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Promoted Skin Wound Healing by Tail-Amputated *Eisenia foetida* Proteins via the Ras/Raf/MEK/ERK Signaling Pathway

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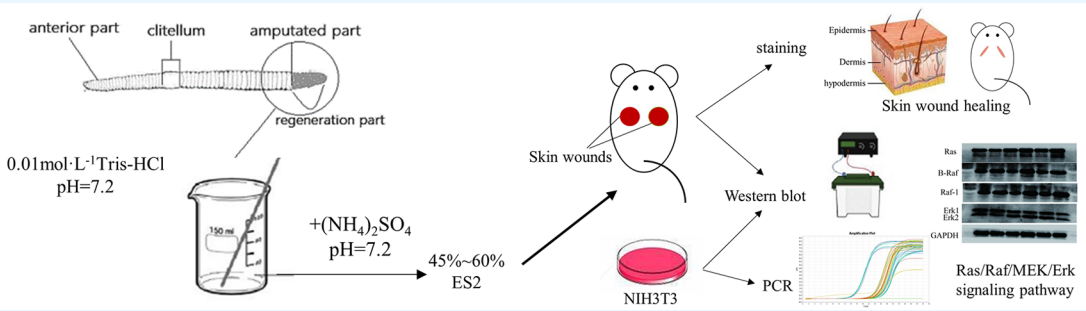

ABSTRACT: Skin wound healing is an important fundamental problem in biological and medical fields. This study aimed to investigate wound healing promotion of protein extract from tail-amputated *Eisenia foetida* (*E. foetida*) and reveal the mechanism correlated with the Ras/Raf/MEK/ERK signaling pathway. Proteins extracted from tail-amputated *E. foetida* were applied on rats' full-thickness excisional wounds to evaluate their regenerative efficacy. Rat skin tissues around surgical defects were analyzed by immunofluorescence staining and Western blot methods. The Ras/Raf/MEK/ERK signaling pathway was further investigated in vitro using the NIH3T3 cell line. A tail-amputated protein extract (ES2) from *E. foetida* significantly accelerated rat wound healing ability via higher re-epithelialization and ECM deposition in the tissue section compared to the blank control and un-amputated earthworm extract groups. Furthermore, ES2 treatment dramatically accumulated the expressions of platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and hydroxyproline (HYP) in wound areas on day 7 without their accumulation on day 21 post-wounding, diminishing excessive scar formation. Accelerated wound healing ability with the ES2 was proved to correlate with the up-regulation of the Ras/Raf/MEK/ERK signaling pathway. The mRNA expression of this pathway increased significantly in NIH3T3 cells after being treated with the ES2 at an appropriate concentration. The tail-amputated *E. foetida* proteins (ES2) can significantly promote skin wound healing better than the un-amputated earthworm tissue extract without excessive scar tissue formation. This effect was related to the up-regulation of the Ras/Raf/MEK/ERK signaling pathway.

INTRODUCTION

Skin wound healing refers to a process that includes a series of complex pathophysiological changes to restore the skin structure and functions. The skin structure is anatomically divided into three layers, namely, epidermis, dermis, and subcutaneous tissue.[1] Fibroblasts are the most abundant cell type in the dermis, whose number and state are important for skin homeostasis. They play an important role in the stages of skin wound healing, including tissue structure reconstruction and scar formation.[2] In addition, the maintenance of skin homeostasis is related to the interdependence and coordination of cell proliferation and extracellular matrix (ECM) deposition.[3] The histological characteristics of scars are the proliferation of fibroblasts with apoptotic disorder, the disorder of collagen fiber arrangement, the excessive deposition of ECM, and the massive production of some cytokines.[4] The skin regenerative healing and scar formation are related to deposition and remodeling of ECM.[5] Collagen and other ECM in wound remodeling are adjustable according to fibroblasts in the process of wound healing.[6] Despite that fibroblasts play a positive role in the wound healing process, microbes met by all human beings live in a non-sterile environment, which makes tissue repair crucial. Once microbes invade during the process, the rate and quality of skin wound healing will be appalling. Common bacteria in skin wound are often multidrug-resistant.[7] Given that the healing process can be interfered by many factors including growth factors, infection, and

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medications,[8] other aspects for accelerating wound repair should be considered besides tissue regeneration.

Earthworms can survive and even regenerate after being amputated. Studies have found that the substances from *E. foetida* have multiple biological activities including anticancer[9] and hepatoprotection.[10] The lumbrokinase from earthworm significantly attenuated fibroblast migration and adhesion.[11] Earthworm extract has been shown to improve healing quality and can be a propitious wound healing agent.[12] It played a role in suppressing fibrosis, epithelial regeneration, inhibiting scar formation, and ultimately contributing to wound healing.[13] Natural proteins from earthworm may be used with materials such as antibacterial hydrogel.[14,15] The bioactive substances can be made into synthetic agents or used in targeted drug delivery systems to exert effects and avoid toxic.[16−19]

We noted that the materials and effects are different in the recovery stage of earthworm. The preliminary study of our group showed that the tail-amputated *E. foetida* promoted wound healing without scarring and the effect was better than that of un-amputated earthworm.[20] The effect was best of proteins from *E. foetida* on day 3 after being tail-amputated. Moreover, it can increase the expression of basic fibroblast growth factor (bFGF) in skin. The principle and application of antimicrobial drugs are also studied in skin wound treatment. Proteins from tail-amputated *E. foetida* may be used with antimicrobial agents in sophisticated therapeutic systems. The mechanism of protein effect should be defined for appropriate application. Although the proteins from tail-amputated earthworm were proved to accelerate wound healing, its mechanism remains unknown.

The Ras/Raf/MEK/ERK signaling pathway is involved in a lot of biomedical studies about cell activities. The effect of drug on skin may be related to the regulation of the Ras−Raf−MEK−ERK signaling pathway.[21] Lots of studies on the Ras/Raf/MEK/ERK signaling pathway are meaningful for the treatment of human diseases but may cause skin adverse reactions.[22] Blocking the Ras/Raf/MEK/ERK signaling pathway results in the inhibition of cell proliferation and migration.[23] The activation of the RAS−RAF−MEK−ERK signaling pathway is an important molecular basis for the skin disease and cure.[24] When skin is wounded, the Ras/Raf/ERK/MEK signaling pathway is also obviously damaged.[25] Skin wound is a strong stimulus, which can be used as a signal to activate the Ras/Raf/MEK/ERK signaling pathway. The ERK−MAPK signaling pathway plays a key role in treating skin diseases.[26] They participate in the synthesis of cytoskeletal proteins. The regulation of the Ras/Raf/MEK/ERK signal cascade affects fundamental cell functions. It is essential for intracellular and intercellular communication.[27] The Ras/Raf/MEK/ERK signaling pathway also relates to other pathways; associations between pathways have effects on growth, apoptosis, and cell cycle of cell lines.[28] This study provides a basis for the association of the Ras/Raf/MEK/ERK signaling pathway with skin tissue repair and regeneration.

MATERIALS AND METHODS

Preparation of Earthworm Extract. All processes were carried out at low temperature under ice bath conditions. The proteins were extracted and separated from *E. foetida* that had been tail-amputated and grown naturally for 3 days. The ES2 protein extract was prepared as in our previous study.[10] 1 cm tissue part of the earthworm at one-third length to the tail was obtained to homogenize. The homogenate was prepared with a weight to volume (10 mM, pH 7.2 Tris−HCl buffer) ratio of 1/4. It was placed at 4 °C for 24 h and centrifuged at 4 °C, 1000 r/min for 30 min. The supernatant was made into lyophilized powder. Un-amputated proteins were also extracted from whole *E. foetida* tissue as negative control in the same method. The protein concentration was confirmed by BCA Protein Assay Kit (Solarbio, China).

Cell Culture. The mouse embryo fibroblasts (NIH3T3) were cultured in Dulbecco's modified Eagle's medium (Solarbio, China) supplemented with 10% newborn calf serum (Sijiqing, China) and 1% penicillin−streptomycin (Solarbio, China). Cells were seeded 100 μL per well (5×10^3 cells) on 96-well plates and cultured at 37 °C in a humidified incubator at 5% CO_2.

Animals. The animal procedures and maintenance were conducted strictly in accordance with the protocols approved by the local Animal Care and Use Committee of Beijing University of Chinese Medicine. Seventy-five male SPF Wistar rats (250−300 g) were obtained from SPF Biotechnology Co., Ltd. (Beijing, China), and kept separately in an appropriate environment (specific-pathogen-free, 20−25 °C, 50% humidity, and 12 h light/dark cycle) for 7 days with free access to standard diet. The license number was SCXK (Beijing) 2016-0002. Rats were anesthetized by intraperitoneal injection of urethane (1.5 g/kg) and euthanized by cervical dislocation under deep anesthesia finally. A sterile 1.5 cm hole punch was used to create two full-thickness wounds on both sides of the rat's back after hair removal. Forty-five rats that were used for observation and sampling of skin wounds were randomly divided into three groups after adaptive feeding for 7 days, 15 in each group. Rats in the blank control, un-amputated, and tail-amputated groups were received 100 μL of 0.9% physiological saline, 100 μL of the un-amputated *E. foetida* extract solution (1.12 mg/mL), and the tail-amputated *E. foetida* extract solution (ES2) (1.12 mg/mL), respectively, for each wound once daily.

Thirty rats that were used for Western blot were randomly divided into five groups: control; tail-amputated protein extract (ES2, 1.12 mg/mL) in low, medium, and high concentrations; and un-amputated *E. foetida* extract (1.13 mg/mL). The rats in the low-, medium-, and high-dose groups were given 50, 100, and 150 μL tail-amputated earthworm protein solution, respectively.

Histology, Staining, and Immunohistochemistry. Rats that were used for observation of their wound morphology were recorded daily for 21 days. Macroscopic images were collected at 3, 5, 7, 10, 14, and 21 days after modeling. Changes in the wound areas over time were calculated in comparison with the initial wound by using ImageJ software (version 8.0). The calculation formula of the wound healing rate is as follows: $p = \left(1 - \frac{A_1}{A_0}\right) \times 100\%$ (p: wound healing rate; A_1: existing wound area; A_0: original wound area).

Five rats were sacrificed in each group on days 7, 14, and 21 after surgery. Skin wounds with surrounding healthy skin were collected. After washing with phosphate-buffered saline (PBS), skin tissues were fixed with 4% paraformaldehyde, dehydrated with a graded alcohol series, and then embedded in paraffin. Thin tissue sections (4 μm thick) were prepared and stained with hematoxylin−eosin staining and Masson's staining (Solarbio, China).

For immunohistochemistry, after antigen retrieval under heated conditions, the sections were cooled and then washed

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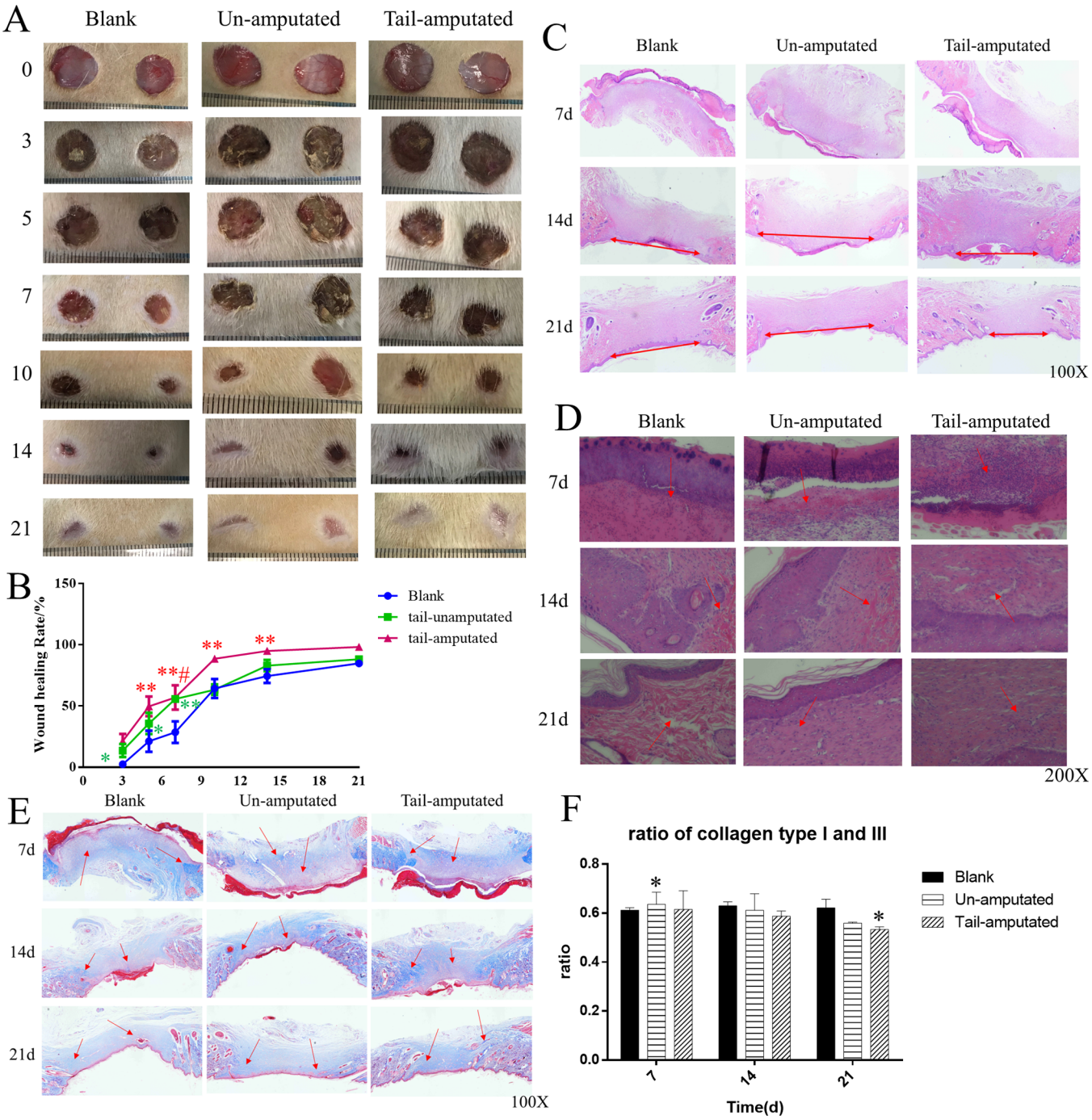

Figure 1. The proteins from tail-amputated *E. foetida* accelerate the wound healing process. (A) Representative images of the healing process in rats on different days. (B) Wound healing rate calculated by wound closure areas (n = 15). (C, D) Representative images of hematoxylin−eosin staining of skin wounds (C: 100×; D: 200×) (n = 5). (E) Masson's trichrome staining of skin wound sections (n = 5). (F) Ratio of collagen type I and collagen type III of rats' skin (n = 5). The results are presented as mean ± S.E.M. compared with the blank control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the un-amputated group, $^{\#}P < 0.05$.

with PBS. Endogenous peroxidase was blocked. Sections were blocked with normal goat serum for 30 min. Tissues were reacted with TGF-β1 Rabbit Polyclonal antibody (1: 6000, Proteintech, China) or PDGF Rabbit Polyclonal antibody (1: 5000, Proteintech, China) at 4 °C overnight. After washing with PBS, the sections were incubated with HRP-conjugated secondary antibodies (DAKO, China) for 50 min. Nuclei were counterstained with hematoxylin for 3 min.

Tissue sections (6 μm thick) were stained by Sirius red staining (Leagene, China) for 1 h to observe type I and III collagen with different colors, and then the ratio of color area was calculated. Nuclei were stained with hematoxylin for 10 min. HYP was determined by alkali hydrolysis according to the manufacturer's instructions (Nanjing Jiancheng Co., Ltd., China).

Cell Viability Assay. Cells were incubated with different concentrations of the proteins from tail-amputated or un-

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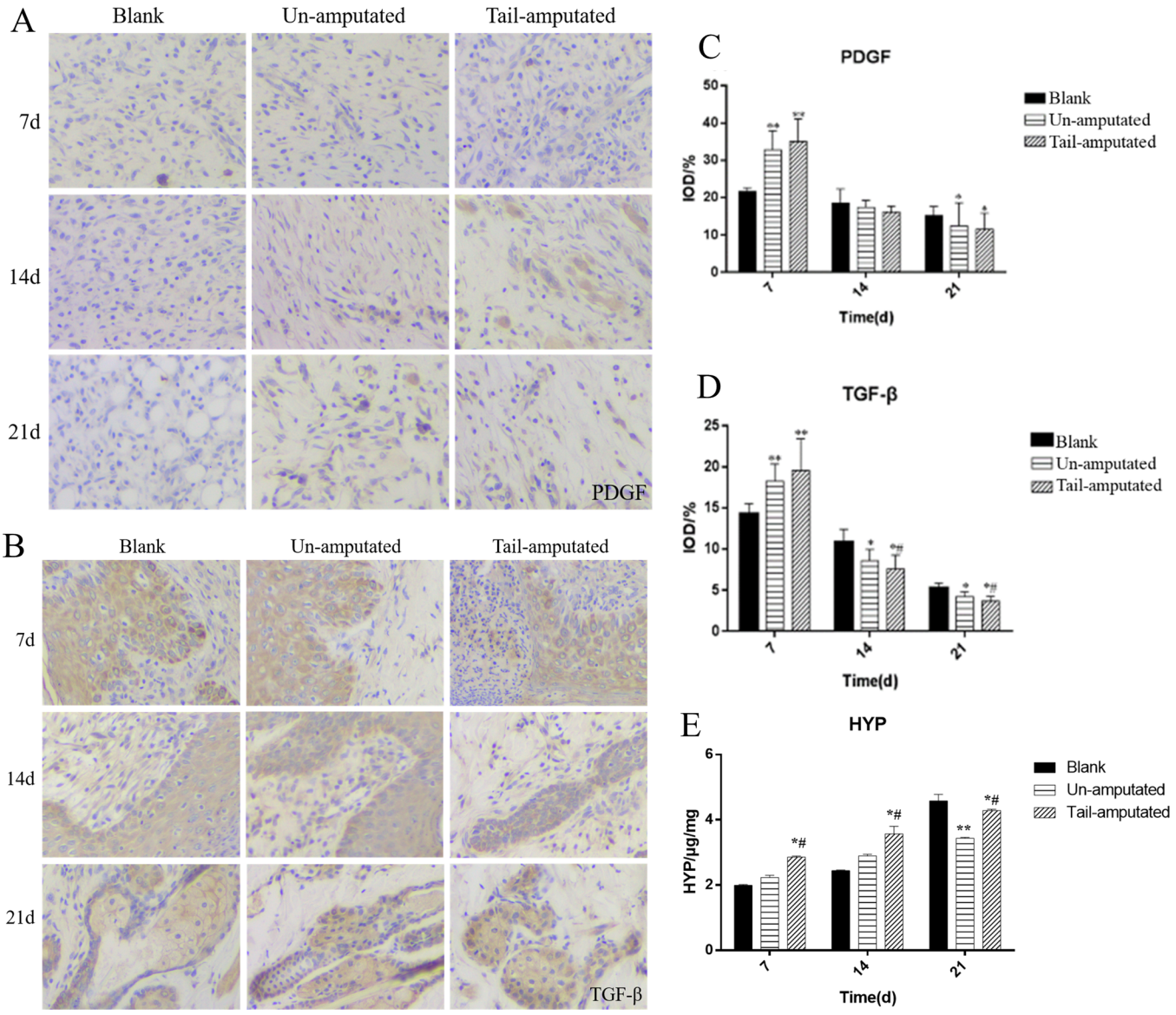

Figure 2. The proteins from tail-amputated *E. foetida* affect secretion of factors about wound healing. (A, B) Representative immunoblot images depict PDGF or TGF-β (n = 5). (C, D) Immunohistochemical results of rat wound. The IOD of PDGF or TGF-β on skin wounds. (E) The HYP expression of rats' skin wounds (n = 5). The results are presented as mean ± S.E.M. Compared with the blank control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the un-amputated group, $^{\#}P < 0.05$.

amputated earthworm at 0.5, 1, 1.5, 2, and 3 μg/mL for 48 h. 110 μL of complete medium containing 10 μL of CCK-8 reagent (Saint, China) was added to each well and was incubated for 1.5 h. bFGF (0.1 μg/mL) was used as the positive control. Absorbance was measured at 490 nm with a microplate reader (BMG LABTECH, Germany).

Real-Time Fluorescence Quantitative PCR to Measure the mRNA Expression of the Ras/Raf/MEK/ERK Pathway in Cells. Cells were incubated with the proteins from tail-amputated earthworm at 0.8, 1, and 1.2 μg/mL or with the proteins from un-amputated earthworm at 1 μg/mL for 48 h. bFGF (0.1 μg/mL) was used as the positive control. Total RNA was extracted from 1 million cells by RNeasy^{TM} Plus Animal RNA Spin Column Extraction Kit (Beyotime, China). The RNA solution was measured for absorbance at 260 and 280 nm. RNA is considered to be of high purity when the A260/A280 ratio is between 1.7 and 2.1. We used 0.05−5 μg of RNA to obtain a cDNA library through the reverse transcription process by TransScript First-Strand cDNA Synthesis SuperMix Kit (TransGen, China). To amplify the cDNA of *K-Ras*, *Raf-1*, *B-Raf*, *Erk1*, and *Erk2*. The primer sequences are as follows with *GAPDH* as the internal control:

K-Ras, 5′-GCGCTGACCTAGGAATGTTG-3′,
and 5′-AGGAGTAGTACAGTTCATGAC-3′;
Raf-1, 5′-CATCAATGGAGCACATACAG-3′,
and 5′-AGGCAGTCATGTAAGCTCAT-3′;
B-Raf, 5′-ATTGTTACCCAGTGGTGTGAG-3′,
and 5′-TCTTGAGGTCTCTGTGGATG-3′;
Erk1, 5′-CCAAGTCAGACTCCAAAGCC-3′,
and 5′-GGTCATAGTACTGCTCCAGG-3′;
Erk2, 5′-GAAGCACCATTCAAGTTCGAC-3′,
and 5′-AAGATCTGTATCCTGGCTGG-3′;
GADPH, 5′-CTTTGGTATCGTGGAAGGACTC-3′,
and 5′-GTAGAGGCAGGGATGATGTTCT-3′.

Western Blot Analysis. Cells in the blank control group were given complete medium. Cells in the un-amputated group

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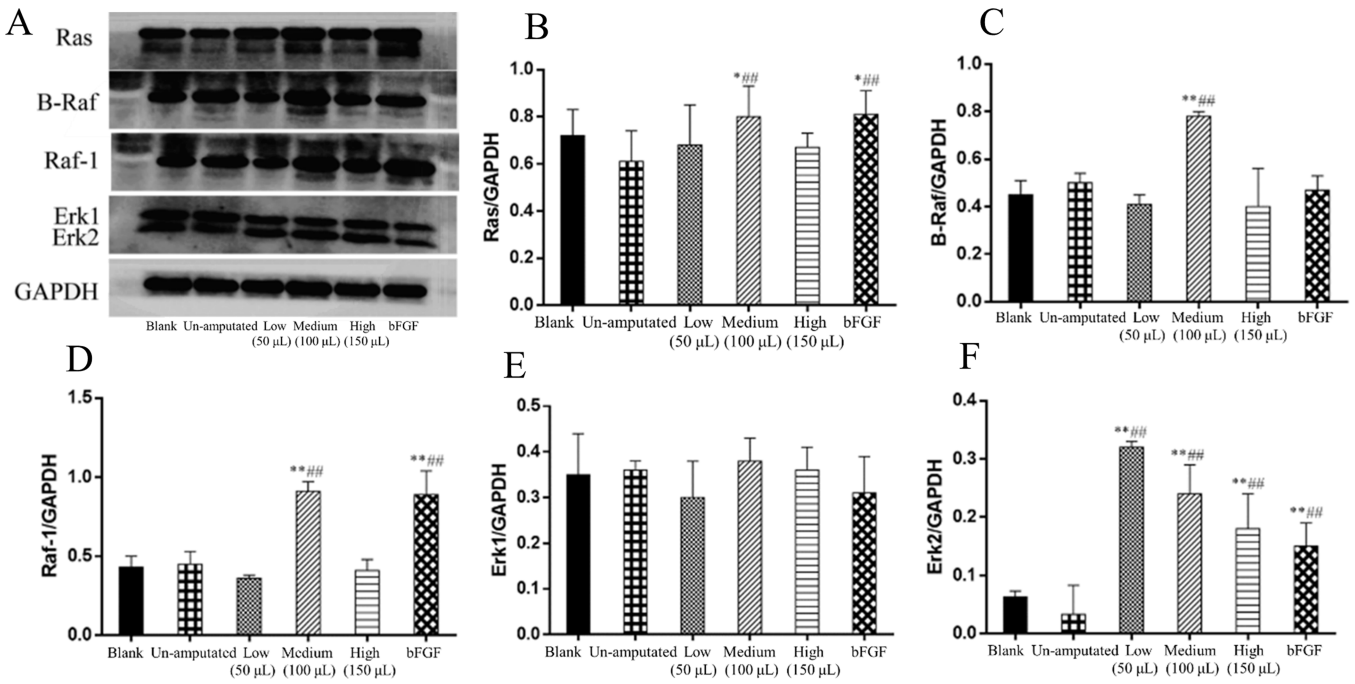

Figure 3. The proteins from tail-amputated *E. foetida* up-regulate the Ras/Raf/MEK/ERK signaling pathway in rats' skin wounds. (A) Representative Western blot analysis of Ras, B-Raf, Raf-1, Erk1, Erk2, and GAPDH in rats. (B−F) Densitometric results of Ras, B-Raf, Raf-1, Erk1, Erk2, and GAPDH in wounds as determined by Western blot. The results are presented as mean ± S.E.M. (n = 6). Compared with the blank control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the un-amputated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

were given complete medium with the un-amputated earthworm proteins (1 μg/mL). Cells in the low-, medium-, and high-dose groups were given complete medium with the tail-amputated earthworm proteins (0.8, 1.0, and 1.2 μg/mL). Cells in the positive control group were given complete medium with bFGF (0.1 μg/mL). After treatment for 48 h, those cells were harvested for Western blot analysis.

Proteins of rat skin wounds were extracted at day 10 post-wounding. The BCA method was used to measure protein concentrations. Equal amounts of protein samples were loaded on SDS-PAGE gels and then transferred onto nitrocellulose membranes. After blocking for 1 h and washing with Tris-buffered saline with Tween (TBST) for three times, the membranes were incubated at 4 °C for 20−22 h with primary antibodies anti-K-Ras (1: 5000, Proteintech, China), anti-B-Raf (1: 5000, Abcam, USA), anti-Raf-1 (1:1000, CST, USA), anti-Erk1/2 (1:2000, Proteintech, China), and anti-GAPDH (1:10000, Proteintech, China). The membrane was placed in TBST and shaken slowly to wash three times for 10 min. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody solution (1:10000, Proteintech, China) in the dark at room temperature for 1 h. The membranes were washed with TBST. The chemiluminescence signals were visualized and the blots quantified.

Statistical Analysis. SAS 7.0 statistical software was used to perform statistical analysis on the data. One-way ANOVA analysis, LSD test, and T test were used for the comparison of means between multiple groups, uniform variance, and uneven variance, respectively. $P < 0.05$ was considered to be statistically significant.

RESULTS

The Proteins from Earthworm Promoted Skin Wound Healing in Rats. To evaluate the therapeutic efficacy of the proteins from tail-amputated *E. foetida* on skin wound healing, we generated full-thickness skin excision wounds on rats. As shown in the images, wound area decreased over time (Figure 1A). On day 14, wound areas treated with the proteins from tail-amputated *E. foetida* were approximately healed with small scars and soft texture. The skin wound healing rate of rats increased time-dependently (Figure 1B). On day 3, the wound area of rats in the tail-amputated group diminished significantly by 20%, while those in the blank control group were generally unchanged. On day 5, the wound healing rate of rats in the treated groups (un-amputated and tail-amputated groups) was highly improved, with their wound healing rates reaching 40 and 50%, respectively. The wounds of rats in the tail-amputated group were approximately closed on day 10. By the end of the 21-day period, skin wounds in all groups were completely healed. Eventually, the skin structure returned to normal and was similar.

Histopathological study by HE staining can visualize the wound morphology in each treated group. As shown in Figure 1C, the wound gap, which represented re-epithelialization ability, was calculated in each group via measuring the distance between two epithelial layers. Wound closure was one of the indicators of wound healing. We then further observed the microscopic details and material change of skin wound.

Fibroblasts as well as capillaries showed more change in the tail-amputated group than in other groups at day 14 post-wounding, further proving the accelerated physiological function in ES2 treatment (Figure 1D). In addition, the results of Masson staining showed obvious collagen fibers in the dermis (Figure 1E). Compared with the blank control and un-amputated groups, the area and number of collagen fibers in the injured area of the dermis increased in the tail-amputated group, with the collagen fibers arranged parallel and close in this group. In the early stage of skin wound healing,

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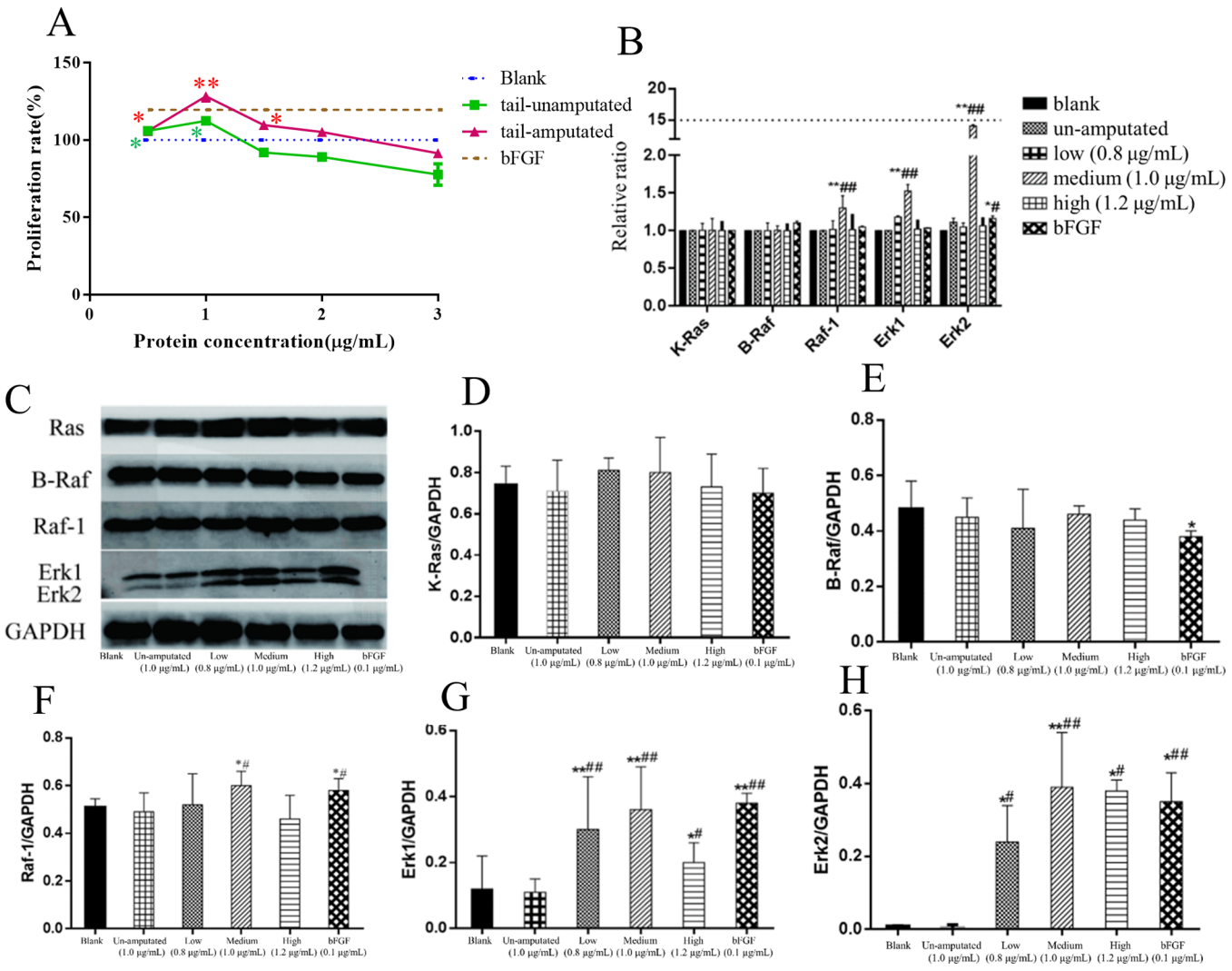

Figure 4. The proteins from tail-amputated *E. foetida* up-regulate the Ras/Raf/MEK/ERK signaling pathway in NIH3T3 cells. (A) Effects of the proteins on proliferation activity of NIH3T3 cells. (B) Representative real-time qPCR of *K-Ras*, *B-Raf*, *Raf-1*, *Erk1*, and *Erk2* in cells. (C) Representative Western blot analysis of expression of the Ras/Raf/MEK/ERK signaling pathway protein in NIH3T3 cells of each group. (D−H) Densitometric results of Ras, B-Raf, Raf-1, Erk1, Erk2, and GAPDH in cells as determined by Western blot. The results are presented as mean ± S.E.M. (n = 6). Compared with the blank control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the un-amputated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

the deposition of ECM was accelerated in the tail-amputated group, which contributed to the rapid healing. As time went by, wound healing slowed down and the ratio of type I/III collagen with two colors in the tail-amputated group reduced significantly (Figure 1F). The proteins from tail-amputated earthworm had multiple effects in different periods on cells and ECM in the dermis.

Effect of the Proteins from Earthworm on PDGF, TGF-β, and HYP Secretions. To investigate factors that were involved in the wound healing process, we analyzed the expression of PDGF, TGF-β, and HYP in the wound area. The PDGF (Figure 2A,C) and TGF-β (Figure 2B,D) in the tail-amputated group on day 7 were the highest compared with the blank control group. However, these growth factors were decreased dramatically and lower than that in the blank control group on days 14 and 21. The results revealed that the proteins from tail-amputated earthworm increased the PDGF and TGF-β in the early stage of healing without excessive accumulation in the late period. This observation was coincident with the result in our previous studies.[10] HYP reflected the collagen metabolism of connective tissue. Increased levels of HYP indicated increased synthesis and accumulation of collagen in tissues. As shown in Figure 2E, the HYP expression in the treated groups on days 7 and 14 was higher than that in the blank control group, while this accumulation decreased on day 21. This phenomenon indicated that the proteins from tail-amputated earthworm heal wound without scar formation.

The Proteins from Earthworm Up-Regulate the Ras/Raf/MEK/ERK Signaling Pathway. The Ras/Raf/MEK/ERK signaling pathway is widely involved in the regulation of cell growth, proliferation, differentiation, and many other physiological and pathological processes. To better understand the mechanism of skin wound healing after treating with tail-amputated earthworm, the expression of proteins associated with the Ras/Raf/MEK/ERK signaling pathway was evaluated on the wound area in the tail-amputated group. As shown in Figure 3A, the Ras/Raf/MEK/ERK signaling pathway in skin tissues was affected by the proteins from tail-amputated earthworm. The expressions of B-Raf, Raf-1, and Erk2 in the medium-dose proteins (100 μL, 1.12 mg/mL) from the tail-

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amputated earthworm group were increased significantly (Figure 3C,D,F). The medium-dose group displayed similar Ras and Raf-1 expressions as the positive control group (0.1 μg/mL bFGF) (Figure 3B,D). However, as shown in Figure 3E, Erk1 expression showed no significant difference in each group.

To further analyze the wound healing mechanism of tail-amputated earthworm correlated with the Ras/Raf/MEK/ERK signaling pathway, we evaluated the expression of those mRNAs and proteins in vitro using the NIH3T3 cell line. As shown in Figure 4, the results indicated that the proteins had impacts on NIH3T3 and the Ras/Raf/MEK/ERK signaling pathway. The proteins from tail-amputated earthworm enhanced cell viability and promoted cell proliferation especially on 1 μg/mL (Figure 4A). The mRNAs of *Raf-1* and *Erk1/2* increased significantly in the 1 μg/mL proteins from the tail-amputated earthworm group, reaching the ratio of 1.3, 1.5, and 15 to blank control (Figure 4B). The mRNA of *B-Raf* in the bFGF group increased slightly, while the mRNA of *Erk2* in the medium group was the highest among all groups (Figure 4B). As to protein expression in this pathway, Raf-1 and Erk1/2 in the bFGF and 1 μg/mL tail-amputated earthworm groups were similar to those in vivo results (Figure 4F−H). The K-Ras and B-Raf in groups were similar (Figure 4D,E). Based on the detected protein, the effects of tail-amputated earthworm proteins on the Ras/Raf/MEK/ERK signaling pathway were analyzed. We identified the concentration that showed the most significant effect. These results obtained in vitro did not exactly correspond to animal experiments, as other unknown influencing factors were involved in the regulation of the skin wound healing process in vivo. Therefore, further studies need to be conducted to precisely reveal this mechanism.

DISCUSSION

Protein products will contribute to the treatment for emergency skin wound immediately.[29] Based on the proteins extracted from *E. foetida* that had positive effects on skin wound healing, we further discovered that the therapeutic effect of tail-amputated earthworm was better than that of the un-amputated one. In our previous studies, we discovered higher bFGF expression in skin wounds after treating with tail-amputated earthworm homogenate, indicating the therapeutic mechanism for this extract.[20] Apart from bFGF, several other factors including PDGF, TGF-*β*, HYP, and collagen are crucial for wound healing and scar formation. PDGF is a basic protein stored in platelet alpha particles, which adheres to damaged parts of blood vessels and promotes wound healing. It is a strong mitogen and is mainly secreted by platelets, macrophages, and keratinocytes. It can significantly promote the growth and proliferation of fibroblasts.[30] PDGF and its preparations have a good therapeutic effect on promoting wound healing.[31] TGF-*β* plays an important regulatory role in some biological activities and is involved in various studies, including skin fibrosis.[32,33] There is less TGF-*β* in chronic hard-to-heal skin wound tissue than in normal tissue.[34] The effect of TGF-*β* on the proliferation of epidermal cells is bidirectional. It inhibits cell proliferation in vitro and promotes cell proliferation in vivo; it can promote the synthesis of ECM and induce the formation and change of granulation tissue. The increased synthesis of TGF-*β* causes the increase in collagen levels and abnormal metabolism, which eventually leads to hypertrophic scars. HYP accounts for 13.4% of collagen; there was little or no HYP expression in elastin and other proteins. However, collagen is mostly distributed in the skin, blood vessels, etc., and the distribution location is limited. Therefore, HYP can reflect the collagen metabolism of connective tissue. The increase of the HYP level in the wound indicates the increase of collagen synthesis and accumulation in the wound tissue. Collagen plays an extremely important role in the speed and quality of healing. The collagen and its proportion in wound tissue are related to the quality of wound repair. The collagen involved in wound repair is mainly type I and type III collagen. Type I collagen acts as a scaffold. Type III collagen determines the diameter and elasticity of collagen fibers. The ratio of the type I and III collagen is crucial to the quality and results of wound repair; the imbalance of collagen metabolism is one of the reasons for the slow wound healing.[35] Increasing the ratio of type III collagen is an important reason that affects the quality of wound healing and reduces scars.

The results of factors in each group showed a similar trend of change. Growth factors play an important role in tissue repair and tissue regeneration after skin wound.[36] The proteins had an effect on the changes of growth factors, but they showed fluctuations of up and down compared to the blank control group. Therefore, we speculated that there were other mechanisms for the protein effect.

The Ras/Raf/MEK/ERK signaling pathway is one of the mitogen-activated protein kinase (MAPK) signaling pathways. The MAPK pathway is associated with diseases including excessive fibrosis of human body tissues.[37] Substances acting on the RAS/MAPK pathway can be designed for the purpose of curing diseases and improving health.[38] *Ras* is a proto-oncogene of the GTP/GDP binding protein. It is located upstream in the Ras/Raf/MEK/ERK pathway. There are three *Ras* genes encoding four highly homologous proteins with a molecular weight of 21 kDa: N-Ras, H-Ras, K-Ras4A, and K-Ras4B.[39] Raf is an important factor in the Ras/Raf/MEK/ERK pathway, which connects the Ras enzyme and MAPK cascade reaction and is considered to be a molecular switch that controls growth and differentiation signal transduction pathways. Raf is a serine/threonine protein kinase with three main subtypes: A-Raf, B-Raf, and Raf-1. Raf can phosphorylate and initiate the downstream MAPK cascade after receiving activation of activated Ras. Erk1/2 plays a major role in the signal pathway to mediate cell activities. The relationship between the genes in the Ras/Raf/MEK/ERK pathway is as follows: when the extracellular stimulus signal is transmitted to the cell, when Ras receives the signal, it converts to activated Ras and then activates Raf. Raf activates MEK to phosphorylate it to p-MEK. p-MEK further phosphorylates Erk1/2. Phosphorylated Erk1/2 enter into the nucleus. The cascade reaction transmits the extracellular signals to the nucleus through the cell membrane and cytoplasm. The ERK pathway drives cell division by acting throughout the cell cycle.[40] Studies have shown that down-regulation of Erk1 in fibroblasts leads to an increase in Erk2-dependent signaling pathways and cell proliferation, while down-regulation of Erk2 can completely inhibit cell proliferation. Substances inhibit cell proliferation, migration, and invasion by inhibiting the MEK/ERK pathway and downstream key proteins.[41]

CONCLUSIONS

In summary, the proteins from tail-amputated *E. foetida* have the effect of promoting skin wound healing. The effect is

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related to many factors including the activation of the Ras/Raf/MEK/ERK signaling pathway. The proteins from tail-amputated *E. foetida* have a therapeutic potential for treating skin wounds.

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Notes

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