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Original Research

Concentrated Growth Factor Promotes Wound Healing Potential of HaCaT Cells by Activating the RAS Signaling Pathway

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Abstract

Objective: The aim of this study was to explore the effect of concentrated growth factor (CGF) on the wound healing potential of human epidermal cells (HaCaT) *in vitro* and *in vivo*. **Methods**: CGF was extracted from venous blood using the centrifugal separation method. The CGF-conditioned medium was prepared from CGF gel immersed in Dulbecco's Modified Eagle medium. Crystal violet staining and wound healing assay were used to evaluate the proliferation and migration of HaCaT cells, respectively. Lipopolysaccharide (LPS) was used to test the anti-inflammatory function of CGF. An ELISA kit was employed to detect the concentration of growth factors and interleukins in CGF medium. mRNA and protein levels of angiogenic biomarkers (Angiopoietin-1 (ANGPT-1), vascular endothelial growth factor-A (VEGF-A) and Angiopoietin-2 (ANGPT-2)) were determined by quantitative polymerase chain reaction (qPCR) and Western blot, respectively. A dorsal excisional wound model was recruited to test the wound healing effect of CGF in mice. **Results**: Three-day treatment of HaCaT cells with CGF significantly promoted cell proliferation, which was followed by an increase in Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) levels in the medium. Cytokines (IL-6, IL-8 and TNF-α) were increased in LPS-stimulated HaCaT cells after 3 days, and CGF slightly inhibited the mRNA expression of these cytokines. The RAS signaling pathway was activated upon CGF treatment. Both RAS knockdown and an inhibitor of RAS (zoledronic acid) could block the migration of HaCaT cells after CGF treatment. Protein expressions of CD31, ANGPT-1, and VEGF-A were up-regulated in a dose-dependent manner upon CGF exposure. The protein level of ANGPT-2 was down-regulated after CGF treatment. CGF could promote wound healing *in vivo*, as demonstrated using the full skin defect model in nude mice. **Conclusions**: CGF was shown to promote wound repair *in vitro* and *in vivo*. The RAS cell signaling pathway was responsible for CGF stimulating the wound healing potential of HaCaT cells.

Keywords: concentrated growth factor; wound healing; RAS signaling pathway; HaCaT cell

1. Introduction

Based on the healing theory of a slightly-humid environment and guided by the TIME mode, the clinical treatment of chronic wounds using debridement technology, negative pressure wound therapy, and multifunctional dressings has significantly improved wound healing rate, reduced amputation rate as well as fatality rates [1]. Despite great progress having been made in skin wound care in the past decades, many challenges still remain in clinical practice. With the acceleration of population aging process and the increasing incidence of diabetes as well as kinds of ulcers, chronic wounds exert huge pressure on public health services in both developed and developing countries [2]. It has been estimated that 2.4–4.5 million people suffer from chronic wounds, which cost over 31.7 billion dollars annually in the United States [3]. Therefore, novel biomaterials and active growth factors have been developed as potential and effective alternatives for skin wound healing in the clinic.

As the first physiological line of defense, skin is constantly exposed to potential injury. Wound healing is a conservative, complicated and multicellular biological process that retains skin integrity after trauma [4]. It is a sequential yet overlapping multiphase process consisting of hemostasis, inflammation, proliferation, and remodeling [5]. Chronic wounds generally are stalled in the inflammation phase, during which macrophages fail to turn from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype [6]. The overexpressions of pro-inflammatory cytokines secreted by macrophages induce the influx of neutrophils, which further release metalloproteinases and elastases [3]. These abnormalities indeed impede wound healing activity and several mechanisms account for the dysregulated tissue repair including loss of endogenous extracellular matrix (ECM), impaired growth factor activity and bacterial infection [3]. Consequently, growth factors promoting tissue repair have been established in numerous studies [7].

Different growth factors have been found to be effective in promoting the wound healing process, including epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor-A (VEGF-A), and platelet-derived growth factor (PDGF) [8,9]. These growth factors are key to wound closure, because they participate in the regulation of cell communication and cellular responses which trigger the proliferation, migration, and differentiation of damaged cells, as well as events that occur during neovascularization, in the ECM and inflammatory response homeostasis [10].

In this study, we used concentrated growth factor (CGF), which belongs to the third generation of blood extractions following platelet-rich plasma (PRP) and platelet-rich fibrin (PRF). It has been well known for some time that PRP can be used therapeutically to promote wound healing in several scenarios [11–13]. It has been demonstrated in several studies that CGF plays an important role in the healing of difficult wounds, which is thought to be related to the high level of growth factors that CGF contains [14,15]. However, the underlying mechanism is still unclear. To address this issue, the HaCaT cell line was selected and subsequently subjected to CGF exposure to test its effective roles in wound healing *in vivo* and *in vitro*, which may provide a potential therapeutic alternative to chronic wound healing.

2. Materials and Methods

This study was performed according to the principles recommended for the experimentation on humans and animals determined by the Institutional Review Board of Guangdong Medical University. Ethics committee approval was also obtained (No. GDY2102255). All enrolled subjects were informed about the procedures and objectives of the study before signing a consent form.

2.1 Cell line and Cell Culture

An immortalized human epidermal cell line (HaCaT) was purchased from the American Type Culture Collection (Manassas, VA, USA). It was cultured in Dulbecco's modified Eagle medium (DMEM) medium (Life Technologies, Carlsbad, CA, USA) enriched with 10% fetal bovine serum (FBS) (Gibco, Australia) in an incubator which provides a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2 CGF Preparation

Venous blood was collected from four healthy adult volunteers of East Asian origin consisting of three males and one female. One sterile additive-free Vacuette tube containing autologous venous blood (9 mL) was centrifuged immediately in a fixed-program device (Silfradent, Forli, Italy). CGF clots were suspended in the middle layer in the Vacuette tube, and placed on gauze to eliminate excess serum and transferred to another tube for freezing. The frozen CGF clots were minced, homogenized, and placed in a refrigerator (–80 °C) for 1 hr, following with centrifugation (3000 g, 10 min) at room temperature. The supernatants, dissolved in 9 mL FBS-free DMEM medium, were considered as 100% CGF medium and stored at –80 °C for future usage. The final CGF concentrations (2%, 5% and 10%) in medium, which was determined in a previous study [16], was calculated based on the CGF volume that was added to the total volume of the culture medium without FBS and administered to the experimental arms. The control group only received DMEM medium.

2.3 Western Blot Analysis

We collected, washed and then resuspended the treated cells in NP-40 lysis buffer (Beyotime Biotechnology, Cat# P0013F, Shanghai, China) containing a protease inhibitor to prevent degradation for 1 hr. A tissue protein extraction reagent kit (Thermo, Cat# 78510, CLD, USA) was employed to extract the excision wound model samples. After complete reaction, samples were centrifuged at 13,000 rpm, 4 °C for 30 min and supernatants were transferred to new protease-free tubes. Protein concentration was measured employing the Bradford Protein Assay Kit (Bio-Rad, Berkeley, CA, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with an 8%–15% gradient was used to load protein samples (30–60 μg) for separation and subsequently electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Cat# IPFL00010, Tullagreen, Carrigtwohill, Ireland). The membrane was immersed in 5% non-fat dry milk dissolved in Tris-buffered saline with Tween 20 TBSTfor blocking, following with incubation with primary antibodies (Table 1) at 4 °C overnight. The membrane was washed 3 times for 10 min each time with PBST. Horseradish peroxidase (HRP)-conjugated secondary antibodies were added to the membrane and incubated for 1 hr at room temperature. Signal detection was performed by applying an enhanced chemiluminescence kit (Thermo Scientific Pierce, USA). Quantification was conducted using Image J software 1.42q (National Institutes of Health, Bethesda, MD, USA).

2.4 Analysis of Cell Viability

The cells were cultured in a 96-well plate at a moderate density (approximately 10^4 per well). Treated cells were exposed to 10 μL of WST-8 reagent (CCK8 kit, HY-K0301, MedChemExpress) followed by incubation for 2 hr. Optical density was read at 450 nm by using a Multimode Plate Reader (Perkin Elmer Victor Nivo, Pontyclun, UK).

2.5 RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) Assay

The TRIzol reagent bought from Invitrogen was used for RNA extraction from harvested samples. Briefly, the TRIzol reagent and chloroform were added orderly and vortexed vigorously for thorough reaction. After incubation

Table 1. List of primary antibodies used in the study.

Name	Species	Manufacture	Cat#	Molecular weight (kDa)	Dilution factor
RAS	Rabbit	Cell Signaling Technology	3965	21	1:1000
p-C-RAF	Rabbit	Cell Signaling Technology	9427	74	1:500
C-RAF	Rabbit	Cell Signaling Technology	9422	74	1:1000
p-ERK	Rabbit	Cell Signaling Technology	4370	44	1:1000
ERK	Rabbit	Cell Signaling Technology	4695	44	1:1000
CD31	Rabbit	Cell Signaling Technology	3528	130	1:500
ANGPT-1	Rabbit	Abcam	ab183701	70	1:1000
ANGPT-2	Rabbit	Abcam	ab36014	66	1:1000
VEGF-A	Rabbit	Abcam	Ab46154	26	1:1000
β-Actin	Mouse	Beyotime Technology	AA128	42	1:2000

for 15 min, samples were centrifuged at 12,000 g for 15 min at 4 °C. The upper colorless aqueous phase was transferred to an RNase-free tube and isopropanol (200 μL per tube) was added to precipitate RNA. The mixture was incubated for another 10 min at room temperature, followed by centrifuging at 12,000 g for 15 min at 4 °C. The pellets in the bottom of tube were saved as RNA and re-dissolved in Diethyl Pyrocarbonate (DEPC)-treated water. Reverse transcription of RNA was conducted using PrimeScript RT Master Mix (Takara, Cat# RR036A, Dalian, China) according to the manufacturer's instructions. RT-qPCR assay was performed with the TB Green Premix Ex Taq kit (Takara, Cat# RR420A) based on the manufacturer's protocol. The relative fold gene expression in the samples was based on $2^{-\Delta\Delta CT}$ method as previously described [17]. The universal primers detected in this study were as follows:

IL-6-F: 5'-CCGCTCGAGAGGAGCCCAGCTATGAACTC-3',
IL-6-R: 5'-CCGGAATTCGACCAGAAGAAGGAATGCCC-3',
IL-8-F: 5'-GAATGGGTTTGCTAGAATGTGATA-3',
IL-8-R: 5'-CAGACTAGGGTTGCCAGATTTAAC-3',
TNF-α-F: 5'-CCTGCCCCAATCCCTTTATT-3',
TNF-α-R: 5'-CCCTAAGCCCCCAATTCTCT-3',
GAPDH-F: 5'-GGTGGTCTCCTCTGACTTCAACA-3',
GAPDH-R: 5'-AGCTTGACAAAGTGGTCGTTGAG-3'

2.6 Wound Healing Assay for HaCaT Cell Migration

HaCaT cells were cultured in six-well plates at a moderate cell density (5×10^5 per well) and allowed to develop cell monolayers within 24 h. A 200-μL sterile plastic tip was employed to make a wound line across the surface of plates, and the suspended and scratched cells were removed with PBS. The cells were re-cultured in reduced-FBS DMEM medium in a humidified incubator containing 5% CO_2 at 37 °C for 48 hr. Subsequently, photos were captured with a phase-contrast microscope and from three independent experimental replicates.

2.7 Transwell Assay for HaCaT Cell Migration and Invasion

Around 1×10^5 HaCaT cells were added to the upper chamber (covered with Matrigel, BD Bioscience, Bedford, MA, USA) of a transwell insert (Cat# 3428, Corning, NY, USA) in serum-free medium. And DMEM medium enriched with 20% FBS was loaded in the lower chamber as a chemoattractant. After general incubation for 48 hr, the transwell chambers were opened, the medium in each well was discarded, and the cells were washed with calcium-free PBS. Subsequently, the cells were fixed with methanol for 30 min following with stained with crystal violet (0.1%) for 20 min. The upper unmigrated cells were gently wiped off using a cotton swab, and the cells passed through the filter were photographed by an inverted fluorescence microscope.

2.8 Short Hairpin RNA (shRNA) Transfection

Knockdown RAS was performed by recruiting a lentiviral particles kit purchased from Santa Cruz (Cat# sc-29340-V, DAL, USA). The step was carried out according to the manufacture's protocol. In brief, the cells were pretreated with polybrene (5 μg/mL) before incubation with lentiviral particles for 24 hr. The cells were further incubated with a complete fresh medium for 48 hr after the removal of unbound viral particles. A stable RAS-silenced cell line was selected by using puromycin dihydrochloride (10 μg/mL). The protein level of RAS was tested by Western blot after cell viability confirmation by CCK8 assay.

2.9 Excision Wound Model

BALB/c-nu nude mice (Laboratory Animal Centre of Guangdong Province, Guangzhou, China) were assigned into two groups randomly. The first group (n = 8) was received saline (0.9%) only. The second group (n = 8) was given CGF gel. The animal research study was conducted in compliance with guidelines approved by the Animal Use and Care Administrative Advisory Committee at Shenzhen Longhua Central Hospital, Guangdong Medical University. The full-thickness excision wound model was generated in this study for the evaluation of wound healing performance of the CGF gel. All mice were anesthetized with isoflurane, and the wound area was sterilized with 70% ethanol. A 10 mm biopsy punch was used to cut out a full-thickness skin with a 10 mm diameter. Images of the wound were

captured by a professional camera immediately. The mice were housed individually to avoid additional effects on the wound. Changes in the wound site were recorded on days 2, 4, 6, and 8. Wounds areas were selected after anesthesia for histological analysis. The wound healing rate was measured using the following equation:

Wound healing (%) = $(A_n / A_0) \times 100$

(A_0: the original wound site; A_n: the wound site at day 2, 4, 6, and 8 time points).

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α in cell supernatants were quantified with commercially available ELISA kits (Abcam, Cambridge, UK). The procedure was carried out according to the manufacture's directions. The measurement ranges were TNF-α: 15.63–1000 pg/mL, IL-6: 7.8–500 pg/mL, and IL-8: 3.91–250 pg/mL. Measured data below the limit of detection were considered as zero. Recombinant human IL-6, IL-8 and TNF-α were used as standards to calculate the interleukin concentrations accordingly. The absorbance was measured at 450 nm (BMG LabtechGmbH, Ortenberg, Germany).

2.11 Statistical Analysis

All data were obtained from at least three to four independent experiments and were expressed as the mean $\pm$ standard deviation (SD). One-way ANOVA statistics was employed for multiple groups, and the Student's two-tailed unpaired *t*-test was also carried out for variables measured at a single time point where appropriate by Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 CGF Induced the Proliferation of HaCaT Cells with FGF and VEGF Accumulation

HaCaT cells were exposed to different concentrations of CGF (0, 2%, 5% and 10%) for 3 days, and crystal violet staining was used to test cell viability. As shown in Fig. 1A,B, CGF promoted HaCaT cell growth in a dose-dependent fashion. CGF contained mounts of growth factors and the levels of VEGF and FGF in the CGF medium were significantly up-regulated upon CGF treatment (Fig. 1C,D). A total of 5% CGF was considered as appropriate concentration to perform the following study.

3.2 CGF Stimulated the Migration and Invasion of HaCaT Cells

Cell migration is one of the key aspects in revascularization. The wound healing assay indicated that HaCaT cells had a higher migration rate after CGF treatment for 24 hr and 48 hr. The wound healing rate of HaCaT cells treated with CGF for 24 hr and 48 hr was increased sharply to 47.2 $\pm$ 6.3% and 84.7 $\pm$ 7.6%, respectively, compared to that of untreated cells (23.3 $\pm$ 13.4% and 19.3 $\pm$ 14.5%). Representative pictures are shown in Fig. 2A. Meanwhile, the

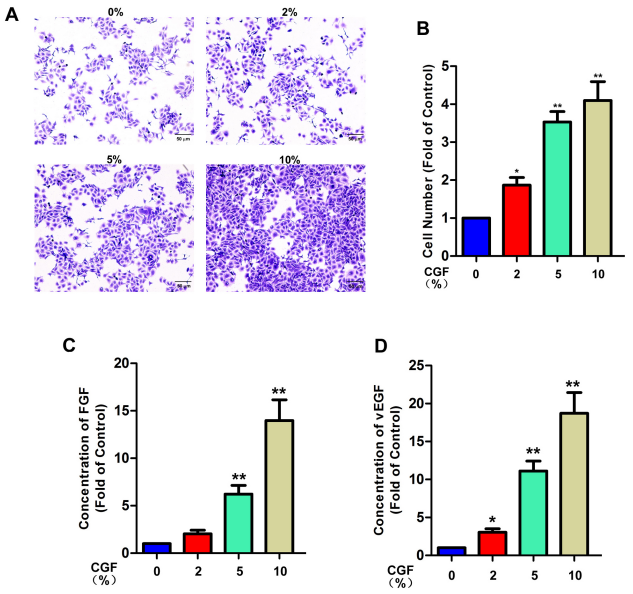

Fig. 1. The promoting effect of CGF on the proliferation of HaCaT cells. (A) Crystal violet staining was used to test the proliferation of HaCaT cells upon CGF exposure for 3 days. (B) The comparison of cell numbers in each group. (C,D) ELISA kits were used to test FGF and VEGF in the medium after cells were exposed to different concentrations of CGF for 3 days. Data are displayed as the mean $\pm$ S.D. from three independent assays. *$p < 0.05$ and **$p < 0.01$ compared with the control group.

transwell assay also indicated HaCaT cells exposed to CGF treatment were more invasive (OD values, 0.22 $\pm$ 0.017 compared with 0.10 $\pm$ 0.009) as shown in Fig. 2B.

3.3 Effects of CGF on the Levels of Inflammation-Related Cytokines in Cell Culture Supernatant with or without Lipopolysaccharide (LPS)

LPS (Escherichia coli O55:B5) is a well-known tool to activate cellular inflammation. As displayed in Fig. 3, the level of cytokines was significantly accumulated after LPS treatment for 1 and 3 days, and CGF could weakly inhibit the concentrations of cytokines after 1 and 3 days. The effect of LPS and CGF on HaCaT cell proliferation is demonstrated in **Supplementary Fig. 1**.

3.4 CGF Activated the RAS Cell Signaling Pathway

HaCaT cells were exposed to different concentrations of CGF for 3 days. RAS, p-C-RAF and p-ERK were up-regulated in a dose-dependent fashion, as displayed in Fig. 4A,B. HaCaT cells were transfected with RAS lentiviral particles to knock down the endogenous expression of RAS. The wound healing assay indicated that silencing RAS could remarkably block the migration capacity of HaCaT cells. In detail, the wound healing rate of HaCaT dropped sharply from 46.1 $\pm$ 5.7% to 24.4 $\pm$ 3.9% after RAS silencing at 24 hr time point, while a similar phenomenon was observed in 48 hr as the wound healing rate

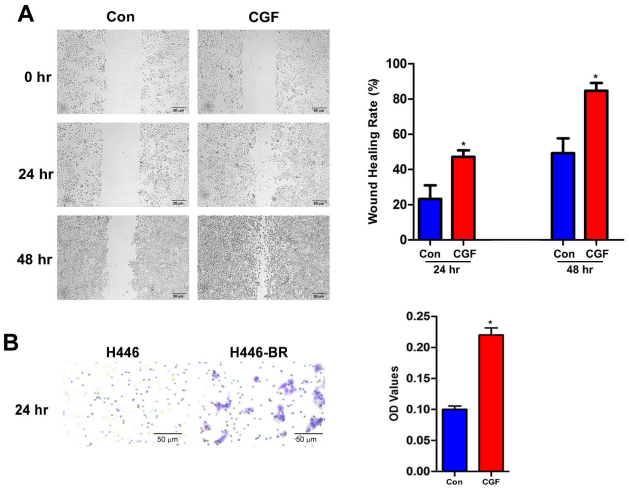

Fig. 2. CGF stimulated the migration and invasion ability of HaCaT cells. (A) Wound healing assay was employed to assess the migration of HaCaT cells after CGF at different time points (0, 24, 48 hr). (B) Transwell assay was recruited to determine the invasion ability of HaCaT cells at 24 hr after CGF treatment. OD values for the crystal violet-stained cells were analyzed using Image J software. All data are displayed as the mean ± S.D. from three independent assays. $*p < 0.05$ compared with the control group.

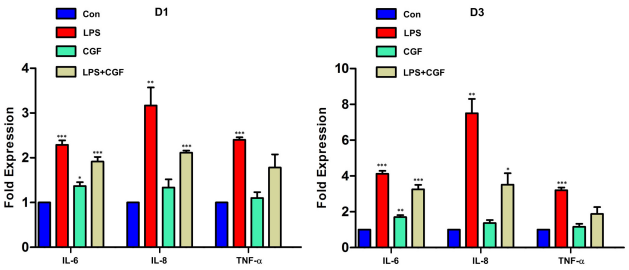

Fig. 3. Effects of CGF on the production of proinflammatory cytokines in LPS-stimulated HaCaT cells. HaCaT cells were exposed with or without LPS (1 μg/mL) in the presence of CGF for 1 and 3 days. Data are displayed as the mean ± S.D. from at least three independent experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the control group.

decreased from 79.2 ± 3.8% to 52.6 ± 7.1% (Fig. 4C,D). Additionally, RAS inhibitor zoledronic acid was employed to test the combined effect with CGF. As displayed in Fig. 4E,F, zoledronic acid blocked the migration of HaCaT cells after CGF treatment at both 24 hr and 48 hr time points. The wound healing rates at 24 hr in the control, zoledronic acid, CGF and combination group were 19.9 ± 10.1%, 17.2 ± 10.1%, 57.5 ± 6.6% and 33.6 ± 3.8%, respectively. Furthermore, the wound healing rates at 48 hr in corresponding groups were 46.7 ± 5.8%, 58.0 ± 8.4%, 95.0 ± 5.0%, and 75.9 ± 5.2%, respectively.

3.5 Angiogenic Biomarkers were Induced by CGF Treatment

Our results above indicated that CGF treatment promoted cellular migration ability, which is an essential factor in angiogenesis. We found that angiogenic biomarkers (CD31, Angiopoietin-1 (ANGPT-1) and VEGF-A) were up-regulated in a dose-dependent fashion upon CGF treatment, meanwhile ANGPT-2 was down-regulated after CGF exposure in protein level (Fig. 5A,B). Similarly, the mRNA levels of these biomarkers also displayed similar trends (Fig. 5C–F).

3.6 CGF Gel Promoted Excision Wound Healing in Vivo

Wound closure was significantly improved in mice receiving CGF gel treatment compared with that in mock-treated mice as displayed in Fig. 6A. Additionally, the wound closure rate of mice was increased after CGF treatment from 18.6 ± 7.4% (mock-treated control group) to 43.6 ± 6.8% (CGF gel treatment group) on day 4. The wound healing rates on day 6 in control group and CGF treatment group were 44.6 ± 10.8% and 73.1 ± 6.5%, respectively. On day 8, the corresponding data were 67.9 ± 7.1% and 95.0 ± 4.4%, respectively. The wound healing rates grew to 85.4 ± 8.0% and 98.3 ± 4.1%, respectively, on day 10 (Fig. 6B). The wound bed tissue on day 6 was collected to determine the levels of angiogenic biomarkers including Next, ANGPT-1, ANGPT-2 and VEGF-A. Consistent with our *in vitro* findings shown above, the mRNA expressions of ANGPT-1 and VEGF-A were sharply elevated, while the mRNA level of ANGPT-2 was demonstrated to be down-regulated (Fig. 6C–E). Similarly, the protein expressions of ANGPT-1 and VEGF-A were up-regulated, and those of ANGPT-2 were decreased (Fig. 6F).

4. Discussion

In this study, concentrated growth factor (CGF) was shown to effectively facilitate the proliferation as well as migration ability of HaCaT cells *in vitro* and excision wound model *in vivo*. The underlying mechanisms were elucidated to mainly involve activating the RAS signaling pathway and increasing the wound healing potential. Thus, this study provides novel findings on the therapeutic effects of CGF in wound healing.

CGF is considered as a novel natural biomaterial, which contains high levels of platelets, cytokines, as well as growth factors to promote wound healing activity; however little information has been obtained from skin wound healing studies [18]. Although CGF contains more growth factors compared with PRP and PRF, the best advantage of CGF might be the unique structure that provides a soft texture with elasticity that allows malleable shaping to form a three-dimensional structure consisting of fibrin to fill variable wound defects [19,20]. Several studies have demonstrated the characteristics of CGF by using scanning electron microscopy (SEM) analysis, which revealed that CGF

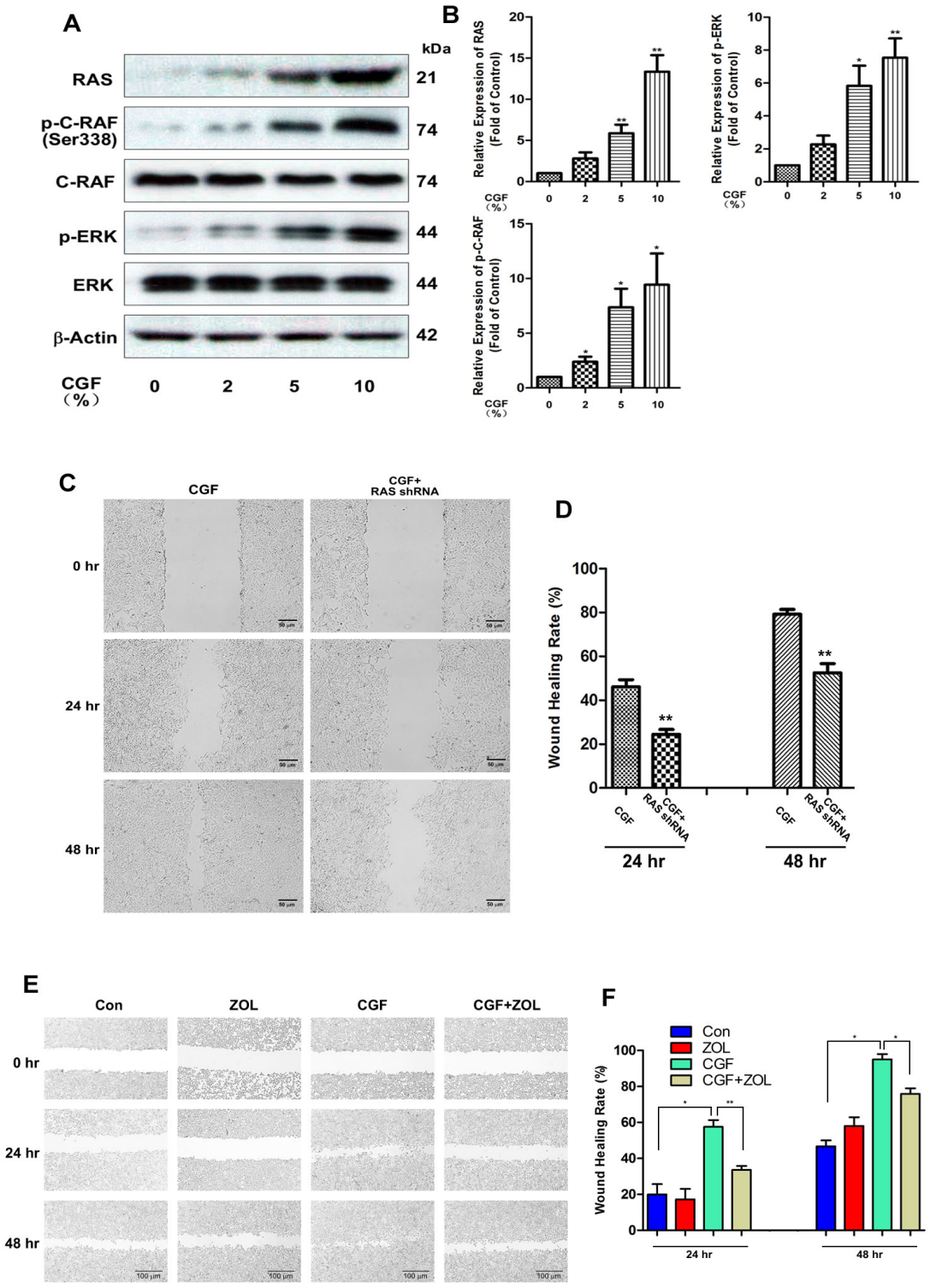

Fig. 4. The RAS cell signaling pathway was induced upon CGF treatment. (A) CGF-treated HaCaT cells were harvested at 3 days. RAS, p-C-RAF, C-RAF, p-ERK, ERK, and β-Actin were tested by Western blot. (B) Quantifications of RAS, p-ERK and p-C-RAF in different treatment groups were displayed by Graphad Prism software. (C) Wound healing assay was employed to assess the migration ability of CGF and CGF-silenced groups at 0, 24, and 48 hr. (D) Quantification of the wound healing rate in CGF and CGF-silenced groups. (E) The wound healing assay was employed to test CGF in cells incubated with or without the RAS inhibitor zoledronic acid (ZOL, 2 mM) at 0, 24, and 48 hr. (F) Quantification of the wound healing rate in different treatment groups. All data are shown as the mean $\pm$ S.D. from at least three independent assays. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the control group.

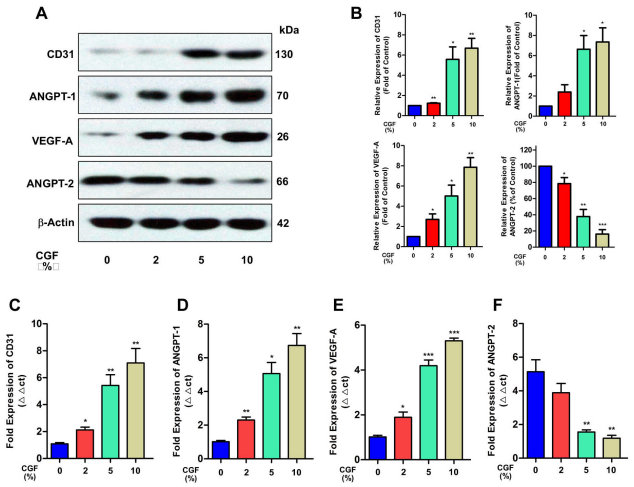

Fig. 5. Angiogenic biomarkers were activated upon CGF treatment. (A) CD31, ANGPT-1, VEGF-A, and ANGPT-2 were measured using western blottingafter cells were treated with different concentrations of CGF for 3 days. (B) Quantification of CD31, ANGPT-1, VEGF-A and ANGPT-2 in western blot were displayed. (C–F) qPCR was used to evaluate the expressions of CD31, ANGPT-1, VEGF-A, and ANGPT-2 in cells treated with different concentrations of CGF (0%, 2%, 5%, and 10%) for 3 days. All data are shown as the mean ± S.D. from at least three independent assays. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with the control group.

had a fiber-like appearance with a 0.1–1.0 μm pore. The diameter of the fibers was 0.36 ± 0.14 μm, and the percentage porosity was 40.44 ± 2.97% [21,22]. The dense random mesh-like network consisted of discrete fibers which support platelet aggregation [20]. This structure protects growth factors from proteolysis thus leading to a slow and sustained release of growth factors from fibrin mixtures. Besides, this bio-scaffold is ideal for promoting proliferation, migration, adhesion, and/or differentiation of the incorporated cells in tissue regeneration strategies.

In the present study, LPS alone was displayed to benefit the proliferation of HaCaT cells, and the up-regulated level in the combination arm was even higher, suggesting CGF could still promote the proliferation HaCaT cells under LPS-stimulated conditions (**Supplementary Fig. 1**). Meanwhile, CGF contains varying amounts of platelets, cytokines, fibrins, and growth factors such as VEGF, FGF, EGF, PDGF, insulin-like growth factor (IGF) and transforming growth factor beta 1 (TGF-β1), which facilitate angiogenesis, fibroblast proliferation, endothelial cell migration and proliferation, re-epithelialization and granulation tissue regeneration [21,23]. A previous study has reported that bioinspired hydrogels covered with basic fibroblast growth factor could greatly facilitate cell proliferation, wound re-epithelialization, as well as collagen deposition in dermal fibroblast cells [24]. Moreover, VEGF, TGF-β1, and its receptor played critical roles in a curcumin promoting human gingival fibroblast wound healing model [25]. Furthermore, Zhu *et al.* [26] demonstrated that roxadustat promoted angiogenesis via activating the HIF-1α/VEGF/VEGFR2 signaling pathway and accelerated cutaneous wound healing in diabetic rats. Therefore, the cytokines and growth factors released from CGF are vital elements in wound healing.

Moderate inflammation is essential in the wound healing process, and both CGF and LPS could lead to the production of proinflammatory cytokines and chemokines including IL-6, IL-8, and TNF-α, which trigger numerous immune responses in human dental stem pulp cells [23]. Consistently, we observed that IL-6, IL-8 and TNF-α were substantially boosted upon LPS stimulus, and CGF regulated the excessive LPS-induced inflammation to a normal level. This might account for the fact that patients with chronic wounds receiving CGF treatment had fewer hypertrophic scars after discharge [14]. However, more evidence is needed to support our viewpoint.

As a classic cell signaling pathway, the RAS/RAF/MEK/ERK (MAPK) cascade is vital for inter and intra-cellular communication, regulating fundamental cellular functions including proliferation, migration, survival, senescence, and differentiation [27]. The RAS family includes H-RAS, N-RAS, and K-RAS subtypes. Under normal conditions, RAS binds to GDP in an inactivated state. Subject to external stimulation with the guanine nucleotide exchange factor (GEF), RAS binds to guanosine triphosphate (GTP), thus becoming activated. GTP-RAS dimers/nanoclusters phosphorylate RAF, leading to the activation of downstream MEK/ERK. The activated MAPK cascade generates a response to regulate downstream cell signals [27]. EGF is one of the most important components in CGF, and triggers the RAS/RAF/MEK/ERK cascade through binding to its receptor located on the cell membrane. EGF signaling has a direct effect on promoting the migratory capacity of keratinocytes [28]. EGF encapsulation in gelatin-alginate coacervates could enhance efficacy in treating chronic diabetic wounds [29]. The FGFR1/ERK signaling pathway accounts for Kanglexin accelerating diabetic wound healing by promoting angiogenesis [30]. In our present study, we found that the RAS/RAF/MEK/ERK signaling pathway was activated upon CGF exposure, and the RAS inhibitor zoledronic acid stalled the migration of HaCaT cells after CGF treatment, which is consistent with our hypothesis. Therefore, the RAS/RAF/MEK/ERK cascade appears to play a critical role in CGF treatment for chronic wound healing and deserves in-depth study in the future.

As a fundamental physiological activity, wound healing could be divided into several stages including immediate hemostasis, acute inflammation, proliferation and maturation [31]. Angiogenesis is a vital accompanying process in the proliferation phase to form new blood vessels, which provide essential nutrients and oxygen for cell prolifera-

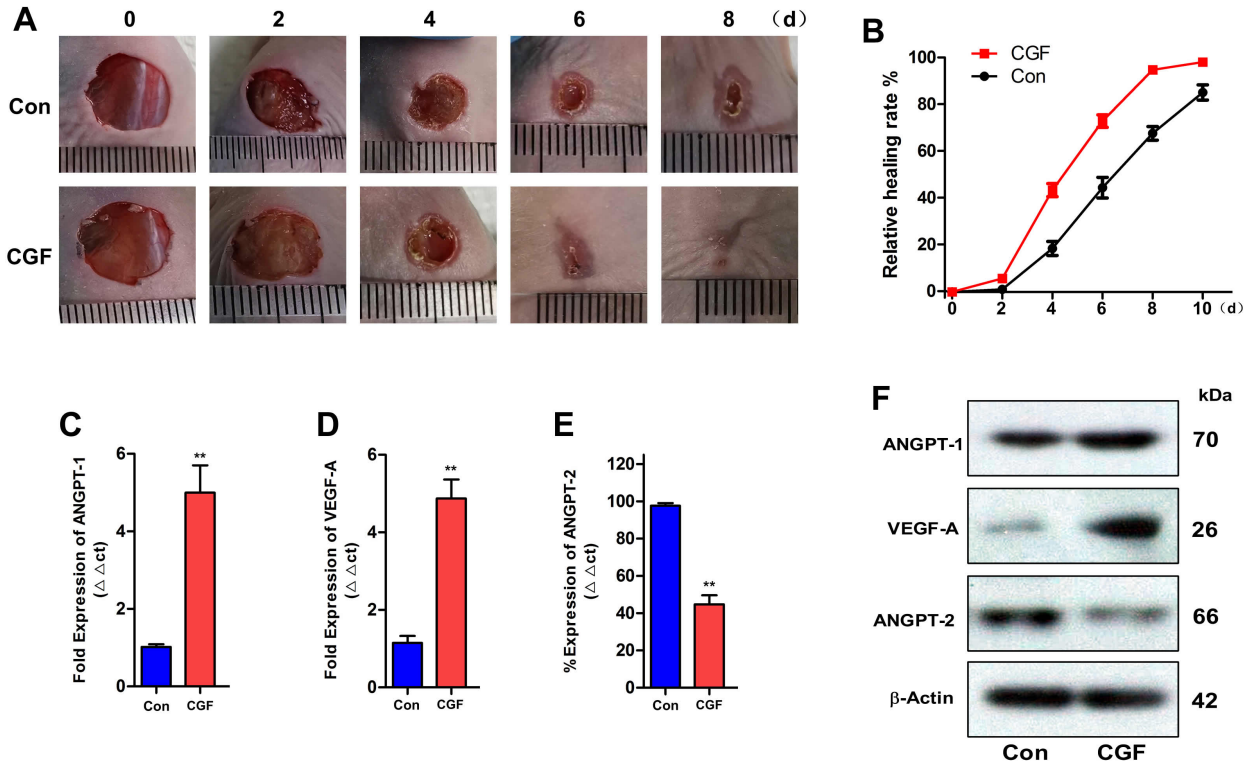

Fig. 6. The *in vivo* effect of CGF in a wound mouse model. (A) Photographs of skin wounds treated with CGF on Days 0, 2, 4, 6, and 8. (B) Quantitative analysis of the wound closure area in the CGF and control groups over time. Eight mice were recruited for each treatment group. (C–E) qPCR was used to evaluate the expressions of ANGPT-1, VEGF-A, and ANGPT-2. (F) Western blot was used to test the expressions of ANGPT-1, VEGF-A and ANGPT-2. β-Actin was used as loading control.

tion. Currently, novel therapeutic strategies for accelerating angiogenesis in healing cutaneous wounds include single and/or dual growth factors, gene and stem cell therapy, drug and drug-like compounds, and skin substitutes [32]. Intensive studies have reported that endogenous growth factors play a key function in benefiting angiogenesis and are essential for effective wound healing [33]. However, akin to our present study, delivery of exogenous growth factors has produced positive results in wound healing and angiogenesis in an animal model of full-thickness skin defect. Most of growth factor-based drugs for wound closure have not displayed clear benefits for promoting wound healing in clinical trials. One postulation for the failure of these trials is that patients acquire resistance to angiogenic growth factors in the context of disease [32,34]. These mixed results from clinical trials have hindered the application of growth factors. CGF, obtained from a patient's autologous venous blood, is characterized by rich growth factors and fibrin, which have concert effect in treatment thus leading to beneficial outcomes [14,35].

It is necessary to unravel the *in vivo* healing effects of CGF using a full-thickness excisional wound model, which offers a systemic effect with the microenvironment of the wound bed being present under the CGF gel. The CGF gel was observed to accelerate the wound closure rate compared with the normal control group. In line with our *in vitro* study, angiogenic biomarkers were activated in the CGF treatment arm. Not only promoting wound healing, CGF can benefit skin appearance, promote pathological changes, and alter the elastic fiber structure of the skin, thus effectively delaying skin senescence in a mouse model [36]. Similarly, CGF along with nanofat could significantly improve the content of collagen and elastin, and has important function in fighting skin aging. And CGF combined with nanofat may provide a more reasonable alternative to skin anti-aging treatment [19].

Although we demonstrated that CGF has encouraging potential clinical applications, our study had several limitations. First, there is no conclusive data on the specific components of CGF mixtures. Moreover, the underlying interaction mechanism of growth factors in CGF has not been examined. Besides, high quality clinical randomized controlled studies are urgently needed to establish guidelines for the application of CGF as well as other blood extracts in wound healing.

5. Conclusions

CGF displayed a great enhancing wound healing effect *in vitro* and *in vivo* via activating the RAS cell signaling pathway. However, more studies will be required to validate the efficacy and safety of CGF in wound healing in the

clinic.

Abbreviations

CGF, concentrated growth factor; LPS, lipopolysaccharide; PRP, platelet rich plasma; PRF, platelets rich fibrin; TGF-β1, transforming growth factor beta 1; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor.

Availability of Data and Materials

The data generated in this study are available from the corresponding author upon reasonable request.

Author Contributions

CK, and SX designed the research study. YueL and YanL conducted the experimental assay. YueL, YanL, CZ, and WL were responsible for writing of manuscript. SX contributed to the revised manuscript. All authors approved the final submitted manuscript.

Ethics Approval and Consent to Participate

This study was performed according to the principles recommended for the experimentation on humans and animals determined by the Institutional Review Board of Guangdong Medical University. Ethics committee approval was also obtained (No. GDY2102255).

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Conflict of Interest

The authors declare no conflict of interest. SX is serving as one of the Editorial Board members of this journal. We declare that SX had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to MF.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2712319.

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