

Fejervarins, Novel Anionic Octapeptides, Promote Cutaneous Wound Healing by Targeting TGFBR2 to Relieve Inflammation and Activate Tissue Repair

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Abstract

Background

Cutaneous wound healing proceeds through a tightly regulated sequence of four phases. Disruption of this process, particularly prolonged inflammation, often leads to delayed repair and the development of chronic wounds.

Materials and methods

In this study, we focused on the Fejervarin (Fej) family peptides, which were isolated from the skin secretions of *Fejervarya moodiei*, *Fejervarya multistriata*, *Hylarana cubitalis*, and *Duttaphrynus melanostictus*. By integrating molecular cloning, in vitro cellular functional assays, animal models, molecular interaction studies, and multi-omics technologies, we established a research framework encompassing: molecular identification - druggability evaluation - *in vivo* efficacy - target validation - pathway analysis.

Results

In murine models of full-thickness wounds, Fej peptides exhibit regulatory efficacy during both the inflammatory and proliferative phases, achieving 99% re-epithelialization by day 12, compared to 91% with human epidermal growth factor (EGF) gel (REFOO®). Biophysical analyses revealed that Fej-1a and Fej-1b directly bind to the transforming growth factor- β receptor II (TGFB β RII) with dissociation constants (K_d) of 0.387 μ M and 5.78 μ M, respectively. During the inflammatory phase, Fej-1a and Fej-1b modulate inflammatory responses through the TGF- β -activated kinase 1 - nuclear factor-kappa B (TAK1-NF- κ B) axis, thereby suppressing M1 macrophage activation while promoting M2 polarization, which accelerates the transition to the proliferative phase of wound healing. Concurrently, these peptides enhance fibroblast migration and collagen deposition by activating the TGF- β /Smad2/3 signaling pathway. Notably, Fej peptides exhibit negligible hemolytic activity and no detectable cytotoxicity *in vitro* and *in vivo*, underscoring their biocompatibility for topical application.

Conclusion

Our findings elucidate a dual-functioning family of amphibian anionic octapeptides that effectively bridge immune resolution and tissue remodeling, highlighting promising prospects for the development of peptide-based therapeutics in wound healing.

Introduction

The skin, serving as the body's primary defense against environmental challenges is particularly vulnerable to damage, which can undermine both its structural integrity and physiological functions. Under normal conditions, acute wounds undergo a self-regulated healing process comprising four partially overlapping phases: hemostasis, inflammation, proliferation, and remodeling, which collectively facilitate effective tissue restoration. However, inadequate management and patient-specific factors, such as malnutrition, uncontrolled diabetes, advanced age, and frailty, can disrupt this process, leading to chronic non-healing wounds (CNHW) [1–3]. Approximately 2% of hospitalized patients worldwide are affected by CNHW, particularly among the elderly patients, and up to 70% of these wounds recur [4]. In the United States, the cases of skin wounds rose to 10.2 million in 2019, with annual healthcare expenditures surpassing \$22 billion [5]. CNHW has high incidence, mortality, and disability rates [6], with its prevalence comparable to that of cardiovascular diseases [7]. For specific wound types, such as diabetic foot ulcers, the mortality rate parallels that of certain cancers [7]. Furthermore, the challenges of CNHW are exacerbated by the growing aging population, the increasing prevalence of chronic diseases like diabetes and obesity, and more drug-resistant wound infections. Consequently, the treatment of CNHW has become increasingly complex, necessitating prolonged care and incurring substantial costs, thereby imposing a significant burden on patients, healthcare systems, and society.

CNHW primarily arise from the dysregulation of inflammatory resolution and tissue remodeling processes. In the context of acute wound healing, inflammatory responses are meticulously regulated in a spatiotemporal manner, facilitating the phenotypic transition of macrophages from a pro-inflammatory (M1) state to a reparative (M2) state, which is crucial for initiating proliferative signaling and matrix remodeling [8]. Conversely, CNHW are characterized by a sustained hyperinflammatory microenvironment, maintained by factors like bacterial biofilms, persistent hypoxia, and oxidative stress. These factors collectively stimulate the overproduction of pro-inflammatory cytokines and matrix-degrading enzymes, including matrix metalloproteinases (MMPs) [9]. The consequent degradation of the extracellular matrix (ECM) disrupts the critical reparative signals, transforming growth factor-beta (TGF- β) and vascular endothelial growth factor (VEGF), resulting in fibroblast dysfunction, compromised angiogenesis, and inadequate collagen deposition [5, 10, 11]. These pathological processes establish a self-perpetuating cycle that impedes tissue repair and prevents wound closure.

Due to their high prevalence and significant clinical impact, CNHW continues to present substantial therapeutic challenges. Existing treatment modalities, including small-molecule drugs, growth factors, antibiotics, and topical formulations, often demonstrate limited efficacy and are hindered by issues such as bioactivity instability, complex production processes, and excessive scarring [11, 12]. Therefore, there is an urgent need for innovative strategies that effectively integrate the resolution of inflammation with tissue repair.

Amphibians, residing in environments with frequent exposure to physical injuries and pathogens, have evolved a unique defense and repair mechanisms. Their skin demonstrates rapid regenerative capacity. Beyond serving as a physical barrier, amphibian skin secretes a variety of bioactive peptides with antimicrobial, immunomodulatory, and pro-healing properties, providing a fundamental molecular basis

for protection against external insults [12]. A growing number of wound-healing peptides have been identified, predominantly originating from the *Ranidae* family and primarily found in the genera *Odorrana*, *Rana*, and *Nanorana* [13, 14]. Peptides derived from *Odorrana* are the most abundant, exhibiting both antimicrobial and regenerative properties. Cathelicidin-OA1 (27 amino acid residues (aa)) [15] and OM-LV20 (20 aa) [16] accelerate wound closure by promoting keratinocyte and fibroblast migration. CW49 (11 aa) [17] contributes to tissue repair through its combined anti-inflammatory and pro-angiogenic effects. Ot-WHP (24 aa) activates macrophages and stimulates cytokine release, thereby initiating the inflammatory phase of healing [18]. In contrast, peptides from the *Rana* genus primarily enhance epithelial migration and remodeling. Temporins A and B (11 aa) directly or indirectly activate the epidermal growth factor receptor (EGFR) and its downstream cascades to stimulate keratinocyte proliferation [19]. RL-QN15 (15 aa) simultaneously activates MAPK and Smad signaling pathways while selectively modulating cytokine production in macrophages [20]. In the *Nanorana* genus, SC17-2 [21] (17 aa) facilitates angiogenesis and cell migration, whereas Cathelicidin-nv (24 aa) plays major roles in immune regulation and neovascularization during tissue repair [22].

Most amphibian wound-healing peptides are composed of 10–25 aa, typically containing lysine (Lys, K) and arginine (Arg, R), resulting in net charges ranging from + 1 to + 5 [13, 14]. This pronounced cationicity facilitates electrostatic interactions with negatively charged cell membranes, phospholipids, and glycosaminoglycans, thereby promoting membrane penetration and receptor binding [13]. A limited number of peptides contain acidic residues aspartate (Asp, D) and glutamate (Glu, E), exemplified by RL-QN15 (-1) [23] and OA-GL12 (-1) [24], constituting only a minor subset of amphibian wound-healing peptides. Current research predominantly emphasizes cationic peptides, leaving the biological properties and therapeutic potential of anionic wound-healing peptides largely unexplored.

In this study, we identified and characterized a novel family of anionic octapeptides from *Fejervarya moodiei* collected on Hainan Island, China, which we designated as Fejervarin (Fej) (Fig. 1). We identified and characterized a new family of anionic octapeptides from *Fejervarya moodiei* collected on Hainan Island, China, designated as Fejervarin (Fej) [25]. Interestingly, homologous sequences were also detected in other amphibian species, including *F. multistriata*, *Hylarana cubitalis*, and *Duttaphrynus melanostictus*, suggesting a conserved biological function. The mature Fej peptides consist of eight amino acids, are enriched with two to three glutamic acid (E) residues, and exhibit a net charge of – 1 to – 2 at physiological pH. This study investigates the wound-healing properties of Fej-1a and Fej-1b, emphasizing several critical aspects: (i) as short, structurally defined anionic octapeptides, Fej-1a and Fej-1b demonstrate favorable biosafety profiles, indicating their therapeutic potential; (ii) during the inflammatory phase, they attenuate excessive inflammation by modulating the TAK1–NF- κ B signaling pathway, which suppresses M1 macrophage activation and promotes polarization toward the M2 phenotype, thereby facilitating the transition from the inflammatory phase to the proliferative phase of wound healing; and (iii) they bind to the TGFBR2 receptor, activating the TGF- β /Smad2/3 signaling pathway, which subsequently enhances fibroblast migration and collagen deposition to accelerate tissue repair and remodeling (Fig. 1). This study lays a foundation for the development of amphibian bioactive

peptide-based wound therapeutic strategies and offers novel candidate molecules for precision intervention in cutaneous wound healing.

Methods

Identification of Fej Peptides

Total RNA was extracted from the skin secretions of four frog species: *Fejervarya moodiei*, *F. multistriata*, *Hylarana cubitalis*, and *Duttaphrynus melanostictus* by using TRIzol reagent (TsingKe, China), followed by purification via oligo(dT) cellulose chromatography (Life Technologies). RNA concentration and purity were assessed using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was synthesized with the Goldenstar™ RT6 Strand cDNA Synthesis Kit, version 2 (TsingKe, China), according to the manufacturer's instructions. Gene-specific primers were designed, and cloning procedures were performed as described previously. Amino acid sequence comparisons were conducted using BLASTp through the National Center for Biotechnology Information (NCBI) database (<https://blast.ncbi.nlm.nih.gov/blast.cgi>). Nucleotide translation and sequence analyses were performed using DNAMAN software. Physicochemical properties of Fej peptides, including theoretical molecular weight, isoelectric point, net charge, and average hydrophobicity, were calculated using the ProtParam tool. Signal peptide prediction and secondary structure analysis were carried out using SignalP-6.0 (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>). The molecular mass of the mature Fej peptides was confirmed by mass spectrometry (Fig. S7A, B). Synthetic peptides with a purity exceeding 95% were commercially synthesized by GenScript (Jiangsu, China).

Full-thickness Skin Wounds in Mice

All *in vivo* animal experiments described herein were strictly performed following the guidelines issued by the International Council for Laboratory Animal Science (ICLAS). All animal experimental protocols were reviewed and fully approved by the Animal Ethics Committee of Laboratory Animals at Hainan University, with the official ethical approval reference number HPIACUC2023005. All experimental operations were designed to minimize animal pain and suffering throughout the study. To assess the impact of Fej peptides on cutaneous wound repair, a full-thickness skin wound model was generated in 96 female KM mice (aged 6–8 weeks, 20 ± 4 g). Following acclimatization under controlled environmental conditions, the mice were anesthetized with Shu Tai 50 (50 mg/kg). After hair removal and disinfection, circular full-thickness excisional wounds (10 mm in diameter) were surgically created. The mice were subsequently housed individually in sterilized cages. The experimental design comprised 8 groups (n = 12 per group), including three concentrations of Fej-1a/b (12.5, 25, and 50 μ M in PBS), positive controls treated with Human Epidermal Growth Factor gel REFOO (EGF, 0.1 g/cm²), and PBS-only negative controls. Test solutions or EGF (50 μ L) were applied topically to the wounds daily. Body weight was monitored over a 9-day period, and the progression of wound healing was documented photographically on days 0, 3, 6, 9, and 12. The process of epithelialization was quantitatively evaluated

using Image-Pro Plus 6.0 software, with the healing rate calculated as: Healing rate (%) = $[(S_0 - S_t)/S_0] \times 100$, where S_0 represents the initial wound area and S_t denotes the residual unhealed area at time t .

Lentiviral Production and Stable Cell Line Establishment

Lentiviral particles were produced by transfecting HSF cells with MISSION® shRNA plasmids (Sigma-Aldrich) targeting the human TGFBR2 (NM_001024847.4) transcript. Supernatants were collected 48 h post-transfection for cell infection or stored at -80°C . For the establishment of stable cell lines, cells were infected at 20% confluence for 48 h using diluted lentiviral supernatants supplemented with 4 ng mL^{-1} polybrene (Sigma-Aldrich, #H9268). After infection, cells were selected with $2 \mu\text{g mL}^{-1}$ puromycin (Sigma-Aldrich, #P8833) for one week before passaging. Quantitative real-time polymerase chain reaction (RT-qPCR) identified TRCN0000197056 and TRCN0000195606 as the most effective constructs, achieving a knockdown efficiency of 60–75%; these constructs were used in all subsequent experiments. The complete shRNA sequences are provided in Supplementary Table S3.

Surface Plasmon Resonance

To assess the interaction between TGFBR2 and Fej-1a and Fej-1b, we used a BIAcore 8K instrument (Cytiva) equipped with a CM5 sensor chip (Cytiva). The procedures for flow cell activation, deactivation, and coupling, as well as ligand-binding assays, were performed as previously described [57]. Briefly, TGFBR2 in sodium acetate buffer ($310 \mu\text{g mL}^{-1}$, pH 4.5) was immobilized on the CM5 sensor chip, and Fej-1a/b was tested at various concentrations. All experiments were conducted in phosphate-buffered saline, with analytes injected at a flow rate of $30 \mu\text{L min}^{-1}$. The association and dissociation phases were set to 160 s and 300 s, respectively. Binding constants were determined using a 1:1 Langmuir binding model via the BIAcore evaluation software.

Statistical Analysis

Statistical analyses were conducted with GraphPad Prism 10 (GraphPad Software, La Jolla, CA, USA). The data are presented as means $\pm$ S.E.M and showed a normal distribution. A two-tailed Student's t-test was used for two-group comparisons, and one-way ANOVA for multiple group comparisons (vs. PBS control/LPS). (N.S., not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

Results

Identification and characterization of the novel anionic Fej peptide family

Two complementary DNA (cDNA) sequences were successfully amplified, each containing a 108 bp open reading frame (ORF) encoding a precursor peptide of 35 aa (Fig. 2A). These peptides exhibited no

homology with any known sequences in the NCBI BLASTp database, thereby warranting their classification as a novel peptide family, designated as Fejervarin (comprising Fej-1a and Fej-1b). Sequence alignment revealed conserved structural features between the two peptides. The precursor peptide is composed of three regions: a signal peptide (15 aa), a spacer peptide (8 aa), and a mature peptide (8 aa), which includes a signal-peptidase cleavage site and a KR protease recognition site motif (Fig. 2B). The mature peptide is produced through the cleavage glycine-lysine (GK) by carboxypeptidase hydrolysis, thereby exposing a terminal E residue essential for C-terminal amidation [26, 27].

Both Fej-1a and Fej-1b are anionic peptides with net charges of -1 and -2, respectively (Table.S1), and possess molecular weights of 963.18 and 1007.23 Da (Fig.S7A, B). Structure analysis via three-dimensional modeling (Fig. 2C) and circular dichroism (CD) (Fig. 2D) spectroscopy revealed that Fej peptides assume an α -helical conformation, exhibiting solvent-dependent structural plasticity. In aqueous solution, the peptides are predominantly disordered, characterized by a negative minimum at 198 nm, whereas they form amphipathic α -helices in PBS containing 50% trifluoroethanol (TFE). Acute toxicity assays indicated no weight loss or mortality in mice, and no pathological changes in vital organs, confirming the non-toxicity of Fej peptides (Fig. S1A, B). Hemolysis assays demonstrated negligible cytotoxicity, with less than 0.5% hemolysis at concentrations up to 800 μ M (Fig. S1C).

To evaluate the therapeutic potential of Fej peptides in promoting skin wound healing, a murine cutaneous wound model was established. After 9 days of treatment, mice administered with Fej-1a or Fej-1b exhibited significantly faster wound healing, achieving about 99% closure, compared to 82.27% in the PBS group and 88.02% in the EGF group (Fig. 2E, F). The 25 μ M concentrations of Fej-1a and Fej-1b displayed the highest wound closure rates throughout the healing process, outperforming the EGF group.

Fej peptides accelerate cutaneous wound healing and tissue regeneration

To investigate the impact of Fej peptides on tissue repair during cutaneous wound healing, histological analyses were performed using hematoxylin and eosin (H&E) staining (Fig. 3A and C). In the PBS group, wounds continued to display signs of inflammation and abnormal repair. The EGF group demonstrated improvement; however, tissue reconstruction remained incomplete. In contrast, the groups treated with low and medium dose of Fej-1a and Fej-1b exhibited superior healing outcomes, as evidenced by distinct tissue architecture, diminished inflammatory infiltration, more intact granulation tissue, and enhanced re-epithelialization. By day 12, the regenerated epidermis was significantly thinner in the Fej-1a (54.7 μ m) and Fej-1b (90.0 μ m) groups compared with the PBS group (250.1 μ m; $P < 0.001$). Masson's trichrome staining (Fig. 3B and D) demonstrated a significant increase in collagen deposition in both the epidermis and dermis in the Fej-1a, Fej-1b, and EGF groups ($P < 0.005$), indicating that these treatments effectively promote collagen fiber formation, thereby accelerating wound healing. Immunohistochemical analysis revealed that Fej-1a (7.87%) and Fej-1b (11.13%) significantly upregulated α -smooth muscle actin (α -

SMA) expression in the dermis compared to the PBS group (6.18%), indicating enhanced myofibroblast differentiation (Fig. 3E and F).

Enzyme-linked immunosorbent assay (ELISA) results showed that Fej-1a and Fej-1b significantly reduced the levels of pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α), while concurrently increasing the level of the anti-inflammatory cytokine interleukin-10 (IL-10) relative to the PBS control group (Fig. 3G). Additionally, both peptides enhanced the expression of endogenous epidermal growth factor (EGF) in wound tissues. Collectively, these findings suggest that Fej-1a and Fej-1b exhibit both anti-inflammatory and tissue-regenerative properties.

Fej peptides promote wound healing via activation of the TGF- β /Smad signaling pathway

To elucidate the molecular mechanisms underlying Fej-mediated wound repair, RNA transcriptomic analysis and tandem mass tag (TMT)-based quantitative proteomics were performed. The transcriptomic analysis revealed a strong inter-sample correlation, indicating high data reliability (Fig. S1D). Gene Ontology (GO) enrichment analysis showed that Fej-1a treatment significantly upregulated genes associated with intermediate filament organization and keratin production, both of which are processes closely linked to epidermal regeneration (Fig. 4A). TMT-based proteomic profiling demonstrated low coefficients of variation across samples, confirming excellent reproducibility (Fig. S1E). Compared with the PBS group, 35 proteins were differentially expressed in the Fej-1a group, with 16 proteins upregulated (fold change > 1.2, P < 0.05) and 19 proteins downregulated (fold change < 0.83, P < 0.05) (Fig. S1f). Pathway enrichment analysis identified the transforming growth factor- β (TGF- β) signaling pathway as one of the most significantly affected pathways (P < 0.001; Fig. 4B).

Consistent with these findings, both mRNA (Fig. S2A, B) and protein levels (Fig. 4C, D) of TGFB2, Smad2/3, phosphorylated Smad2/3, and Smad4 were significantly increased in skin tissues treated with Fej-1a- and Fej-1b (25 μ M) compared with the PBS group (P < 0.01). Immunofluorescence analysis further confirmed the upregulated expression of these signaling components within the epidermis, dermis, and granulation tissue of Fej-treated mice (Fig. 4E, F). These results demonstrate that Fej-1a and Fej-1b activate the TGF- β /Smad pathway, thereby facilitating cutaneous wound healing *in vivo*.

Fej-1a and Fej-1b enhance proliferation and migration of RAW264.7 macrophages and human skin fibroblasts.

Based on the results from *in vivo* wound-healing and cytokine studies, we deduced that Fej peptides promote tissue repair through the coordinated regulation of inflammatory responses and cell proliferation. Given the critical roles of macrophages and fibroblasts in inflammation and tissue remodeling (27, 28), the impact of Fej-1a and Fej-1b on these cells were assayed using Cell Counting Kit-8 (CCK-8) and scratch-wound assays. Both peptides significantly enhanced the proliferative and migratory capacity of RAW264.7 macrophages (Fig. S2C, E, and G) and human skin fibroblasts (HSFs) (P

< 0.01; Fig. S2D, F, and H). Fej-1a and Fej-1b (25 μ M) promoted the closure of the scratch area in RAW264.7 cells within 24 h, resulting in wound-healing rates of about 84%. Similarly, in HSF cells, the scratch closure rate exceeded 99%.

Transwell migration assays further confirmed a dose-dependent enhancement in cell motility for both cell types, with Fej-1a and Fej-1b inducing approximately 2.3-fold increases in migratory activity at 25 μ M (Fig. S3A, B). The 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assays demonstrated that Fej-1a (9.62%, 20.12%) and Fej-1b (9.55%, 19.54%) significantly increased the proportion of RAW264.7 and HSF cells undergoing DNA synthesis compared to the control group (5.19%, 1.52%) ($P < 0.001$; Fig. S3C-D and Fig. S2I). These findings indicate that Fej peptides promote wound healing by enhancing cellular proliferation and migration.

Fej peptides suppress M1 macrophage polarization and promote M2 repolarization.

Given that macrophages are pivotal regulators of cutaneous wound healing, contributing to host defense and tissue regeneration through mechanisms such as phagocytosis, inflammatory modulation, and growth factor secretion [28], we subsequently employed lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages as an *in vitro* inflammatory model to investigate the anti-inflammatory properties of Fej peptides. At a concentration of 25 μ M, both Fej-1a (Fig. S4A, B) and Fej-1b (Fig. S4C, D) significantly inhibited LPS-induced nitric oxide (NO) production, with inhibition rates of 13.1% and 7.0%, respectively ($P < 0.001$). Quantitative real-time PCR (RT-qPCR) analysis revealed that both peptides deregulated the expression of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, NF- κ B, p65, and the M1 marker CD86, while upregulating the expression of IL-10, Arg-1, and CD206 ($P < 0.001$; Fig. S4E), as corroborated by ELISA. Fej-1a and Fej-1b (25 μ M) increased IL-10 secretion by 12.41% and 13.86%, respectively, and reduced IL-6 production by 59.76% and 96.39% ($P < 0.001$; Fig. 5A, B).

These molecular alterations were associated with a shift in macrophage polarization. Flow cytometry analysis showed that LPS increased the proportion of M1 macrophages to 71.46%, compared with 13.26% in the control group. Fej-1a and Fej-1b reduced the proportion of CD86⁺ M1 macrophages to 34.80% and 50.7%, respectively, while increasing the proportion of CD206⁺ M2 macrophages to 30.37% and 22.23%, compared to 19% in the LPS group ($P < 0.001$; Fig. 5C, D). Immunofluorescence staining confirmed these findings, revealing decreased CD86 expression and increased CD206 expression in the Fej-1a and Fej-1b groups relative to the LPS group (Fig. 5E to H). These findings indicate that Fej-1a and Fej-1b suppress M1 macrophage polarization while promoting M2 repolarization, thereby contributing to the resolution of inflammation and the promotion of wound healing.

Fej-1a and Fej-1b peptides attenuate LPS-induced inflammation in RAW264.7 macrophages via modulation of the TGFBR2-NF- κ B signaling axis.

Given that Fej-1a and Fej-1b suppressed the production of pro-inflammatory mediator and induced a phenotypic shift in macrophages from M1 to M2, and considering the pivotal role of NF- κ B inhibition in

this transition [29], we subsequently investigated whether these effects were mediated through the TGFBR2–NF-κB signaling axis in LPS-stimulated RAW264.7 macrophages.

Immunofluorescence analysis revealed that, compared to the LPS group, Fej-1a and Fej-1b significantly upregulated TGFBR2 expression by 2.47-fold and 4.90-fold, respectively ($P < 0.001$; Fig. 5I). This upregulation was accompanied by a substantial increase in p-Smad2/3 levels, which rose by 5.29-fold and 4.91-fold, respectively (Fig. 6A), indicating activation of the TGF-β/Smad signaling pathway.

Concurrently, both peptides markedly suppressed canonical NF-κB signaling. Fej-1a and Fej-1b (25 μM) significantly reduced TGF-β-activated kinase 1 (TAK1) expression by 90.93% and 95.37%, respectively ($P < 0.001$; Fig. 6B). Additionally, they inhibited the nuclear translocation of p65 by 69.53% and 82.70%, respectively, which was accompanied by a reduction in p65 expression. Correspondingly, the abundance of inhibitor of κB-α (IκBα) increased by 3.22- and 2.71-fold, indicating a reversal of LPS-induced IκBα degradation ($P < 0.001$; Fig. 6B, C).

These results were further corroborated by Western blot analysis. Fej-1a and Fej-1b increased TGFBR2 and p-Smad2/3 expression in a dose-dependent manner (Fig. 6D, E). Compared with the LPS group, treatment with 25 μM Fej-1a and Fej-1b resulted in a 2.51- and 4.92-fold increase in TGFBR2 expression ($P < 0.01$), while p-Smad2/3 levels were elevated by 5.10- and 4.82-fold, respectively ($P < 0.05$; Fig. 6D). Consistent with the immunofluorescence results, both peptides markedly reduced TAK1 and p65 expression, inhibited p65 nuclear translocation ($P < 0.001$; Fig. 6B), and restored IκBα levels to those comparable to unstimulated controls (Fig. 6F, G).

These findings collectively indicate that Fej-1a and Fej-1b synergistically activate the TGF-β/Smad signaling pathway while concurrently suppressing the NF-κB signaling pathway. By promoting macrophage polarization toward the M2 phenotype, Fej peptides attenuate excessive inflammatory responses and foster a pro-reparative microenvironment.

Anti-inflammatory activity of Fej-1a and Fej-1b in vivo

To evaluate the anti-inflammatory effects of Fej peptides *in vivo*, we established a murine model of full-thickness excisional wounds, focusing on the inflammatory phase of healing (days 1–3 post-injury) (Fig. 7A). Immunofluorescence analysis revealed that Fej-1a and Fej-1b significantly accelerated the phenotypic transition of macrophages at the wound site from an M1 to an M2 state. On day 1 post-injury (Fig. 7B, C), Fej-1a and Fej-1b reduced the proportion of CD86⁺ M1 macrophages by 16.83% and 17.82%, respectively ($P < 0.01$), while increasing the proportion of CD206⁺ M2 macrophages by 35.65% and 6.33%, respectively, relative to the PBS control group ($P < 0.001$). By day 3 (Fig. 7D, E), the proportion of CD86⁺ macrophages were further reduced by 14.80% and 21.03%, while CD206⁺ macrophages remained elevated, showing an increase of 8.01% and 19.39%, respectively ($P < 0.001$), indicating sustained anti-inflammatory reprogramming.

These findings were corroborated by ELISA analysis (Fig. 7F). Compared to the PBS group, Fej-1a elevated the levels of IL-10 and EGF by 1.5-fold and 2.0-fold, whereas Fej-1b increased by 1.5-fold and 2.5-fold, respectively. Concurrently, Fej-1a reduced the levels of IL-1 β , IL-6, and TNF- α by 44.59%, 65.71%, and 38.51%, while Fej-1b reduced them by 24.84%, 58.76%, and 27.76%, respectively ($P < 0.001$). These findings demonstrate that Fej peptides effectively suppress the pro-inflammatory environment while enhancing anti-inflammatory and repair-associated mediators *in vivo*.

Histological analyses corroborated the alterations observed in the inflammatory microenvironment. H&E staining showed a significant reduction in inflammatory cell infiltration in wounds treated with Fej, as indicated by sparse leukocyte accumulation within the granulation tissue and diminished dermal edema relative to the PBS and EGF groups (Fig. 8A). Masson's trichrome staining further revealed denser and more organized collagen bundles in the Fej-treated groups, characterized by compact alignment across the wound bed, indicative of enhanced ECM deposition (Fig. 8A). In alignment with these findings, immunohistochemical analysis showed that TNF- α expression in both the epidermis and granulation tissue was significantly lower in Fej-1a-treated wounds (10.72% and 23.53%) and Fej-1b-treated wounds (9.93% and 18.17%) compared to PBS-treated wounds (27.72% and 33.57%) or EGF-treated wounds (18.66% and 32.85%), indicating attenuated inflammatory responses during the wound healing process (Fig. 8B, C).

To ascertain whether the *in vivo* effects were associated with the signaling mechanism identified *in vitro*, the components of TGFBR2 and NF- κ B pathway were examined in wound tissues. Immunofluorescence analysis revealed a pronounced upregulation of TGFBR2 expression in both the epidermal and dermal layers of Fej-treated wounds (Fig. 8D, E). Western blot analysis further confirmed elevated TGFBR2 protein levels (Fig. 8G). Concurrently, Fej treatment suppressed NF- κ B pathway activation, as indicated by elevated I κ B α protein levels and a reduced p-p65/p65 ratio (Fig. 8F, G), indicative of attenuated inflammatory signaling *in vivo*.

These *in vivo* findings confirm that Fej-1a and Fej-1b promote the polarization of macrophages towards an anti-inflammatory M2 phenotype through the coordinated regulation of TGF- β /Smad and NF- κ B signaling pathways. Specifically, Fej peptides enhanced TGFBR2-mediated TGF- β /Smad signaling while suppressing TAK1-dependent NF- κ B activation, thereby reducing the pro-inflammatory cytokines (TNF- α and IL-6) and increasing the anti-inflammatory mediator IL-10. This dual action led to a shortening of the inflammatory phase, and the promotion of tissue repair.

Fej-1a and Fej-1b activate the TGF- β /Smad signaling pathway in human skin fibroblasts by up-regulating TGFBR2.

Proteomic analysis has indicated that Fej activates the TGF- β pathway to promote wound healing. Given that HSFs are key dermal effector cells responsible for orchestrating extracellular matrix synthesis, migration, and proliferation during wound repair (3), we subsequently investigated whether Fej peptides activate the TGF- β /Smad signaling pathway in HSFs. RT-qPCR analysis (Fig. S5A) revealed that treatment with Fej-1a or Fej-1b significantly up-regulated the mRNA levels of TGF- β pathway-related

genes by 1.85- to 8.41-fold compared to the control group ($P < 0.001$). These findings were corroborated by Western blot analysis (Fig. 9A and B), which confirmed an elevated expression of TGFBR2, Smad2/3, p-Smad2/3, and Smad4 following Fej peptide treatment. Immunofluorescence analysis further supported these results, showing increased fluorescence intensities of TGFBR2 (Fig. S5B; Fig. 9C), Smad4 (Fig. S5C; Fig. 9D), and Smad2/3 (Fig. S5D; Fig. 9E) in Fej-treated groups ($P < 0.001$). These findings indicate that Fej-1a and Fej-1b activate the TGF- β /Smad signaling pathway in HSFs, thereby promoting fibroblast proliferation.

To determine whether the activity of Fej depends on TGFBR2, HSFs to stably express TGFBR2-specific shRNAs were engineered (Fig. S6C, D). Knockdown of TGFBR2 resulted in a $32.3\% \pm 5.1\%$ reduction in receptor expression at the mRNA level (Fig. 9F) and a $43.8\% \pm 6.4\%$ reduction at the protein level (Fig. 9G, H), accompanied by decreased expression of Smad4 and TGFBR2 (Fig. 9I–K). Functionally, scratch-wound assays showed that TGFBR2 knockdown markedly impaired cell migration relative to the wild-type (WT) cells (migration rate of 47.65%, $P < 0.001$). After treatment with Fej-1a or Fej-1b, the migration rate of shRNA1 cells increased from 11.00% to 60.48% and 59.32%, respectively, while that of shRNA2 cells increased from 4.75% to 48.72% and 36.65%, respectively (Fig. 9L). Under these conditions, Fej-1a and Fej-1b partially restored TGFBR2 expression and rescued migratory capacity to approximately 80% of control levels (Fig. 9L, M).

Immunofluorescence analysis further supported this rescue effect. TGFBR2 knockdown significantly reduced the mean fluorescence intensity (MFI) from 7.97% in WT cells to 2.53% and 2.37% in shRNA1 and shRNA2 cells. Upon treatment with Fej-1a and Fej-1b, the MFI in shRNA1 cells increased to 9.98% and 7.71%, while the MFI in shRNA2 cells rose to 6.50% and 7.16%, respectively (Fig. 9N–P). These findings indicate that Fej treatment can partially restore TGFBR2 expression diminished by shRNA-mediated knockdown.

Overall, the findings demonstrate that Fej-1a and Fej-1b restore the functional phenotype of fibroblasts with TGFBR2 knockdown by reinstating TGFBR2 expression. This restoration reactivates the TGF- β /Smad signaling pathway, enhances migration and proliferation, and mitigates the associated impairment in wound-healing capacity.

Molecular Interactions and Functional Validation of Fej-1a and Fej-1b

Our findings demonstrate that Fej-1a and Fej-1b significantly restore the functional phenotype of fibroblasts subjected to TGFBR2 knockdown. Considering the pivotal role of TGFBR2 as a receptor in the TGF- β signaling pathway [30], we investigated the potential direct interactions between Fej peptides and the TGFBR2 receptor.

Utilizing the Molecular Operating Environment (MOE), we identified distinct binding modes for Fej-1a and Fej-1b within the extracellular domain of TGFBR2. Fej-1a established stable interactions with residues Arg57, Asn70, and Asn91, exhibiting a predicted binding free energy of $-9.8 \text{ kcal}\cdot\text{mol}^{-1}$ (Fig. 10A). In

contrast, Fej-1b primarily interacted with Asp92 and Ile95, displaying a lower binding affinity of $-8.3 \text{ kcal}\cdot\text{mol}^{-1}$ (Fig. 10B). Confocal microscopy demonstrated co-localization of FITC-labeled Fej-1a with TGFBR2 in RAW264.7 macrophages and HSFs (Fig. 10C). *In vivo* tracking demonstrated prolonged retention of Fej-1a within granulation tissue and the deep dermis following topical application (Fig. 10D). Co-immunoprecipitation (Co-IP) assays confirmed a direct association between Fej-1a and TGFBR2 (Fig. 10E). Surface plasmon resonance analyses further revealed dose-dependent binding of both peptides to TGFBR2, with a dissociation constant (K_d) of $0.387 \mu\text{M}$ for Fej-1a (Fig. 10F) and $5.78 \mu\text{M}$ for Fej-1b (Fig. 10G), indicating a higher receptor affinity for Fej-1a.

Based on our comprehensive findings, we propose that Fej-1a/b promotes wound healing through interaction with the cell-membrane receptor TGFBR2.

Discussion

Amphibian skin secretes a diverse repertoire of bioactive peptides, predominantly comprising cationic peptides, including dermaseptins (24–34 aa) [31], temporins (10–17 aa) [32], brevinins (24–33 aa) [33], esculentins (31–46 aa) [34, 35], and ranatuerins (23–33 aa) [36, 37] et al. Comparatively, less anionic AMPs (AAMPs) are reported [38]. In this study, we identified and characterized a novel family of bioactive peptides, designated as Fejervarin (Fej) from four amphibian species of Hainan Island, China. The occurrence of Fej peptides across phylogenetically diverse taxa indicates a significant degree of evolutionary conservation on Hainan Island, implying that these amphibians may share conserved physiological functions. The mature Fej peptides consist of eight amino acid residues, containing two to three E residues, which confer a net charge of -1 to -2 under physiological conditions. Research on bioactive peptides has predominantly focused on cationic species. As of Jan 1, 2026, the Antimicrobial Peptide Database (APD6, <https://aps.unmc.edu/AP/>) catalogs 6309 peptide sequences, with approximately 88% being cationic, while neutral and anionic peptides each account for about 6%. These anionic peptides range from 10 to 50 aa residues. Among them, only ten peptides derive from amphibians, ranging from 11 to 45 aa, and are mostly cysteine-rich skin-secretory peptides that primarily exert defensive or anti-inflammatory functions rather than directly promoting wound healing. In contrast, Fej peptides represent the first identified family of cysteine-free anionic octapeptides.

Multiple peptides derived from amphibian skin have demonstrated efficacy in promoting wound healing. Among those peptides characterized by wound-healing or anti-inflammatory properties, the majority are cationic [39, 40], highlighting the under-representation of anionic peptides in functional studies. Cationic peptides primarily exert their effects through electrostatic interactions with negatively charged components on cell membranes [41, 42]. However, these charge-dependent interactions present inherent limitations, as host cell membranes also contain anionic components, potentially resulting in nonspecific interactions, cytotoxicity, or hemolysis [43]. In contrast, Fej peptides exhibit favorable biocompatibility, evidenced by minimal hemolytic activity and an absence of detectable acute toxicity. In murine models, Fej peptides demonstrate therapeutic efficacy in promoting skin wound healing. The distinctive structural features and characteristics of Fej peptides broaden the diversity of anionic peptides and uncover an

unrecognized role for octapeptides in skin regeneration. These attributes highlight the underappreciated functional potential of short anionic peptides and suggest that Fej peptides may serve as promising molecular scaffolds for future research and therapeutic development.

The timely resolution of inflammation is essential for the successful transition from the inflammatory phase to the proliferative and remodeling phases of wound healing, with macrophage polarization serving as a key regulatory node in this process [28]. During the initial inflammatory phase, M1 macrophages secrete pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β , which are instrumental in pathogen clearance and debris removal. However, prolonged M1 macrophage activation can hinder the healing process and contribute to the development of chronic wounds. Conversely, M2 macrophages facilitate the resolution of inflammation and promote tissue regeneration by secreting anti-inflammatory and reparative cytokines, including IL-10, EGF, and TGF- β [28, 44, 45]. The failure to transition from the M1 to the M2 phenotype is a characteristic pathological feature of chronic wounds, such as diabetic foot ulcers and pressure ulcers [9]. Some bioactive peptides have been reported to modulate macrophage polarization. The human antimicrobial peptide LL-37 plays a role in balancing pro-and anti-inflammatory responses and in regulating immune cell recruitment [46]. Peptides such as AW1 and Mast-MO primarily attenuate inflammation by suppressing NF- κ B-dependent cytokine production [47, 48]. Most existing strategies tend to focus on either inflammatory suppression or reparative signaling, often lacking the ability to effectively coordinate both processes. In contrast, Fej peptides concurrently suppress LPS-induced M1 macrophage activation while promoting M2 polarization, resulting in reduced production of TNF- α , IL-6, and IL-1 β , and increased secretion of IL-10 and EGF, mediated through the TGF- β /NF- κ B pathways. By orchestrating this balanced regulation, Fej peptides disrupt the self-reinforcing cycle of chronic inflammation and reprogram the local immune microenvironment towards a pro-resolution and reparative state. Given that persistent M1 polarization is a major obstacle to chronic wound healing, this dual immunomodulatory capacity of Fej peptides highlights their potential for applications in treatment to wound healing.

Beyond immune regulation, Fej peptides also promote tissue regeneration by activating the TGF- β /Smad signaling pathway through the up-regulation of TGFBR2 expression. The proliferative phase of wound healing is characterized by the formation of granulation tissue, deposition of extracellular matrix, and remodeling processes [49], which are critically depend on the activation and function of fibroblast [50]. Upon ligand binding, TGFBR2 recruits TGFBR1 to form an active heterotetramer receptor complex, thereby initiating Smad-dependent transcriptional programs that regulate fibroblast proliferation, migration, and matrix synthesis [51, 52]. Our findings demonstrate that Fej peptides enhance fibroblast proliferation and migration via the activation of TGF- β /Smad signaling, while genetic depletion of TGFBR2 impairs these cellular responses. Importantly, Fej treatment partially rescues these defects, indicating that Fej peptides selectively enhance TGFBR2 expression and receptor responsiveness, thereby amplifying the downstream TGF- β signaling pathway.

Notably, the Fej-mediated up-regulation of TGFBR2 establishes a regulatory nexus between the TGF- β /Smad and NF- κ B signaling pathways. By augmenting TGFBR2 expression, Fej peptides activate the

TGF- β signaling cascade while concurrently suppressing the TAK1-NF- κ B pathway, resulting in the coordinated modulation of inflammatory and reparative signaling processes. This mechanism aligns with previous studies that have demonstrated crosstalk between the TGF- β and NF- κ B pathways, including TGF- β -mediated inhibition of NF- κ B through TAK1-associated adaptor proteins [53] and the disruption of TRAF6-dependent TAK1 ubiquitination [54]. Our findings advance these observations by identifying anionic peptides as upstream regulators of TGFBR2 expression, thereby offering a strategy for balancing these two critical signaling pathways during wound repair.

From a mechanistic perspective, SPR analyses have demonstrated that Fej-1a and Fej-1b directly bind to TGFBR2 with dissociation constants of 0.387 μ M and 5.78 μ M, respectively. These interactions were validated by co-immunoprecipitation, receptor knockdown, and fluorescence co-localization assays. Considering the well-established pivotal role of TGFBR2 in the regulation of fibrosis and inflammation [55, 56], the capacity of Fej peptides to engage this receptor is particularly noteworthy. Compared with the high-affinity binding of TGF- β ligands to TGFBR2 [57], the moderate affinities observed for Fej-1a/b may indicate a distinct mechanism of receptor modulation, potentially involving transient binding, conformational selection, or allosteric regulation. Such dynamic interactions could provide spatiotemporal fine-tuning of downstream signaling pathways and may represent a beneficial characteristic for therapeutic intervention. Interestingly, Fej-1a/b not only function as ligands binding to TGFBR2 but also upregulate the receptor's expression at both the transcriptional and protein levels. This dual effect likely signifies a positive feedback mechanism within the TGF- β signaling cascade. Upon binding, Fej-1a/b activates the canonical Smad2/3 signaling pathway and inhibit auxiliary pathways, such as TAK1-NF- κ B, which can subsequently enhance TGFBR2 promoter activity and receptor stability on the cell surface. Similar ligand-induced receptor reinforcement has been documented for endogenous TGF- β 1 [51], indicating that Fej-1a/b mimics the aspects of natural growth factor signaling. Through this feedback loop, Fej-1a/b both initiate and sustain TGFBR2-mediated regenerative signaling, thereby ensuring prolonged fibroblast activation and efficient wound closure. However, several concerns warrant further investigation: specifically, how Fej peptides coordinate fibroblast-immune cell crosstalk across different phases of wound healing, and whether their bidirectional anti-inflammatory and pro-repair regulation mitigates scar formation by preventing aberrant collagen crosslinking or the persistence of myofibroblasts.

Conclusions

This study identifies Fej as a novel family of anionic octapeptides, thereby contributing to the expanding repertoire of amphibian peptides. Fej peptides promote wound healing by directly targeting and upregulating TGFBR2. They display a distinctive dual regulatory capacity, enhancing TGF- β -mediated repair signaling while concurrently suppressing excessive inflammation mediated by the TAK1-NF- κ B pathway. This orchestrated modulation of immune and reparative responses highlights the potential of Fej peptides as promising candidates for therapeutic development, particularly in diseases characterized by chronic inflammation and impaired tissue repair, such as non-healing wounds and fibrotic disorders.

Abbreviations

Fej
Fejervarin
TGFB2
transforming growth factor- β receptor II
EGF
human epidermal growth factor
TAK1
TGF- β -activated kinase 1
NF- κ B
nuclear factor-kappa B
ECM
extracellular matrix
MMPs
matrix metalloproteinases
VEGF
vascular endothelial growth factor
CNHW
chronic non-healing wounds
 α -SMA
 α -smooth muscle actin
IL-1 β
interleukin-1 β
IL-6
interleukin-6
TNF- α
tumor necrosis factor- α
IL-10
interleukin-10
RAW264.7
RAW264.7 murine macrophage cell line
HSF
human skin fibroblast
SPR
surface plasmon resonance
Kd
dissociation constant
CD86
cluster of differentiation 86

CD206
cluster of differentiation 206
LPS
lipopolysaccharide
NO
nitric oxide
RT-qPCR
quantitative real-time polymerase chain reaction
Co-IP
co-immunoprecipitation
TMT
tandem mass tag
GO
Gene Ontology
CCK-8
Cell Counting Kit-8
EdU
5-Ethynyl-2'-deoxyuridine
MOE
Molecular Operating Environment
CD
circular dichroism
TFE
trifluoroethanol
I κ B α
inhibitor of κ B α
AAMPs
anionic antimicrobial peptides
APD6
Antimicrobial Peptide Database version 6
CNHW
chronic non-healing wounds
ICLAS
International Council for Laboratory Animal Science
I κ B α
inhibitor of κ B α
TGF- β
transforming growth factor-beta.

Declarations

All vertebrate animal experiments in this manuscript strictly comply with the guidelines of the International Council for Laboratory Animal Science (ICLAS). The animal study protocols were reviewed and approved by the Animal Ethics Committee of Laboratory Animals at Hainan University, ethical approval ID: HPIACUC2023005. We adopted standardized breeding, anesthesia and tissue sampling procedures to reduce animal distress in all operations.

Clinical trail number

not applicable.

Consent for publication

All authors read and approved the final manuscript.

Conflict Of Interest

The authors declare no conflict of interest.

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Author Contribution

Yanmei Zha: writing original draft, methodology, investigation, data curation. Shangjun Jiang: methodology, investigation. Ting Zhao: methodology, investigation. Shuangshuang Wei: data curation. Junchen Lyu: methodology, investigation. Wenting Hu: data curation. Yanting Song: data curation. Tianchen An: data curation. Rong Wang: supervision, funding acquisition. Yingxia Zhang: supervision, project administration, funding acquisition.

Data Availability

All experimental procedures involving animals were conducted in strict accordance with the guidelines outlined in the "Guide for the Care and Use of Laboratory Animals". Ethical approval for these procedures was granted by the esteemed Animal Ethics Committee of Laboratory Animals at Hainan University (HPIACUC2023005).

Supporting Information

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Figures

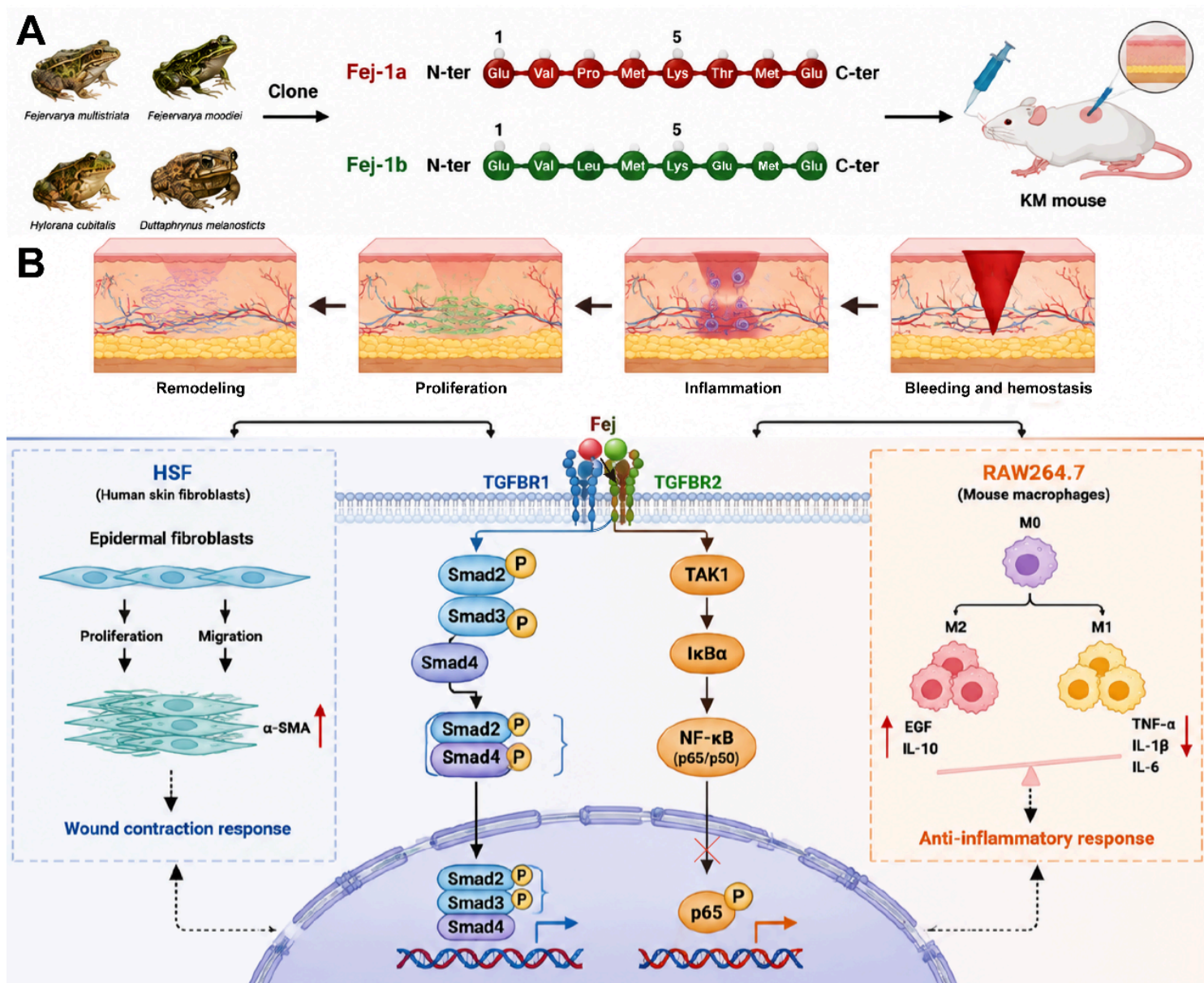


Figure 1

Fejervarin, a novel class of anionic octapeptides, promote cutaneous wound healing by targeting TGFBR2 to suppress inflammation and activate tissue repair. **(A)** Identification and characterization of a novel family of anionic octapeptides, designated Fejervarin (Fej), from four amphibian species. **(B)** Fej-1a/1b binds to TGFBR2 and induces Smad2/3 phosphorylation, thereby promoting macrophage M2 polarization, enhancing fibroblast proliferation and migration, attenuating inflammatory responses, and accelerating collagen deposition and re-epithelialization.

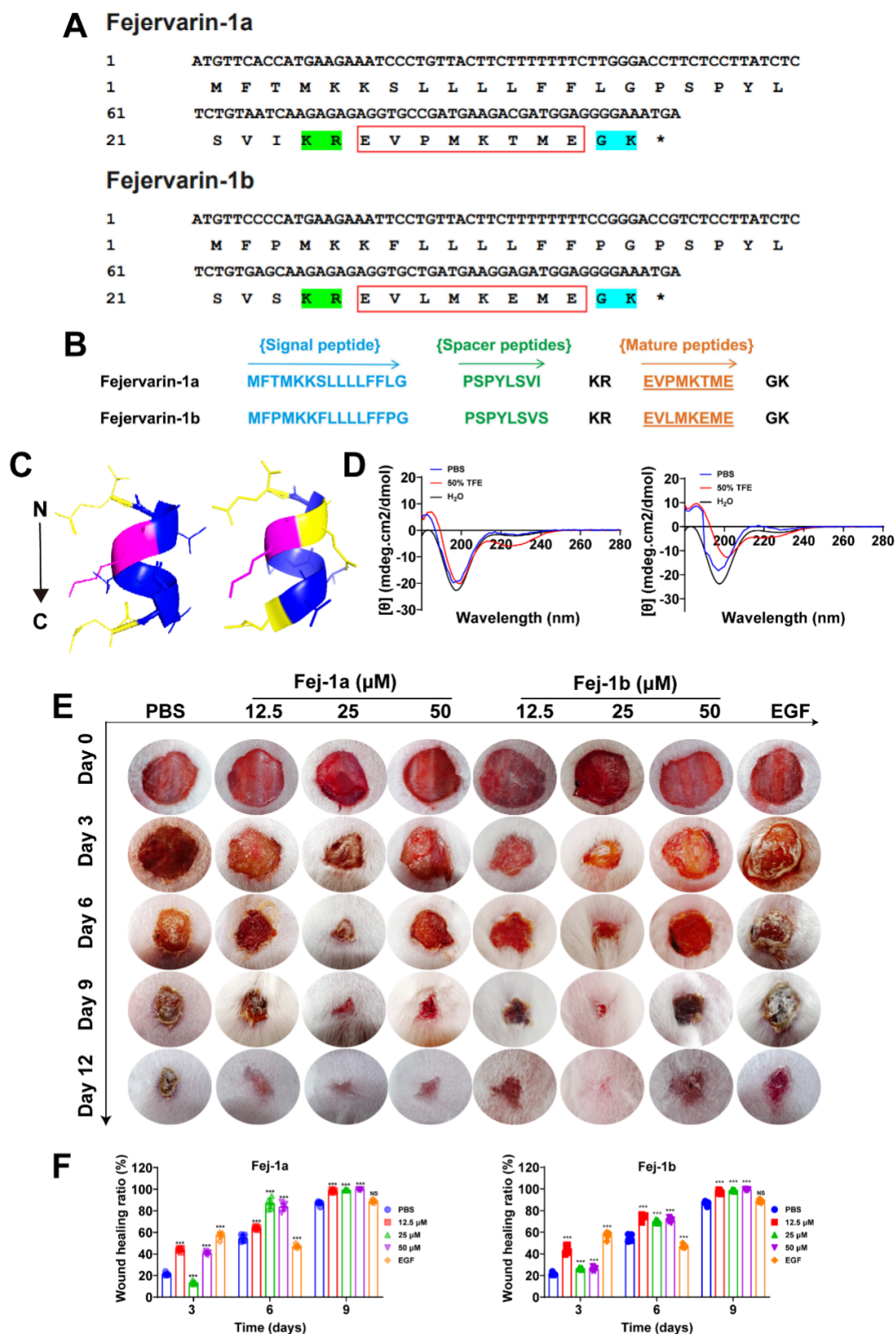


Figure 2

The structural characterization and efficacy promoting wound healing *in vivo* of Fej-1a and Fej-1b peptides. (A) The cDNA sequences encoding Fej-1a and Fej-1b peptides. The stop codon is indicated by an asterisk (*). The green box denotes the cleavage site of the signal peptidase, while the blue-green box indicates the hydrolysis site of the carboxyl peptidase. (B) The precursor peptides of Fej-1a and Fej-1b consisting of three domains: a signal peptide, a spacer peptide, and a mature peptide. (C) The three-

dimensional structures of Fej-1a and Fej-1b were analyzed using PEP-FOLD (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>) and visualized with PyMOL, showing a blue hydrophilic surface and a yellow hydrophobic surface, along with pink basic amino acids, N indicates the amino terminus. **(D)** Secondary structures of Fej-1a and Fej-1b were analyzed by CD spectroscopy. **(E, F)** The representative photographs of full-thickness skin wounds in mice treated with PBS, EGF, Fej-1a and Fej-1b (12.5, 25, and 50 μ M) at 0, 3-, 6-, 9-, and 12 days post-injury. All data are expressed as means $\pm$ S.E.M (n=6). Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test compared to the PBS control group. * P <0.05, ** P <0.01, *** P <0.001; N.S., not significant.

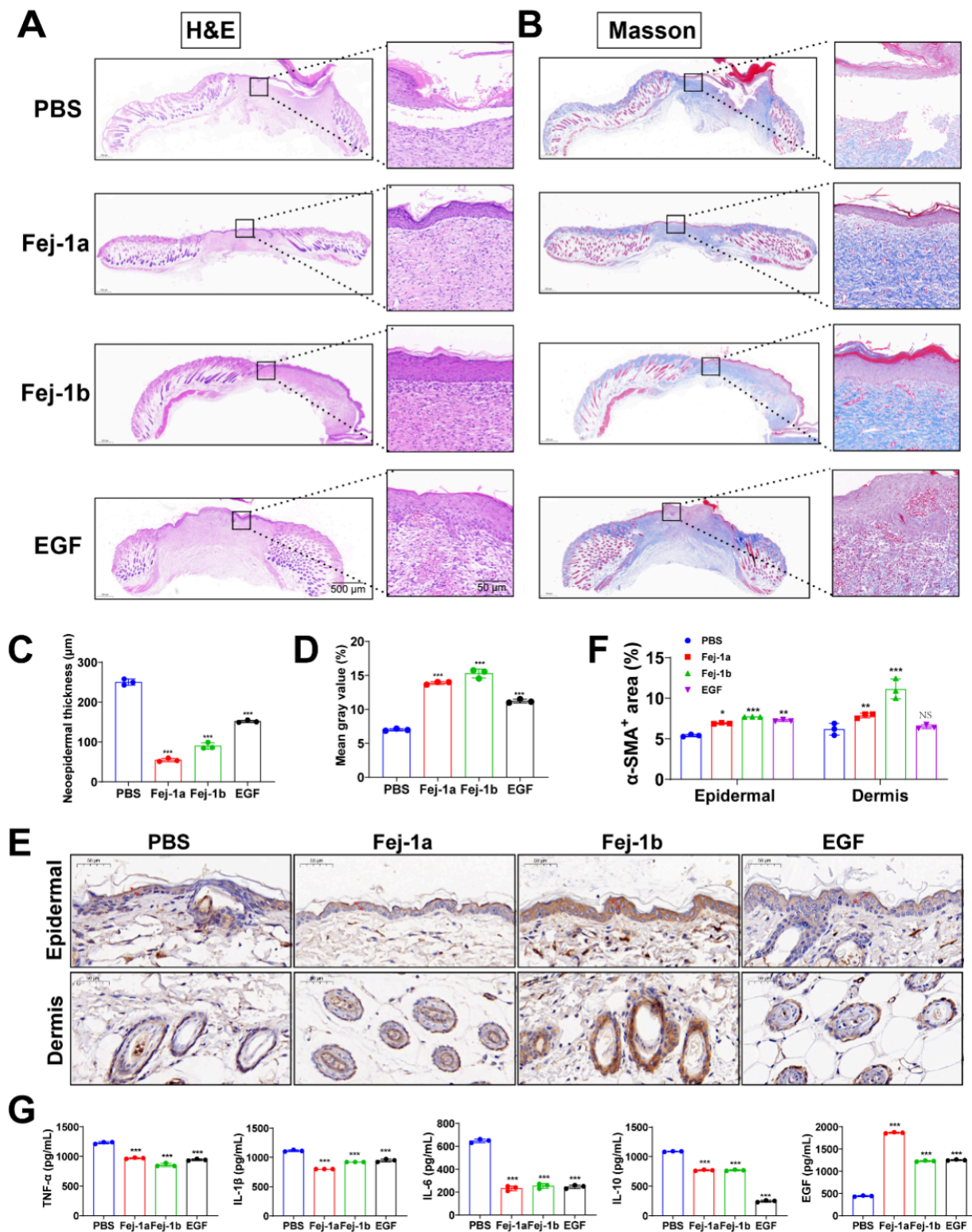


Figure 3

Fej peptides accelerate cutaneous wound healing and tissue regeneration. (A, B) H&E and Masson's trichrome staining of wound tissues on day 12 (scale bars: 500 μm , left; 50 μm , right). (C, D) The quantification of neo-epidermal thickness and collagen deposition (Masson's trichrome) on days 12. (E, F) The representative immunofluorescence images showing $\alpha\text{-SMA}$ expression in the wound epidermis and granulation tissue on day 12 (scale bar = 50 μm), with corresponding quantitative analysis. (G) The

levels of pro-inflammatory (IL-1 β , IL-6, and TNF- α) and anti-inflammatory (IL-10 and EGF) cytokines of wound tissue using ELISA on day 12. All data are expressed as means $\pm$ S.E.M (n = 3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test compared to the PBS control group. * P <0.05, ** P <0.01, *** P <0.001; N.S., not significant.

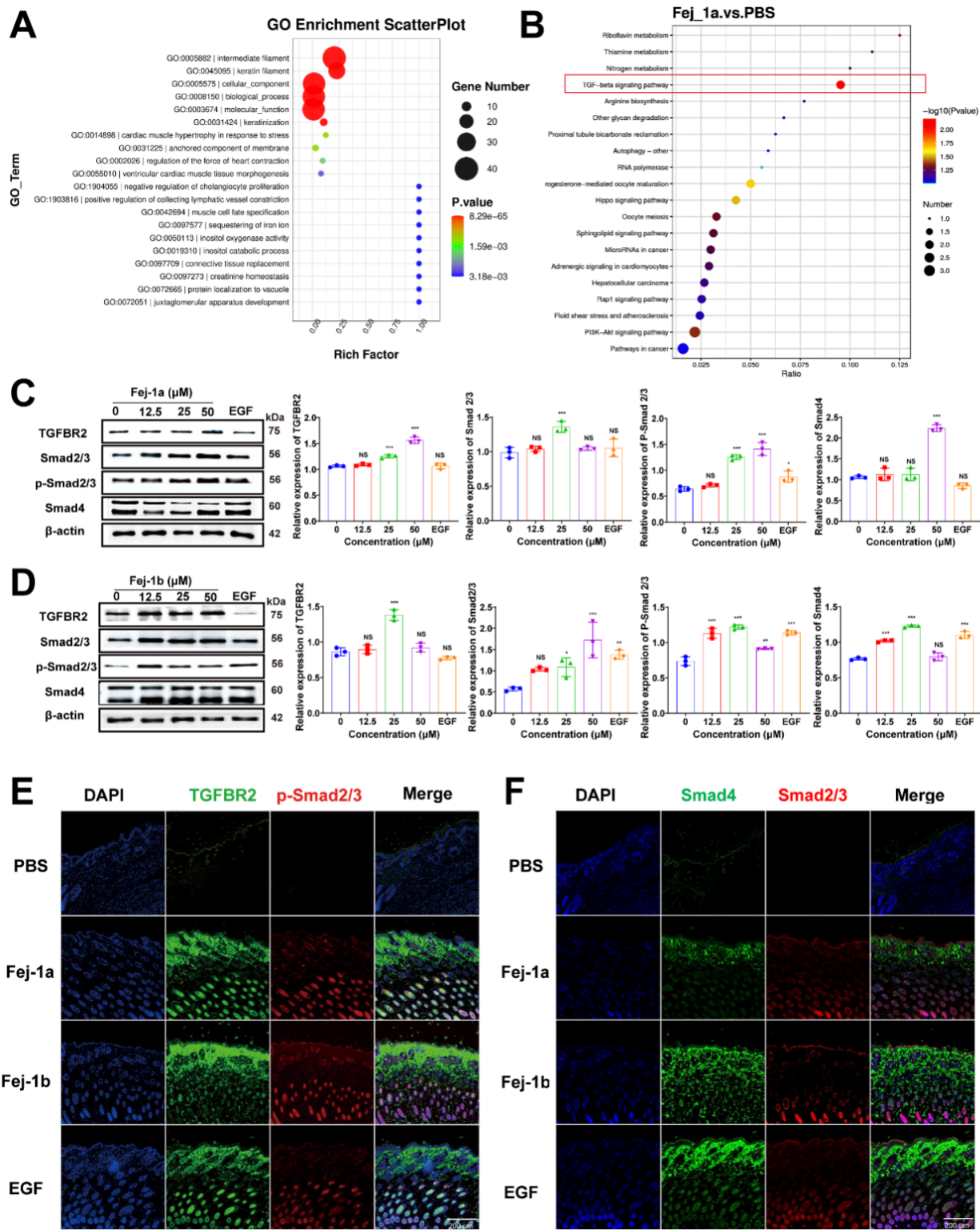


Figure 4

Fej-1a/b promotes wound healing via activation of TGF- β signaling in murine. (A, B) Top 20 enriched biological process termed from RNA-seq and Proteome Analysis (fold change >1.5, $P<0.05$). (C, D) Western blot quantification of TGF- β pathway-related proteins (TGFBR2, Smad2/3, p-Smad2/3, and Smad4) in wound tissues treated with Fej-1a and Fej-1b on day 9. (E, F) The localization of TGFBR2 (green), Smad4 (green), Smad2/3 (red), and p-Smad2/3 (red) in healed skin at day 12 after treatment with Fej-1a and Fej-1b using immunofluorescence assay. The nuclei were counterstained with DAPI (blue). Scale bar = 200 μm . All data are expressed as means $\pm$ S.E.M, ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test compared to the PBS control group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; N.S., not significant.

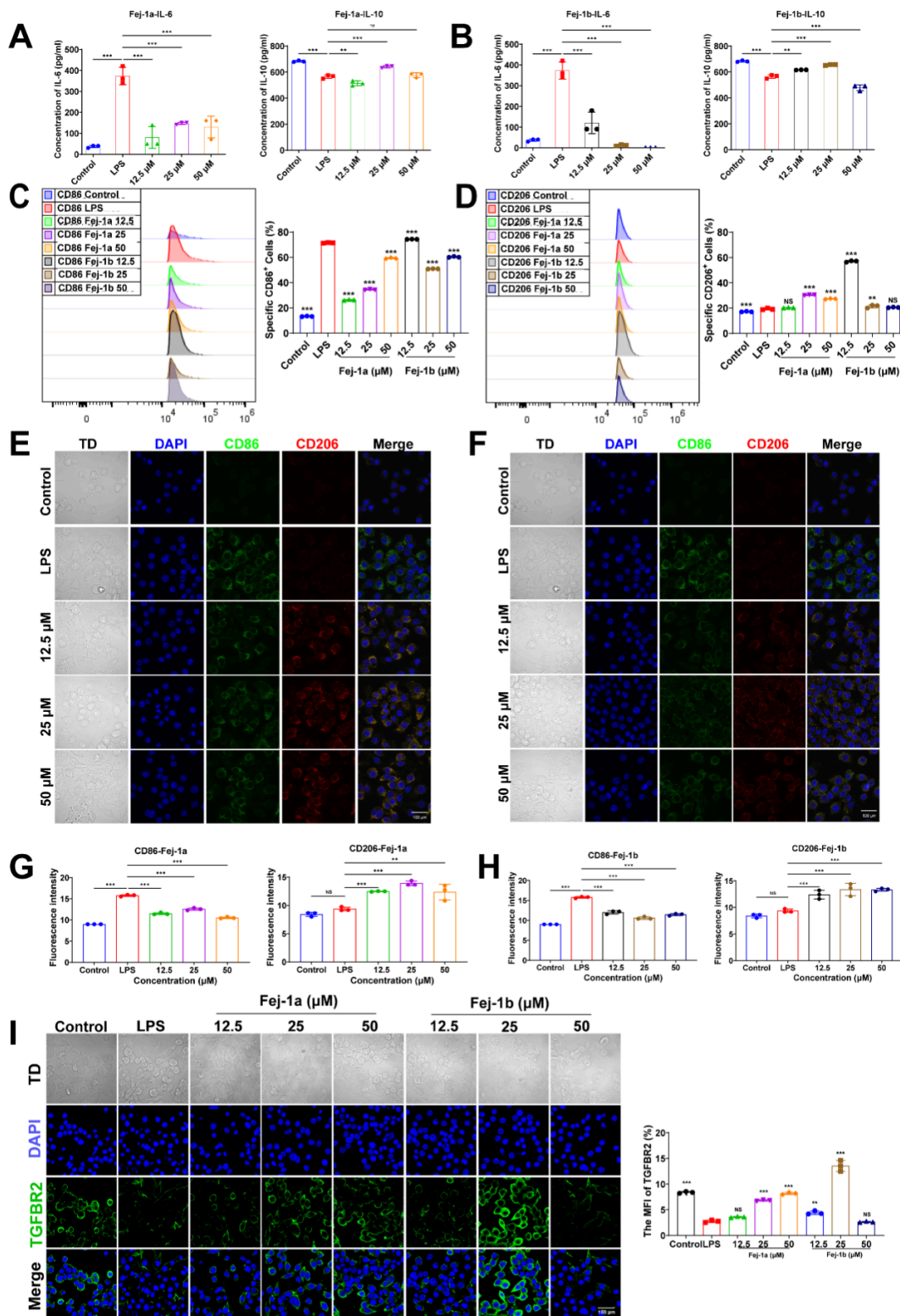


Figure 5

Fej peptides suppress M1 macrophage polarization and promote M2 repolarization.

(A, B) The cytokine secretion profiles of IL-10 and IL-6, as measured by ELISA in LPS-stimulated macrophages, with or without peptide treatment. The data are normalized to the LPS-only group (n = 3). (C, D) Statistical analysis and representative flow cytometry histograms depicting surface expression of

CD86 and CD206 proteins in LPS-induced RAW264.7 cells (n = 3). (E-H) Immunofluorescence analysis and corresponding quantification of M1/M2 polarization markers (CD86⁺ [M1, green] and CD206⁺ [M2, red]) in groups treated with Fej-1a- and Fej-1b (n = 3; Scale bar = 100 μ m). (I) Immunofluorescence analysis and quantification of TGFBR2 expression in LPS-induced RAW264.7 treated with Fej-1a and Fej-1b. Nuclei stained with DAPI (blue) (n = 3; Scale bar = 100 μ m) All data are presented as means $\pm$ S.E.M. Statistical significance was determined by one-way ANOVA (compared to the LPS-only group). * P <0.05, ** P <0.01, *** P <0.001; N.S., not significant.

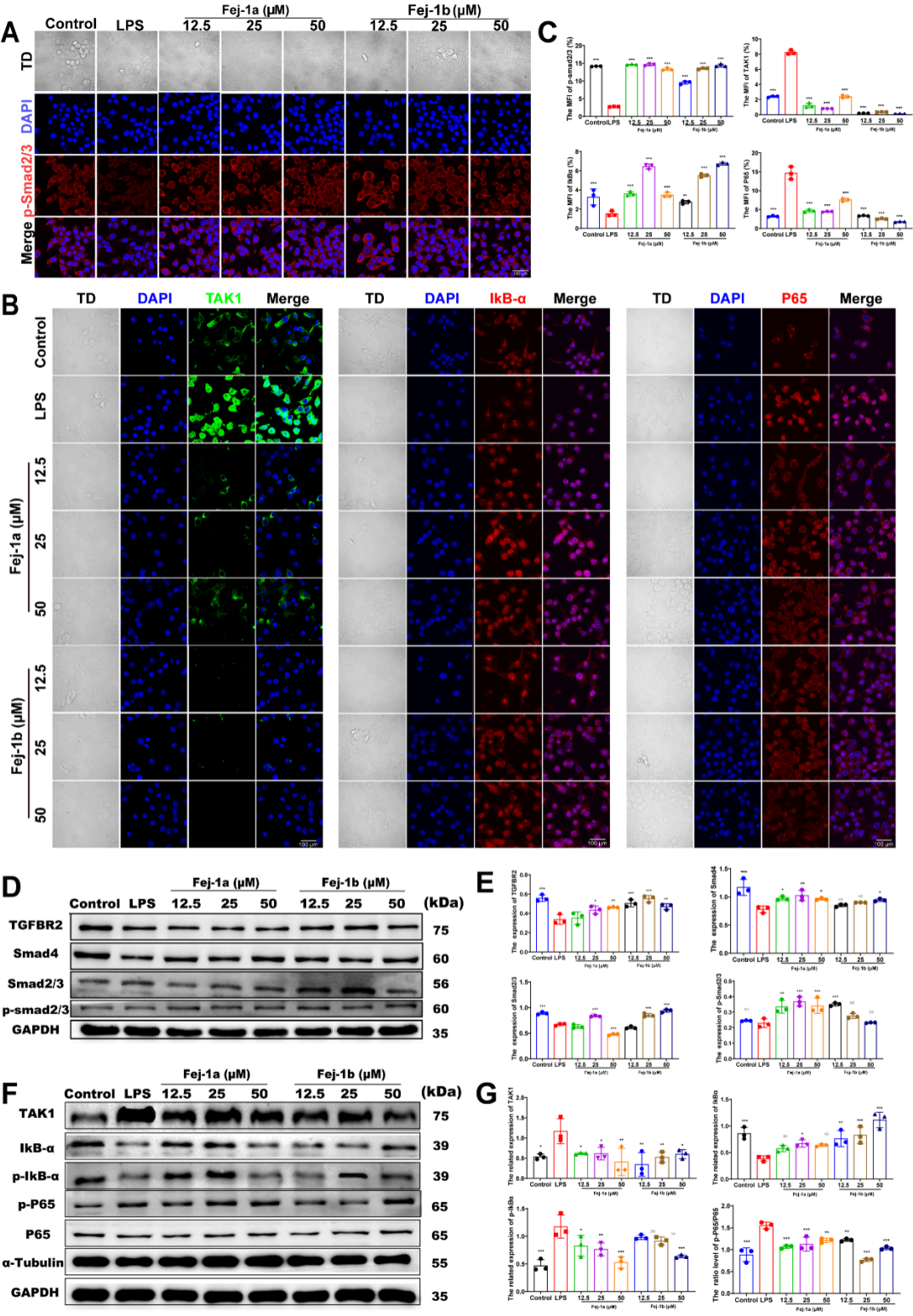


Figure 6

Fej-1a/b enhances I κ B α expression through the TGFBR2-TAK1 pathway, inhibiting NF- κ B nuclear translocation in RAW264.7 macrophages. (A, B) Immunofluorescence analysis of p-Smad2/3, TAK1, I κ B α , and p65 on LPS-induced RAW264.7 treated with Fej-1a and Fej-1b. Nuclei stained with DAPI (blue) (Scale bar = 100 μ m). (C) The quantification of p-Smad2/3, TAK1, I κ B α , and p65 according to the immunofluorescence analysis (n = 3). (D, E) Western blot and quantification of TGF- β signaling components (TGFBR2, Smad4, Smad2/3, and p-Smad2/3) in LPS-stimulated cells. GAPDH served as a loading control (n = 3). (F, G) Western blot and quantification of TAK1, I κ B α , and NF- κ B activation (p-p65/p65 ratio) in LPS-stimulated cells. GAPDH and α -Tubulin served as loading controls (n = 3). Data are expressed as means $\pm$ S.E.M. Statistical analyses were performed using a one-way ANOVA test, with comparisons made to the LPS group. * P <0.05, ** P <0.01, *** P <0.001, N.S. not significant.

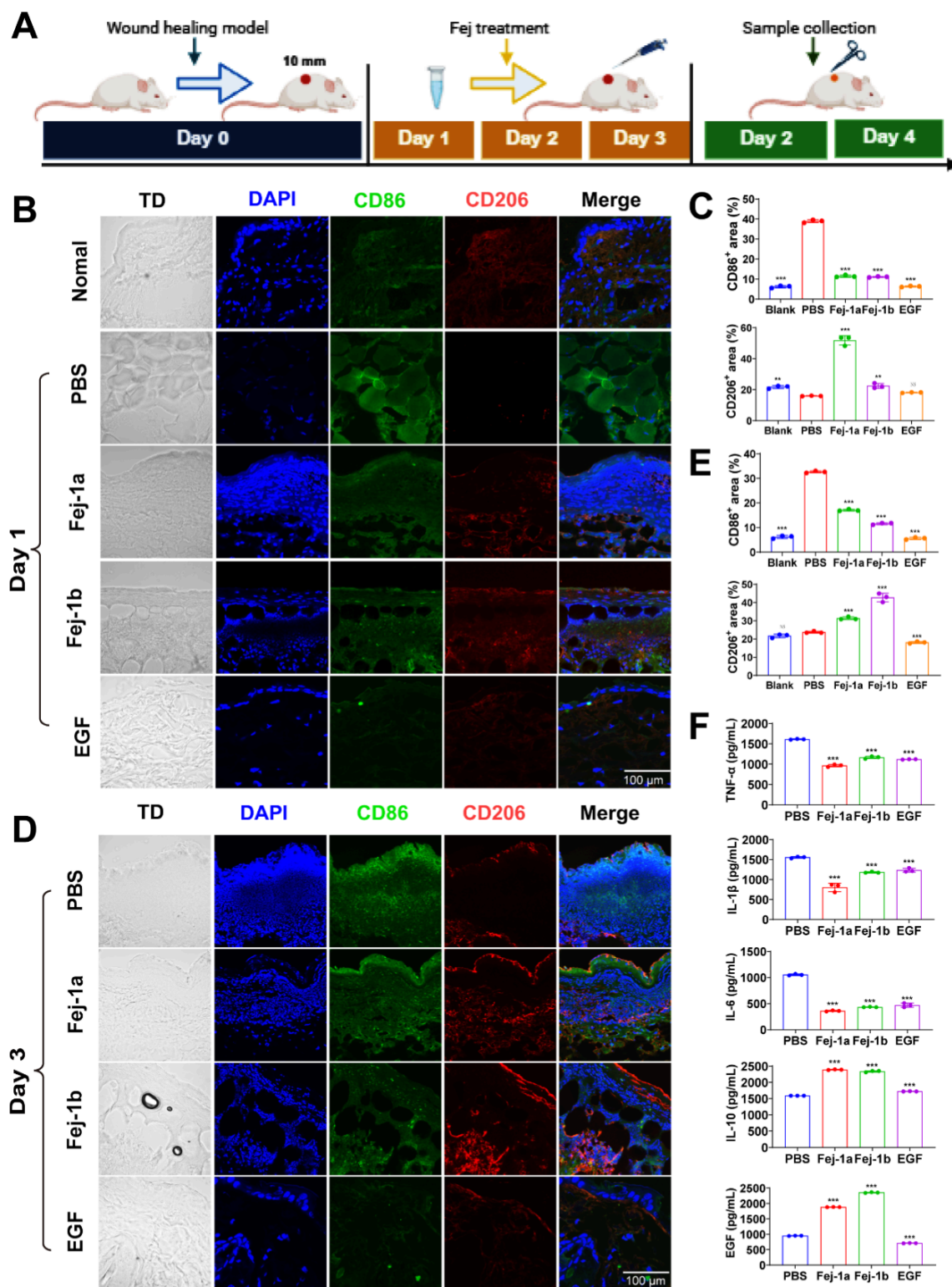


Figure 7

Fej-1a/b enhances $\text{IkB}\alpha$ expression through the TGFBR2-TAK1 pathway, inhibiting NF- κ B nuclear translocation in RAW264.7 macrophages. (A, B) Immunofluorescence analysis of p-Smad2/3, TAK1, $\text{IkB}\alpha$, and p65 on LPS-induced RAW264.7 treated with Fej-1a and Fej-1b. Nuclei stained with DAPI (blue) (Scale bar = 100 μ m). (C) The quantification of p-Smad2/3, TAK1, $\text{IkB}\alpha$, and p65 according to the immunofluorescence analysis (n = 3). (D, E) Western blot and quantification of TGF- β signaling

components (TGFR2, Smad4, Smad2/3, and p-Smad2/3) in LPS-stimulated cells. GAPDH served as a loading control (n = 3). (F, G) Western blot and quantification of TAK1, IκBα, and NF-κB activation (p-p65/p65 ratio) in LPS-stimulated cells. GAPDH and α-Tubulin served as loading controls (n = 3). Data are expressed as means ± S.E.M. Statistical analyses were performed using a one-way ANOVA test, with comparisons made to the LPS group. **P*<0.05, ***P*<0.01, ****P*<0.001, N.S. not significant.

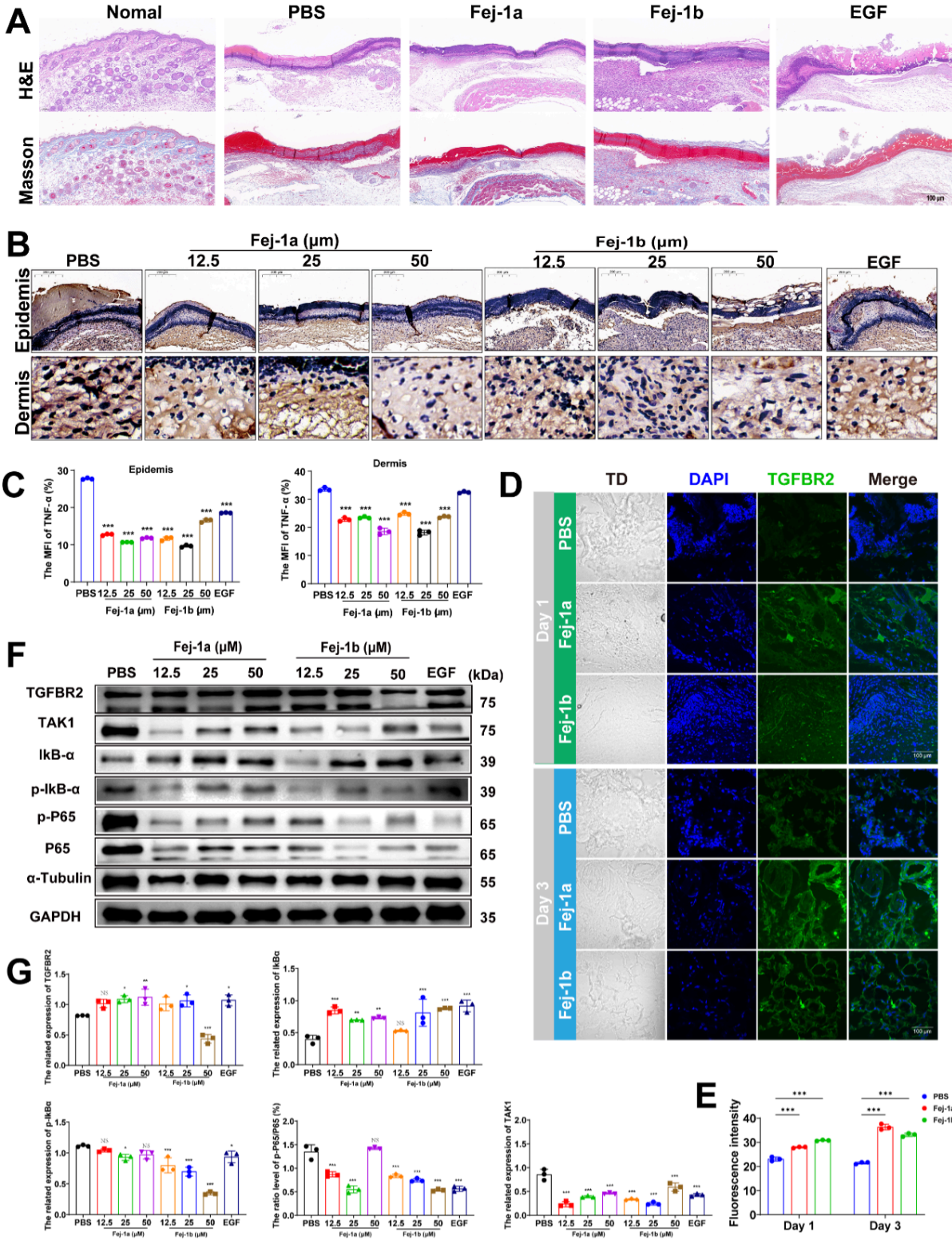


Figure 8

Fej-1a/b accelerates wound healing in mouse skin by regulating the TGFBR2–NF-κB signaling pathway.

(A) Histopathological assessment of day 3 wound tissues by H&E and Masson's trichrome staining (n = 3; scale bar = 100 μm). (B, C) Immunohistochemical detection of TNF-α expression at 3 days post-injury (epidermis: scale bar = 200 μm; dermis: scale bar = 50 μm). (D) Immunofluorescence staining of TGFBR2 (green) in day1 and day 3 wounds treated with Fej-1a and Fej-1b (n = 3; scale bar = 100 μm). (E) Quantification of the TGFBR2-positive area as a percentage of the total wound field (n = 3). (F, G) Western blot analysis of TGFBR2 expression and NF-κB activation in mouse skin tissue after 3 days of Fej-1a/b treatment. GAPDH and α-Tubulin were used as loading controls (n = 3). Data are presented as means ± S.E.M. Statistical significance was determined by one-way ANOVA (vs. PBS control). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; N.S., not significant.

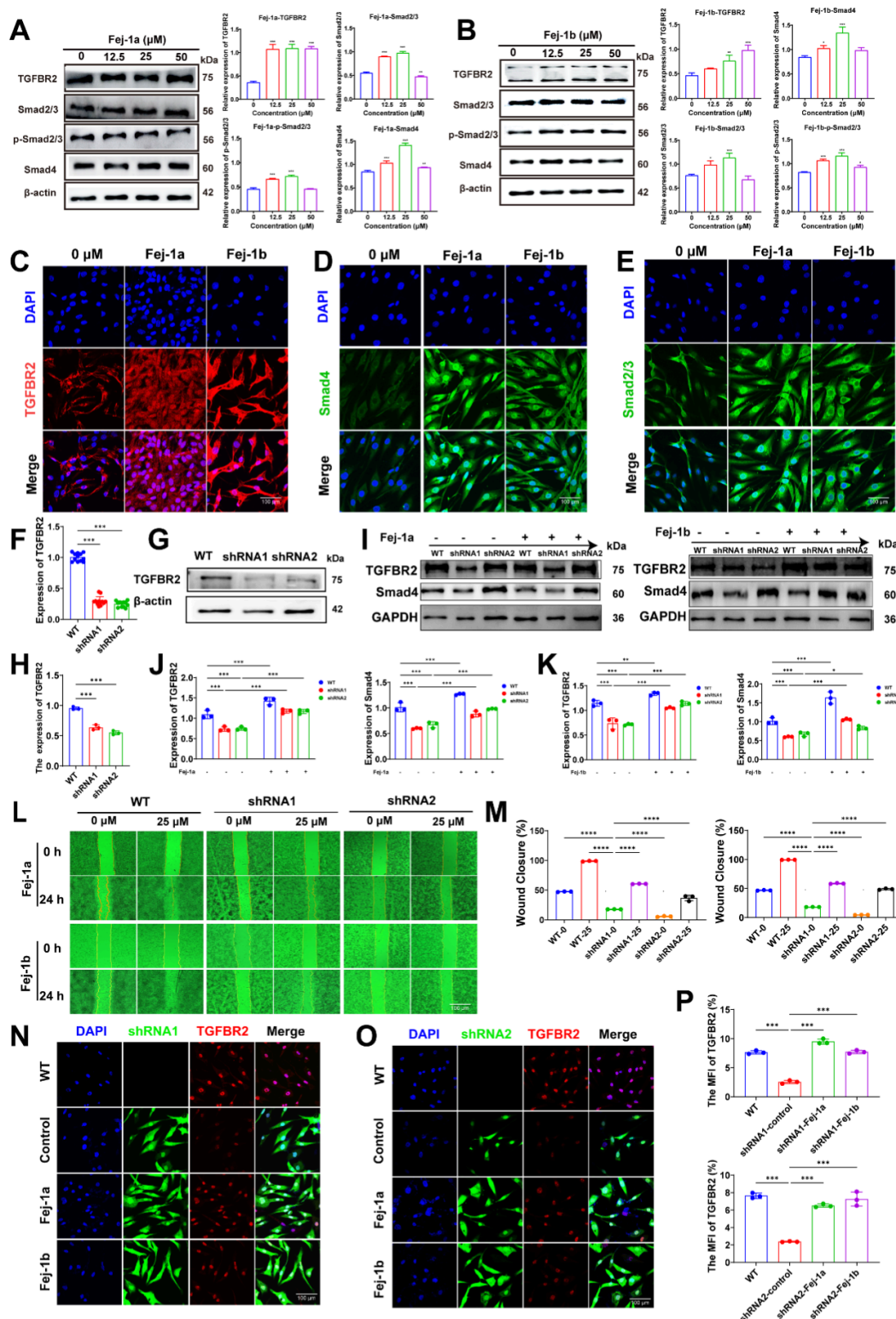


Figure 9

Fej-1a/b rescues TGF- β signaling in TGFBR2-knockdown HSFs. (A, B) Western blot analysis and quantitative assessment of key TGF- β signaling components, including TGFBR2, Smad4, Smad2/3, and p-Smad2/3 in HSFs following treatment with Fej-1a and Fej-1b. β -actin was used as a loading control (n = 3). (C) Immunofluorescence staining of TGFBR2 (red) in HSFs treated with Fej-1a and Fej-1b; nuclei were counterstained with DAPI (blue) (n = 3; scale bar = 100 μ m). (D, E) Subcellular localization of the

Smad4 (green) and Smad2/3 (green) in Fej-1a- and Fej-1b-treated HSFs (n = 3; scale bar = 100 μ m). **(F)** RT-qPCR analysis of TGFBR2 mRNA levels in HSFs following transfection with TGFBR2-knockdown plasmids (shRNA1 and 2), (n = 9). **(G, H)** Western blot analysis of TGFBR2 protein expression in HSFs transfected with TGFBR2-targeting shRNAs (shRNA1 and 2), with β -actin genes as a loading control (n = 3). **(I-K)** Functional rescue experiments in TGFBR2-knockdown HSFs. Western blot analysis of TGFBR2 expression after 24 h treatment with Fej-1a and Fej-1b at 25 μ M. **(L)** Cell migration evaluated by scratch-wound assays. **(M)** Wound closure rates quantified from scratch-wound assays. **(N-P)** Immunofluorescence visualization and quantification of TGFBR2 (red) in TGFBR2-knockdown HSFs treated with Fej-1a and Fej-1b. Nuclei were stained with DAPI (blue). (n = 3. Scale bar = 100 μ m). Data are presented as means $\pm$ S.E.M. Statistical significance was determined by one-way ANOVA (vs. PBS control). * P <0.05, ** P <0.01, *** P <0.001; N.S., not significant.

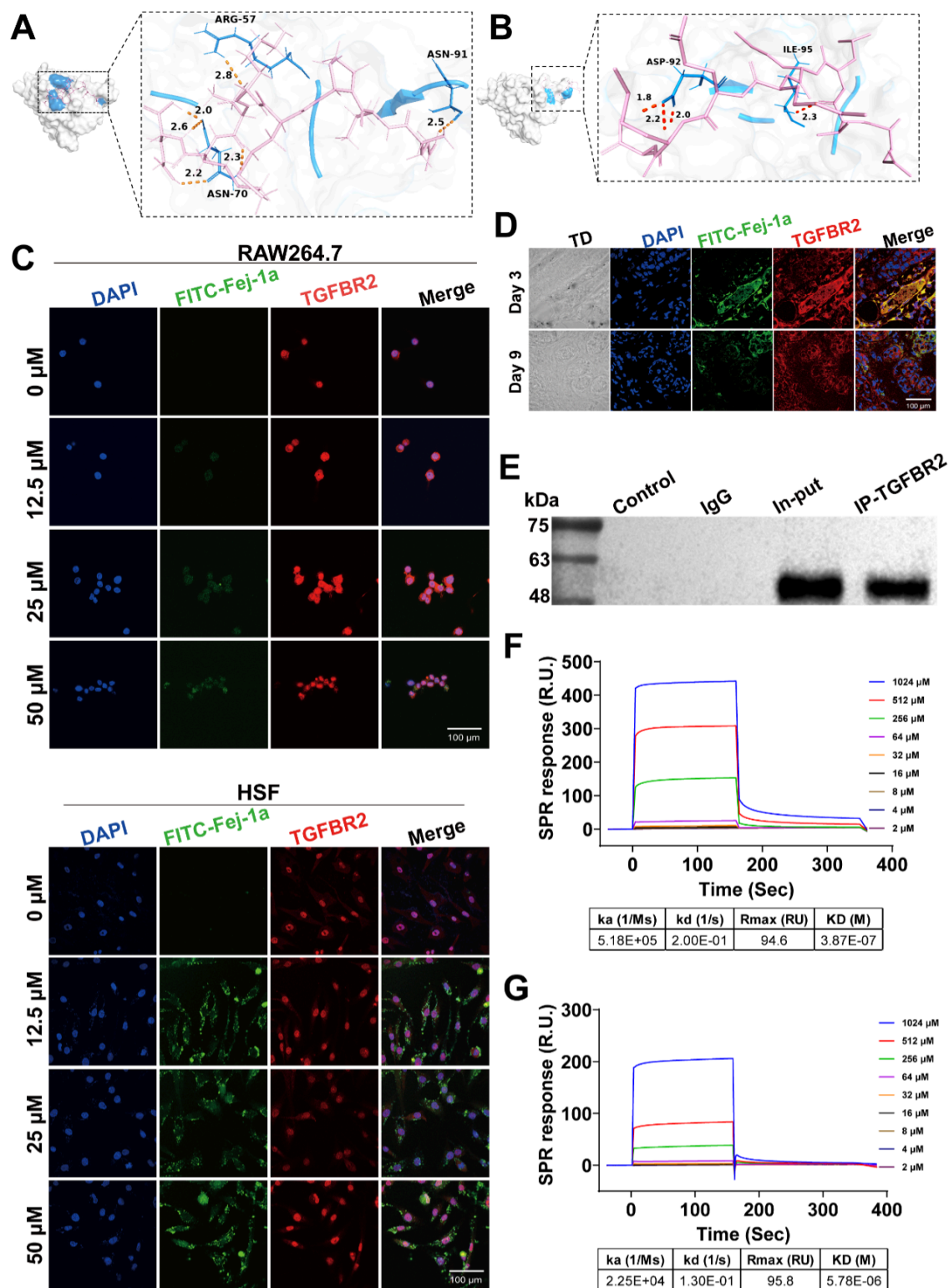


Figure 10

Fej-1a/b promotes skin wound healing by interaction with TGFBR2. (A, B) Computational docking models of Fej-1a and Fej-1b binding to TGFBR2 (PDB: 9E9G), with binding affinities of $\Delta G = -9.8 \text{ kcal mol}^{-1}$ for Fej-1a and $\Delta G = -8.3 \text{ kcal mol}^{-1}$ for Fej-1b. (C) In RAW264.7 and HSF cells, FITC-labeled Fej-1a (green) colocalizes with TGFBR2 (red) after 1 h. Nuclei were stained with DAPI (blue). (n = 3; scale bar = 100 μ m). (D) *In vivo* colocalization dynamics at wound sites were assessed using dual-channel intravital

imaging, revealing a persistent Fej-1a-TGFBR2 interaction between days 3 and 9 after wounding (n = 3; scale bar = 100 μ m). (E) Endogenous TGFBR2 association in HSFs was detected via Immunoprecipitation and immunoblot analysis (n = 3). (F, G) The binding affinity of Fej-1a/b to TGFBR2 was determined using surface plasmon resonance.

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