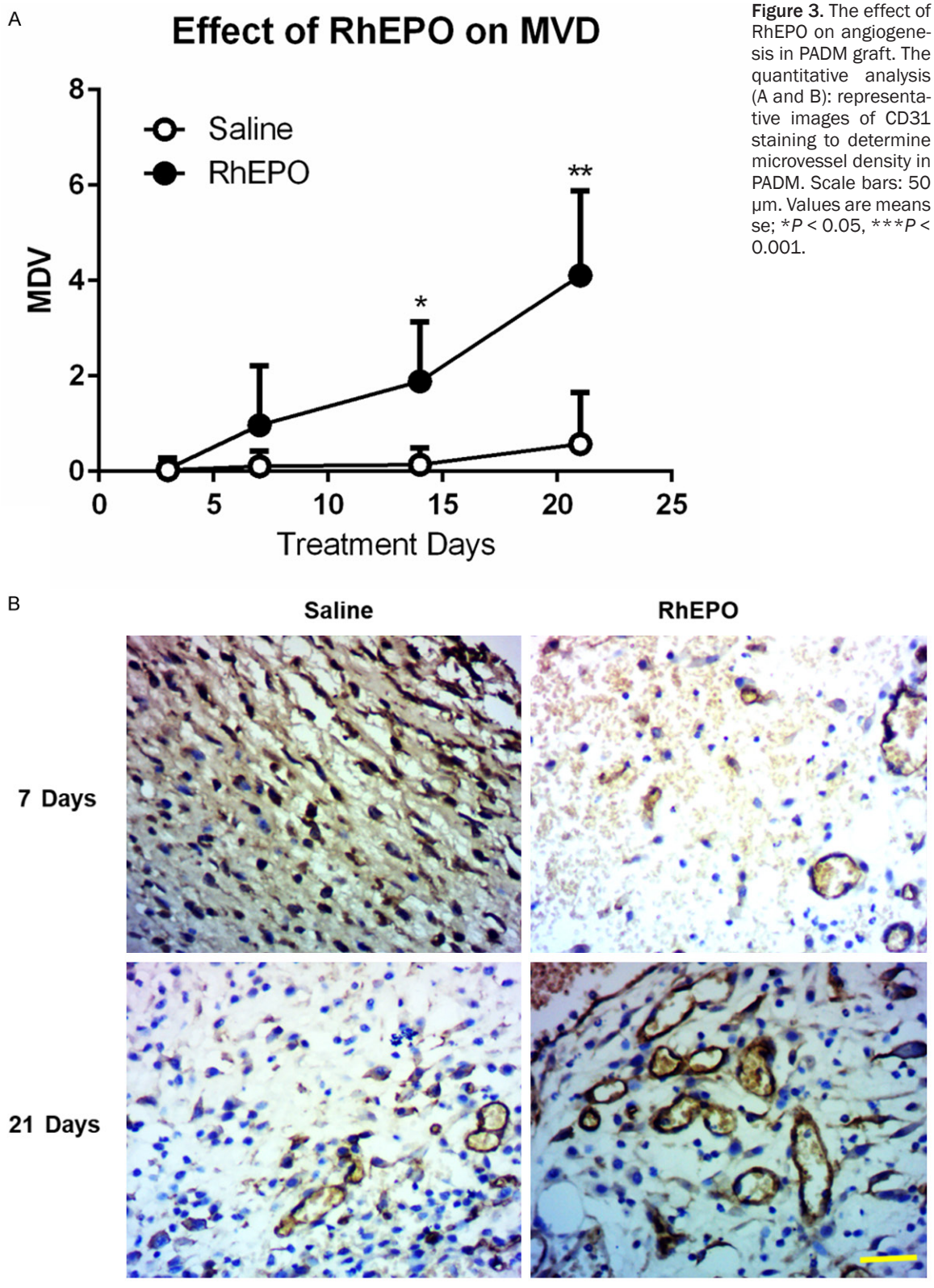

Figure 3. The effect of RhEPO on angiogenesis in PADM graft. The quantitative analysis (A and B): representative images of CD31 staining to determine microvessel density in PADM. Scale bars: 50 μm. Values are means se; $*P < 0.05$, $***P < 0.001$.

These data suggested that RhEPO stimulated EPC proliferation in mice peripheral blood and this mechanism may account for its wound healing effect.

RhEPO promotes revascularization and angiogenesis in implanted porcine a cellular dermal matrix (PADM)

Since revascularization and angiogenesis are essential steps of wound healing we speculated that RhEPO enhances wound healing by promoting angiogenesis in the area. As higher doses of RhEPO proved to be more effective in inducing EPC proliferation, we focused on the high dosage treatment group (1000 IU/kg) and the saline control group in this experiment. After treatment with either saline or 1000 IU/kg RhEPO for 7 days, a piece of PADM was subcutaneously implanted between the two scapula bones on the dorsum. The PADM was harvested on day 3, 7, 14 and 21 after implantation and was subjected to IHC analysis of CD31, which is a marker for blood vessel. MVD was calculated based on the expression level of CD31. The results showed that the MVD increased substantially in the PADM from mice primed by RhEPO from day 3 to day 7 while the level of MVD in unprimed mice remained unchanged (**Figure 3**).

Discussion

Wound healing is a complicated process that involves multiple steps of cell proliferation, engraftment and vascularization. It is important to understand the mechanisms of molecules involved in this processes in order to better device therapeutic agents for future clinical application. Numerous cytokines and growth factors have been shown to hold promises for improvement in wound healing but their applications are withheld by various degrees of side effects. Erythropoietin (EPO) is the regulator of red blood cells development and maturation and is recently implicated in the process of wound healing. The receptor of EPO has been shown to be expressed in both normal and wounded skin [11], suggesting a potential role of EPO as a therapeutic target in wound healing. In this study, we examined the wound healing effect of RhEPO in a mouse model by investigating the degree of epithelialization, stem cell stimulation and revascularization.

Firstly, we found that systemic administration of RhEPO promotes wound healing in mouse at both low and high dosages in a dose-dependent manner, consistent with previous reports. However, no studies have been conducted to deduce the exact molecular mechanism involved. Previous studies suggested that SMAD3, a key molecule that activates transforming growth factor beta (TGF-β) and its downstream signal transduction pathways, may act as a latent nuclear transcriptional activator that regulates cellular functions pivotal to wound repair [12, 13]. In this study we demonstrated that there was indeed a concurrent increase of pSmad3 at the wound healing area after treatment with RhEPO.

In addition, our study demonstrated that RhEPO increases the number of circulating EPCs in peripheral blood. This is consistent with a recent study that showed activated Smad2/Smad3 pathway supporting migration and sprouting of endothelial progenitor cells in peripheral blood [14]. However, previous reports showed that EPO not only enhances EPCs proliferation, migration and tube formation *in vitro* but also improves the survival of transplanted EPCs [15, 16]. Therefore we conclude that RhEPO accelerates wound repair by activating Smad3 which in turn stimulates EPCs in the peripheral blood.

Apart from activating EPCs, SMAD3 also plays a role in the angiogenesis process. It was reported that Smad3 and hypoxia inducible factor α (HIFα) synergistically activated vascular endothelia growth factor (VEGF) transcription [17, 18]. Other studies also reported that SMAD3 promotes angiogenesis via endoglin, a cytokine involved in all phases of the wound healing process [19, 20]. Thus, it is plausible that the observed wound healing effect induced by RhEPO is partly due to the improved vascularization and angiogenesis. An association between increase of MVD and treatment with RhEPO was observed in this study at the wound area, further strengthening the causative relationship between wound healing and RhEPO.

Skin grafting is pivotal to regenerative medicine for chronic and burnt wounds. With increasing incidence of diabetes globally, effective skin grafting becomes even more critical in the clinic for successful management. Diabetes patients tend to develop tissue damages in the limbs as the disease progresses and the quality of their lives can be largely compromised. Finding novel therapeutic agents to improve

clinical outcomes is particular important for this group of patients. While the gold standard solution is to use a patient's own skin in the treatment to avoid tissue rejection or graft-versus-host diseases, the limited amount of skin available from autologous donor restricts the coverage of the wound. Similarly, the availability of allogenous skin graft is scarce and also bears the risk of transmitting infectious diseases [21, 22]. As a result, a number of alternative products are under development, one of which is PADM [23, 24]. Successful engraftment of PADM depends heavily on revascularization and angiogenesis [25, 26]. Our study showed that systemic administration of RhEPO in mouse effectively stimulates EPCs and promotes angiogenesis in PADM engraftment, proving that the combination of RhEPO administration and implantation of PDAM may hold the key for successful skin engraftment in future regenerative medicine.

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Disclosure of conflict of interest

None.

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