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Original Article

Recombinant human acid fibroblast growth factor accelerates skin wound healing in diabetic rats through promoting fibroblast proliferation and angiogenesis

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Abstract: The aim of the present study is to investigate the effect and mechanism of recombinant human acidic fibroblast growth factor (rhaFGF) on skin wound healing of diabetic rats. SD rats were randomly divided into three groups: control, diabetes and rhaFGF groups. Skin wound tissues of rats in the three groups were respectively arranged on days 3 and 7 after establishment of skin wound of diabetic rats. Immunohistochemistry analyses were performed to evaluate the effect of rhaFGF on expression levels of PCNA and CD34 in diabetic rats. Real-time PCR and Western blot assays were used to examine the expression levels of Bcl-2, Bax, TGF-β, CXCL-1, CXCL-2, p-ERK1/2 and p-STAT3 in wound tissues. The results showed that rhaFGF could significantly increased expression levels of PCNA, CD34, TGF-β, CXCL-1, CXCL-2, p-ERK1/2, p-STAT3 and the ratio of Bcl-2/Bax in wound tissues. The preliminary mechanism that rhaFGF accelerates wound healing of diabetic rats is possibly associated with the promotion of fibroblasts proliferation and angiogenesis through ERK1/2 and STAT3 pathways.

Keywords: Wound healing, recombinant human acidic fibroblast growth factor, proliferation, angiogenesis, ERK1/2, STAT3

Introduction

Impaired wound healing is a major diabetic complication and a major clinical problem in diabetes patients. The pathophysiology of poor wound healing in diabetes is characterized by diminished formation of granulation tissues and impaired angiogenesis, and angiogenesis impairment plays an important role in these processes that required for successful wound repair [1]. Patients with diabetes exhibit impaired wound healing accompanied with increased susceptibility to wound infection [2]. Consequently, not recognized and treated early for integument disruption in diabetic patients responsible for the risk of developing complications associated with chronic wounds, which become infected can contribute to limb amputation especially in diabetic foot ulcers [3]. Considering these reasons, early intervention is crucial for successful treatment of poor wound healing in diabetes.

Recently, bone marrow-/adipose tissue-derived stem cells and bioengineered skin have been used clinically and reported to be effective in wound healing [4, 5]. However, the high cost and limited supply of skin substitutes are significant issues for further clinical usage. Therefore, some growth factors have been clinically used for the treatment of chronic skin wounds. Both acidic and basic fibroblast growth factors have (aFGF and bFGF) have many biological activities including stimulating the proliferation of fibroblasts, thus promoting angiogenesis and wound healing [6]. Previous studies demonstrated that recombinant human acidic fibroblast growth factor (rhaFGF) and rhbFGF promoted healing of skin ulcer in diabetic rats and patients [7].

Although there have been many *in vivo* and *in vitro* studies on rhaFGF involved in wound healing of diabetic rats, it is still not known what causes the effects. The particular protein expression patterns in wound healing of diabetic rats may be related to the fibroblasts proliferation and angiogenesis. Transforming growth factor beta (TGF-β) is known to enhance matrix production and has been reported to have differential effects on proliferation rates of fibroblasts in wound healing of diabetic rats versus normal rats [8, 9]. The progression of normal wound healing leads to granulation tissues formation and fibroblasts differentiation into myofibroblasts through different stages. Chemokines are critical for white blood cell recruitment to injured tissues and play an important role in normal wound healing processes [10]. Many different cell types, including endothelial cells and fibroblasts, produce and respond to chemokines, and these interactions are altered in diabetic wounds.

As wounds close there is a decrease in cellularity due to apoptosis that ensures the transition between granulation tissue and scar. Recent findings in a model of regressing granulation tissues have suggested that differentiation into myofibroblasts may not be a prerequisite for apoptosis [11]. Signal transduction pathway involves in many diseases regulating apoptosis and angiogenesis. MicroRNA-99 deregulation has been linked with impaired wound healing via targeting AKT/mTOR signaling pathway [12]. Topical insulin and N-acetylcysteine accelerate wound healing in diabetes via enhancing the Extracellular-signal regulated kinase 1/2 (ERK1/2) and signal transducers and activators of transcription 3 (STAT3) pathway [13, 14]. bFGF promotes the migration of human dermal fibroblasts under diabetic conditions through ROS production via the PI3K/AKT-Rac-1-JNK pathways [15].

As mentioned above, the role and efficiency of rhaFGF in wound healing has been proven, however, it is unclear if rhaFGF is effective in improving the poor wound healing process in diabetes. In our current study, we investigated the effect of rhaFGF on wound healing in diabetic rats and showed that rhaFGF accelerated wound healing in diabetic rats and stimulated the expression of growth and angiogenesis factors and activation of ERK1/2 and STAT3 involved in wound healing.

Materials and methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of People Hospital of Lishui. Four- to five-week-old male Sprague-Dawley rats (80 g~100 g) were randomly assigned to receive either 65 mg/kg STZ intraperitoneally. Rats were categorized as diabetic when the blood glucose exceeded 16.7 mmol/L at 72 h after STZ administration.

The wounds were created 7 days after establishment of diabetic rats as described previously. Briefly, rats were anaesthetized using an intraperitoneal injection of pelltobarbitalum natricum (45 mg/kg, SHANGHAI WESTANG BIOTECH CO., LTD, Shanghai, China). The dorsal surface was shaved and washed with iodophor. Then a disposable 1.8 cm diameter skin biopsy punch tool (Shiny Health Import & Export Co. Pty Limited, Sydney, Australia) was used to create a full thickness excisional wound down to the fascia. The rats were then randomized into six groups (10 rats in each group) for the 3-week experiment and given treatment once a day or not as follows: normal rats without surgery (control), diabetic rats (skin ulcer) and 0.2 ml rhaFGF treatment rats (skin ulcer). All rats had free access to standard rat food and drinking water.

Immunohistochemistry

Wound tissues were initial treatment for deparaffinization and hydration and then heated in EDTA (pH 8.0) and incubated with 3% hydrogen peroxide for 10 min for antigen retrieval. The reaction of PCNA mouse monoclonal antibody (Abcam, Cambridge, MA, USA) and CD34 mouse monoclonal antibody (Abcam) were taken place 1 h at room temperature, following incubated by goat anti-mouse horseradish peroxidase-conjugated IgG (Abcam). Slides were stained with DAB (Shanghai Long Island Biotec. Co., LTD, China) and hematoxylin staining (BASO, China). Immunohistochemical signals were calculated with the positive staining cells. Stained tissue sections on slides were analyzed under identical light microscope at ×200 magnifications.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA). Real-time PCR analyses were performed with SYBR Green (Takara, Dalian, China) and the data collection were performed on ABI 7500. Real-time PCR was performed to detect mRNA levels of TGF-β, CXCL-1 and CXCL-2. GAPDH was used an internal control. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [16]. Primers were list as follows: Bax, 5'-GGACGCATCCACCAAGAAG-3' and 5'-CTGCCACACGGAAGAAGAC-3'; Bcl-2, 5'-GGGATGCCTTTGTGGAAC-3' and 5'-GTCTGCTGACCTCACTTG-3'; TGF-β, 5'-AAGGACCTGGGTTGGAAGTG-3' and 5'-TGGTTGTAGAGGGCAAGGAC-3'; CXCL-1, 5'-TAGAAGGTGTTGAGCGGGAAG-3' and 5'-TGAGACGAGAAGGAGCATTGG-3'; CXCL-2, 5'-GCGCCCAGACAGAAGTCATAG-3' and 5'-AGACAGCGAGGCACATCAG-3'; GAPDH, 5'-GTCGGTGTGAACGGATTTG-3' and 5'-TCCCATTCTCAGCCTTGAC-3'. The following PCR conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s.

Western blotting

Total protein was extracted from wound tissues using radioimmunoprecipitation buffer. An equal amount of protein was subjected to Tris-glycine-SDS polyacrylamide gel (12%) electrophoresis, and Western blotting was performed as described with use of appropriate primary and horseradish peroxidase-conjugated secondary antibodies. Mouse monoclonal antibodies against Bax (1:5000), Bcl-2 (1:500), CXCL1 (1:1000), CXCL2 (1:500), p-STAT3 (1:20000) and STAT3 (1:5000) were purchased from Abcam except mouse monoclonal antibody against p-ERK1/2 (1:2000, Cell Signaling Technology, Danvers, MA, USA), ERK1/2 (1:2000, Cell Signaling Technology) and GAPDH (1:1500, Cell Signaling Technology). Goat anti-mouse secondary antibodies (Beyotime, Nantong, China) were used in this study. ECL chromogenic substrate was used and signals were quantified by densitometry (Quantity One software, Bio-Rad Laboratories Inc. Hercules, CA, USA).

Statistical analysis

The data was presented as the mean value ± S.D. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA, USA). The paired, two-tailed Student's t-test was used to analyze the significance of the difference between groups. *P* value lower than 0.05 was considered to be statistically significant.

Results

rhaFGF treatment accelerates fibroblasts proliferation of wounds in diabetic rats

Having documented significantly thick granulation tissues and re-epithelialisation in rhaFGF-treated wounds, we wonder how rhaFGF influence fibroblasts proliferation. Therefore, proliferating cell nuclear antigen (PCNA), was analyzed in diabetic rats by immunohistochemistry on days 3 and 7. Our results showed that thin expression of PCNA was detected in diabetes group compared with the control group, while strong immunohistochemical appearance of PCNA in rhaFGF-treated diabetic rats compared with the diabetes group (**Figure 1A**). These results suggest that rhaFGF accelerates fibroblasts proliferation through upregulation of PCNA expression in wounds of diabetic rats.

rhaFGF treatment accelerates angiogenesis of wounds in diabetic rats

Since healing of skin wounds requires angiogenesis, we next evaluated the newborn vessels of wound granulation tissues. Wound angiogenesis was analyzed by CD34 immunohistochemistry of 3-μm frozen sections to visualise neovascularisation. CD34 staining of wound granulation tissues on days 3 and 7 in rhaFGF-treated diabetic rats was shown in **Figure 1B**. Narrow and tiny newborn vessels at the wound margin were observed in the diabetes group, whereas thicker and larger vessels were growing widely in the rhaFGF group on days 3 and 7. These results suggest that topical rhaFGF application significantly enhanced wound angiogenesis, as assessed by the strong expression of CD34 in rhaFGF group compared with the diabetes group.

rhaFGF treatment increases angiogenesis-associated factor expression

To further investigate the molecular mechanism of rhaFGF accelerated angiogenesis of wounds in diabetic rats, we evaluated the expression levels of TGF-β, CXCL-1, CXCL-2 and

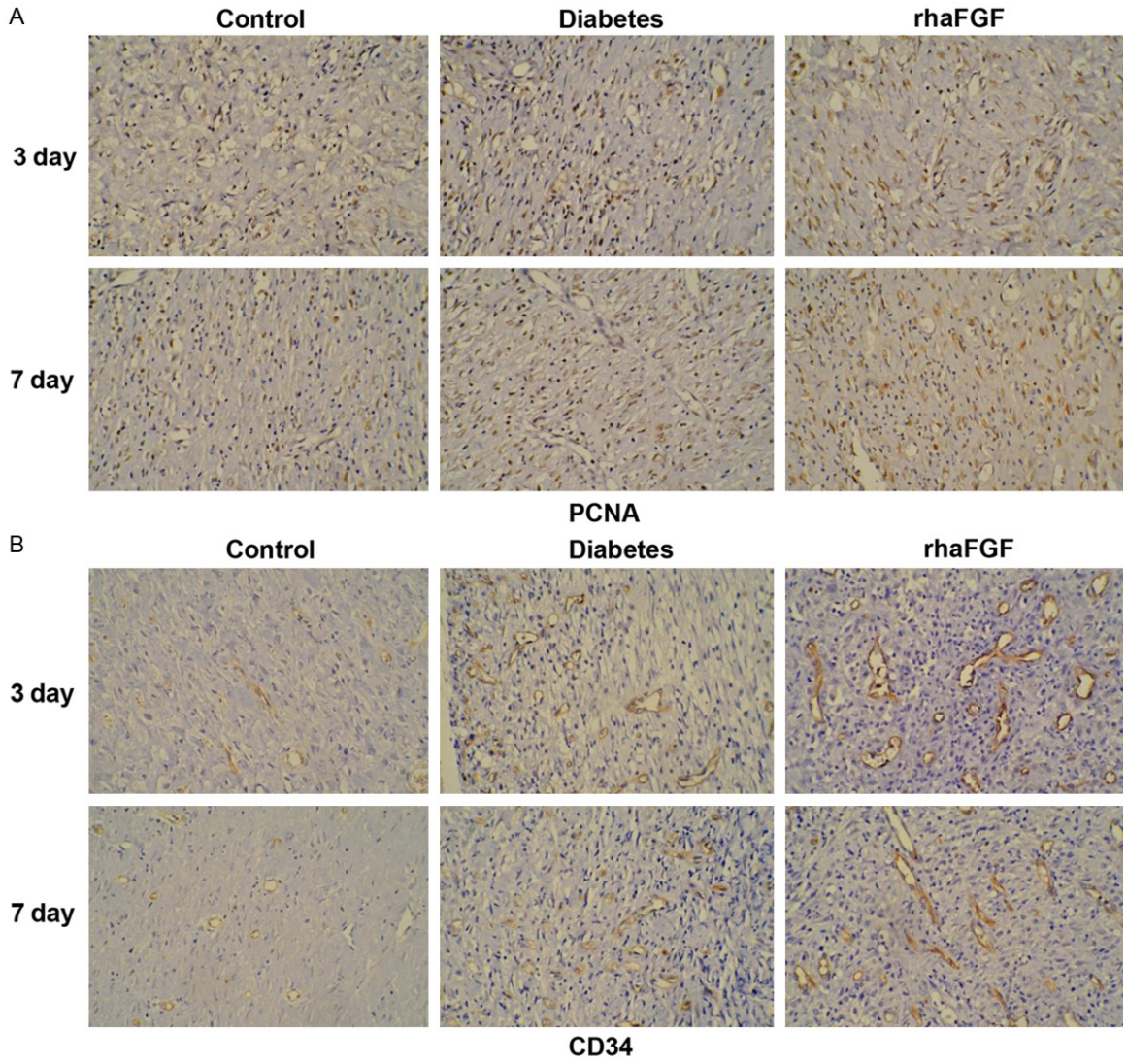

Figure 1. rhaFGF treatment accelerated fibroblasts proliferation and wound angiogenesis. A. Sections from wounds of diabetic rats treated with rhaFGF were stained with an antibody against PCNA. Wound margins on days 3 and 7 showed increased PCNA expression with time for rhaFGF-treated wound. B. Sections from wounds of diabetic rats treated with rhaFGF were stained with an antibody against CD34. On days 3 and 7, tiny and narrow newborn vessels at the wound margin were present in the diabetes groups, while larger and thick vessels were growing widely in the wound in the rhaFGF groups. Original magnification ×200.

the phosphorylation of STAT3 in the diabetic rats after rhaFGF treatment on days 3 and 7. As shown in **Figure 2A-C**, the mRNA expression levels of CXCL-1, CXCL-2 and TGF-β were increased in diabetic rats compared with the control, while significantly increased expression levels of these factors were observed in rhaFGF-treated diabetic rats compared with the diabetes rats. The protein level of these factors was similar to the mRNA level of those (**Figure 2D**).

rhaFGF treatment attenuates apoptosis of wounds in diabetic rats

To investigate the effect of rhaFGF on apoptosis of wounds in diabetic rats, we performed Real-time PCR and Western blot assay to detect the expression levels of apoptosis-related markers, including Bax and Bcl-2, just after wounding. As shown in **Figure 3A**, the mRNA expression level of Bcl-2 was significantly decreased in diabetic rats compared with the

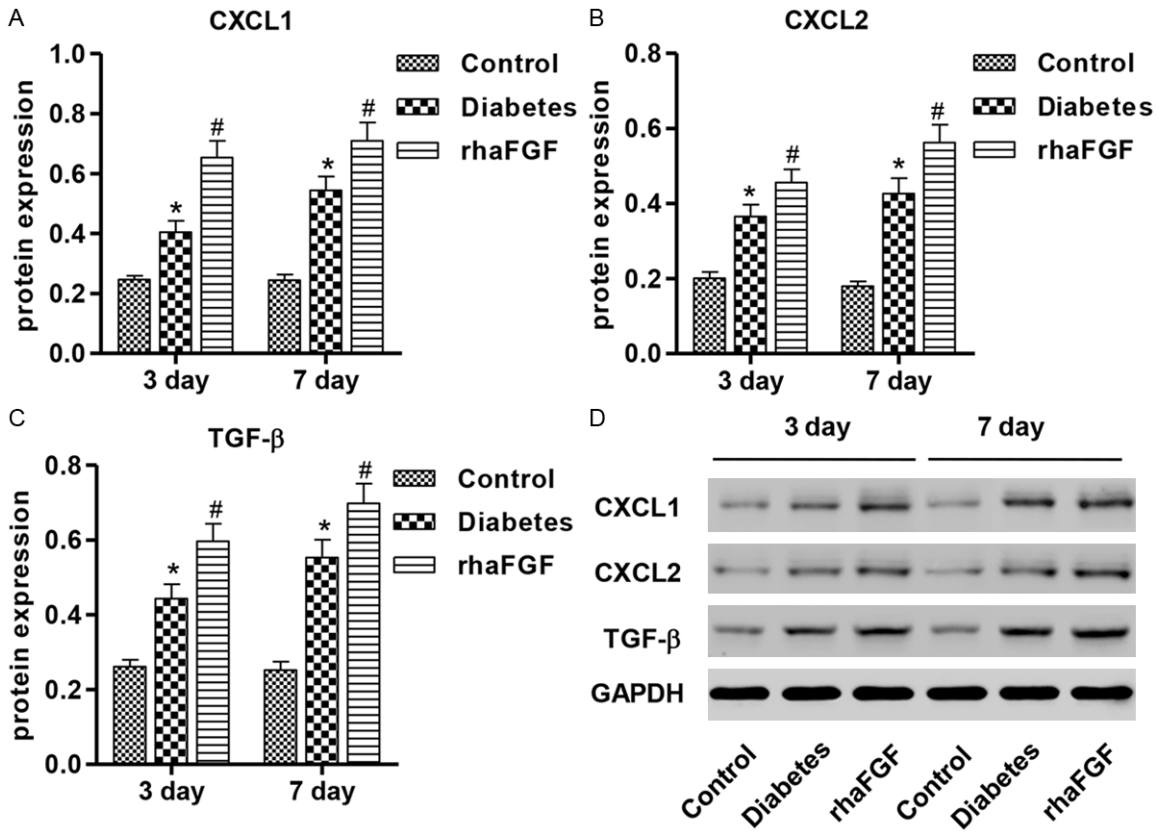

Figure 2. rhaFGF treatment increased angiogenesis-associated factors expression. Real-time PCR (A-C) and Western blotting (D) analyses showed that the mRNA and protein expression levels of CXCL1, CXCL2 and TGF-β were increased in diabetic rats and rhaFGF-treated diabetic rats compared with control rats. $^{*}P < 0.01$ compared with the control group. $^{\#}P < 0.01$ compared with the diabetes group.

control, while increased expression level of Bcl-2 was observed in rhaFGF-treated diabetic rats compared with the diabetes rats. The protein level of Bcl-2 was similar to the mRNA level of it (**Figure 3C** and **3D**). Inversely, the mRNA and protein levels of Bax were significantly increased in diabetic rats, but decreased in rhaFGF-treated diabetic rats (**Figure 3B**, **3C** and **3E**). These findings indicated that rhaFGF treatment attenuates apoptosis via increase in the ratio of Bcl-2/Bax in diabetic rats.

rhaFGF treatment activates ERK1/2 and STAT3

In addition, the activation of ERK1/2 and STAT3 was also measured by Western blot analysis and the results showed that the significantly enhanced phosphorylation of ERK1/2 and STAT3 in rhaFGF-treated diabetic rats compared with the control and diabetes rats (**Figure 4A-C**). These findings indicated that rhaFGF treatment promoted angiogenesis and attenuated apoptosis via activation of ERK1/2 and STAT3.

Discussion

Wound healing in diabetes has become a hot research because of its high cost and the risk of amputation, therefore needs a better alternative healing strategies and agents. Recently, because of the advances in tissue engineering and cell culture techniques, cultured skin substitutes have been used in the wound healing. For example, adipose-derived stem cells, endothelial progenitor cells and mesenchymal stem cells were observed acceleration of wound healing in diabetic rats [17-19]. In addition, many growth factors such as bFGF, VEGF and epidermal growth factor (EGF) have been used for the clinical wound healing [20, 21]. In the present study, we demonstrated that rhaFGF is significantly effective in diabetic wound healing using the diabetic rat models.

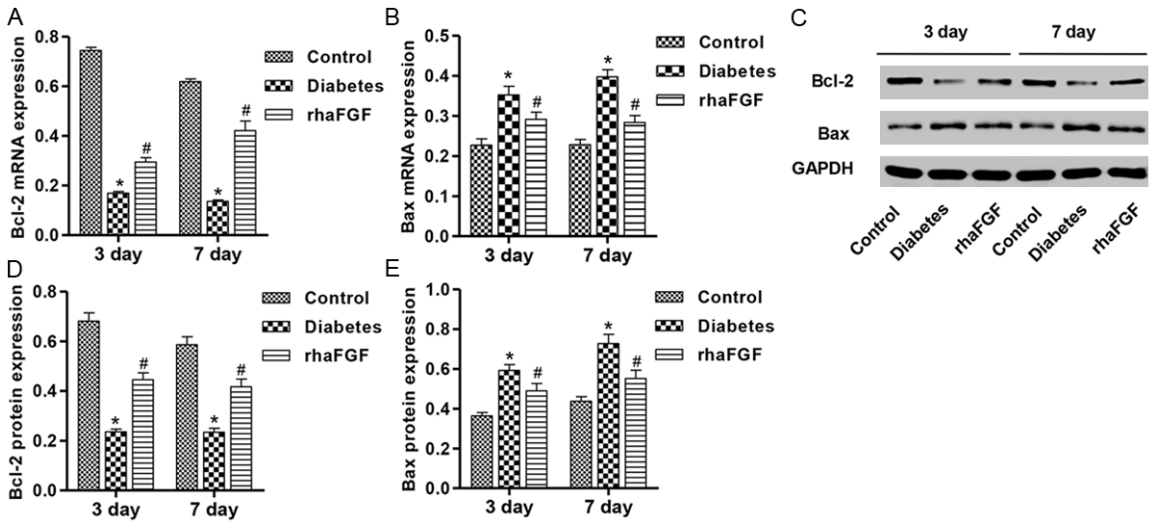

Figure 3. rhaFGF treatment increased the ratio of Bcl-2/Bax. Real-time PCR and Western blotting analyses showed that the mRNA and protein expression levels of Bcl-2 were significantly decreased (A, C and D) and Bax were significantly increased (B, C and E) in diabetes groups, while inverse effects on Bcl-2 and Bax were found in the corresponding rhaFGF groups. $^{*}P < 0.01$ compared with the control group. $^{\#}P < 0.01$ compared with the diabetes group.

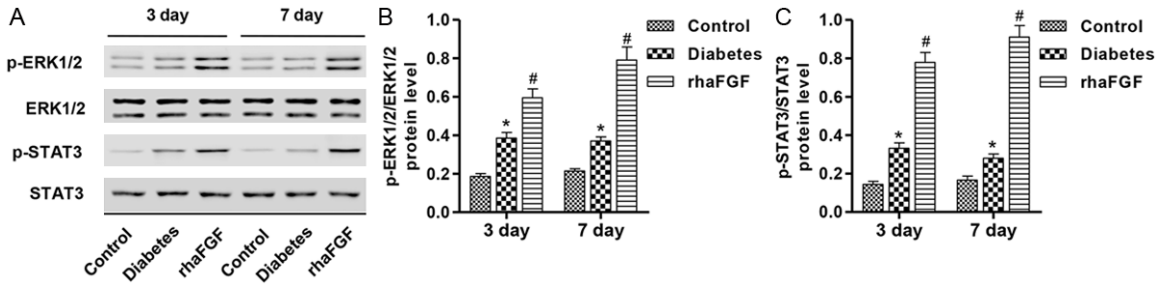

Figure 4. rhaFGF treatment activated the ERK1/2 and STAT3 pathways. Western blotting (A) and quantification of the phosphorylation of ERK1/2 (B) and STAT3 (C) was thin increased in diabetes groups, while the significantly sustained increase in phosphorylation of ERK1/2 and STAT3 was observed in rhaFGF groups. $^{*}P < 0.01$ compared with the control group. $^{\#}P < 0.01$ compared with the diabetes group.

Previous investigations on the role of rhaFGF in wound healing of surgical wounds have shown that rhaFGF does so by accelerating granulation tissue formation, re-epithelialization and tissue remodeling [22]. aFGF was a member of the FGF family and could be released in response to cell injury including disrupted parenchymal cells during wound healing resulting in the stimulation for angiogenesis [23]. *In vitro* experiments demonstrated that bFGF enhances keratinocyte motility during re-epithelialization, promotes fibroblast migration and production of collagenase [24]. In this study, we observed that daily topical application of rhaFGF significantly enhanced the degree of wound healing in the diabetic rats evidenced by increases in PCNA expression and the high level of CD34-postive vessels indicated that rhaFGF increase angiogenesis. As we known, PCNA is a marker of cell proliferation and has been observed highly expressed in response to acidic FGF (aFGF) treatment in healing-impaired STZ diabetic rat [25]. Meanwhile, CD34 positive blood cells were found accelerated vascularization and healing of diabetic mouse skin wounds [26].

To further evaluate the effects of rhaFGF on angiogenesis, we examined TGF-β, CXCL-1 and CXCL-2 expression, since all these molecules are major angiogenesis associated factors in wound healing. The protein analysis of the wound tissues from the rhaFGF-treated diabetic rats showed significant increase in TGF-β,

CXCL-1 and CXCL-2 expression. Delayed wound healing is associated with decreases in most growth factors, including TGF-β, because of proliferation and angiogenesis inhibitions [27]. TGF-β also plays an important role in angiogenesis *in vivo*, although it inhibits growth and proliferation of endothelial cell monolayers *in vitro*. These different effects may be attributable to the capacity of TGF-β *in vivo* to recruit and stimulate macrophages that then produce other active angiogenesis factors [28]. Recent data suggest that bFGF may set the stage for angiogenesis during the first 3 day of wound repair, whereas VEGF may be critical for angiogenesis during granulation tissue formation from day 4 through 7 [20]. However, in this study, the expression of TGF-β sustained increase from day 3 through 7, suggesting its long term role in rhaFGF-treated wound healing of diabetic rats. CXCL1 and CXCL2 are two important members of chemokine family, play critical role in regulating wound healing proven by their angiogenic role. Similar to our study, the increased expression of CXCL1 and CXCL2 were observed in low-dose radiation-treated diabetic wound healing [29].

In addition, we observed that rhaFGF treatment significantly increased the ratio of Bcl-2/Bax and the activation of ERK1/2 and STAT3 pathways. In agreement with our study, previous study showed that pro-angiogenic miR-27b inhibits endothelial progenitor cells apoptosis and improves wound healing in type 2 diabetes via increasing the ratio of Bcl-2/Bax and suppressing p53 [30]. In contrast, other data have shown that the aFGF leads to induction of apoptosis in human lung myofibroblasts by prior treatment with TGF-β *in vitro*, suggesting that the role of rhaFGF in fibroblast and myofibroblasts with significant difference in different cells. We observed that rhaFGF treatment significant activation of ERK1/2 and STAT3, which in agreement with the previous study that Cardiotrophin-1 induced rapid phosphorylation of ERK1/2 and STAT3 that acceleration of wound healing [31]. Other study showed that wound healing was controlled in keratinocytes by the gp130-SOCS3-STAT3 pathway and an imbalance of this pathway resulted in delayed wound healing [32].

Based on these effects, the wound healing potential of rhaFGF may due to its ability to increase fibroblasts proliferation and angiogenesis in diabetic rats. In conclusion, in this study, we provide evidence for the role of rhaFGF in accelerating wound healing in a diabetic rat model and propose rhaFGF to be a good candidate for clinical use because of its multifaceted role in wound healing.

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Disclosure of conflict of interest

None.

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