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Fibromodulin and Its Peptide-based Therapy for Cutaneous and Diabetic Wound Healing

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Fibromodulin and Its Peptide-based Therapy for Cutaneous and Diabetic Wound Healing

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Oral Biology

by

Yao Chen

2022

ABSTRACT OF THE DISSSERTATION

Fibromodulin and Its Peptide-based Therapy for Cutaneous and Diabetic Wound Healing

by

Yao Chen

Doctor of Philosophy in Oral Biology

University of California, Los Angeles, 2022

Professor Shen Hu, Chair

Scarring and wounds are a significant clinical issue which affects up to 100 million people worldwide. More specifically, as one of the most common and devastating sequelae of uncontrolled diabetes, chronic non-healing diabetic wounds affect an estimated 25% of diabetic patients. Wound healing process is significantly impaired in diabetic patients, including imbalanced production of extracellular matrix components, aberrant cellular infiltration, abnormal fibroblast function, and insufficient angiogenesis. A molecular therapeutic for diabetic wound healing that acts to restore the molecular and cellular processes is needed for successful wound healing.

Fibromodulin (FMOD), as a key molecule required for fetal scarless skin repair, can significantly accelerate wound closure, promote angiogenesis, decrease scarring, and improve extracellular matrix organization. Studies have shown FMOD promotes

dermal fibroblast migration, myofibroblast differentiation and contraction, which plays an important role in diabetic wound healing process. F06-C40, a FMOD derived peptide acts similar as FMOD protein and is much faster and easier to generate, which can potentially benefit millions of patients. However, the effect of FMOD and its peptide on diabetic wounds remains unknown. In this study, type II diabetic NONcNZO10 mouse model was used to study the effect of FMOD and its peptide *in vivo*. After full thickness excisional wounds, scar area, capillary formation, and extracellular matrix formation and collagen architecture were determined. *In vitro*, hyperglycemia-induced fibroblasts and primary human dermal diabetic fibroblasts were treated with FMOD and its derived peptide to study the effect of FMOD and its peptide on diabetic dermal fibroblast proliferation, migration, contraction and invasion. To further investigate the mechanism of FMOD on wound healing in a molecular level, the effect of FMOD on cell adhesion was analyzed and the downstream molecule was studied. In addition, TGF-β1 plays an important role in scarring and fibrosis formation. The effect of FMOD on TGF-β1 pathway was determined to further elucidate the role of FMOD on wound healing and scar reduction.

As a result, FMOD and its peptide successfully accelerated wound closure, reduced scar formation and promoted angiogenesis *in vivo*. In addition, they promoted diabetic fibroblast migration, contraction and invasion. FMOD promoted fibroblast cell adhesion via integrin. And FMOD shows a down regulation on TGF-β1 non-canonical pathway, which is the opposite effect on TGF-β1 canonical pathway according to previous studies. Therefore, FMOD and its peptide have the potential to treat diabetic wounds by improving fibroblast characteristics.

The dissertation of Yao Chen is approved.

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2022

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Fibromodulin and Its Peptide-based Therapy for Cutaneous and Diabetic Wound Healing

1. INTRODUCTION

Scarring and wounds are a significant clinical issue which affects up to 100 million people worldwide[1]. It significantly affects patients developmentally, functionally, aesthetically, and psychologically. Moreover, diabetes induced non-healing uncontrolled wounds bring tremendous pains to patients and their family. It has become a public health epidemic with half of the Unites States population diagnosed with either diabetes or pre-diabetes[2, 3]. Among those, an estimated 25% of diabetic patients develop chronic non-healing ulcers in their lifetime[4-7], which are at a relatively high risk for infection given the poor vascularity and immunodeficiency related to diabetes. Amputation and even death are not uncommon, which as a result leads to diabetes as the seventh leading cause of death in the United States[8, 9]. It is an urge for us to find a therapeutic way to treat wounds, especially diabetic non-healing wounds.

However, the characteristics of diabetic non-healing uncontrolled wounds make it much harder to treat compared with other cutaneous wounds. Diabetes delays wound healing process because it impairs each phase of wound healing, such as hemostasis, inflammation, proliferation, and remodeling[9]. It exhibits a persistent inflammatory phase associated with an impediment in the formation of mature granulation tissue and reduction in wound tensile strength[9]. In addition, diabetes causes vascular damage with reduced angiogenesis resulting in ischemia[9-11]. Diabetic wounds also cause destruction of skin and nearby tissues with chances of bacterial infection. Although the mechanism of

delayed healing in diabetic wounds still remain unclear, studies have shown diabetes impairs healing process in both cellular and molecular levels[12, 13]. Cellularly, wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets[13]. Specifically diabetic wounds exhibit reduced dermal and gingival fibroblast proliferation and increased apoptosis[14-16]. Type II diabetic fibroblasts and adult mouse diabetic fibroblasts have been found a reduction in migration compared to the normal fibroblasts[16-18]. Adult diabetic fibroblasts exhibit a sevenfold impairment in vascular endothelial growth factor (VEGF) production compared to wild-type fibroblasts[9]. There is a decrease in fibroblast adhesion during diabetic wound healing process as well[19, 20]. Diabetes also inhibits epithelial cells migration and tight junction formation[13, 21-23], as well as endothelial cell proliferation[24], which leads to delayed wound closure, re-epithelialization[22] and reduced angiogenesis[25, 26]. Molecularly, diabetic wounds exhibit defects in growth factors during extracellular matrix synthesis. Previous studies have shown a significantly decreased expressions of factors regulating extracellular matrix formation during wound healing in diabetic patients, such as MMPs, VEGF, TGF-β, and FGF[9, 27-29]. Putting it together, diabetic patients show impaired cell proliferation, migration, adhesion and defected extracellular matrix formation, which lead to delayed wound healing and increased risk of infection. To improve the prognosis of diabetic wounds, it is crucial to restore cell function and extracellular matrix formation during healing process.

Fibromodulin (FMOD) is an endogenous small leucine-rich proteoglycan (SLRP) in extracellular matrix that is essential for wound healing[30]. Previous studies have shown that FMOD is essential for fetal-type scarless wound healing[31]. Neonatal mice

with FMOD deficiency have delayed wound healing and defected healing process, which are restored with FMOD administration[31]. In adult rodents and porcine models, FMOD has shown to significantly promote wound healing, accelerate re-epithelialization, and reduce scar formation. It reduces scar formation by eliciting a fetal-like type phenotype[32]. During cutaneous wound healing process, FMOD promotes dermal fibroblast migration and invasion[32], improves angiogenesis by increasing endothelial cells proliferation and cell attachment, actin stress fiber formation and tube-like structure network establishment [33]. On a molecular level, high FMOD level is associated with reduced TGF-β1 expression and scar repair[31]. More specifically, FMOD does not just simply inhibit TGF-β1 signaling, but rather uncouple pro-migration/contraction TGF-β1 signaling pathway from pro-fibrotic TGF-β1 signaling pathway. It selectively increases Smad2/3 pathway and downregulates AP-1 TGF-β1-mediated pathway. By delicately orchestrating TGF-β1 pathway, FMOD promotes TGF-β1 mediated fibroblast migration and contraction, and reduces TGF-β1 mediated extracellular matrix accumulation and fibrotic scar formation[32]. FMOD also reduces nuclear factor-κb (NF-κB) activity by delaying the constitutive degradation of IκBα via a JNK-dependent pathway that promotes the activation of c-Jun N-terminal kinase, the inhibition of calpain and casein kinase 2 activity, and the induction of fibroblast apoptosis[34, 35]. In addition, FMOD increases expression of collagen I and III, angiopoietin (Ang)-2, and vascular endothelial growth factor (VEGF). Putting together, FMOD provides a favorable network to reduce pro-fibrotic activities, promote signaling pathways that regulate fibroblast migration and contraction, and mobilize quiescent endothelial cells to an angiogenic phenotype[32, 36]. Therefore, FMOD is a potential therapeutic way for cutaneous scarring. It remains unknown if FMOD

has similar effects on diabetic wound healing, however, if FMOD works similar way on diabetic wound, it would significantly help diabetic patients since one of the reasons for non-healing diabetic wound is the defected fibroblast functions. However, it is not efficient to produce FMOD through bacteria and yeast systems[37], and cell culture for FMOD manufacture is expensive thus not costly efficient[34]. In our lab, we have developed a 40 amino acid length FMOD-based peptide that is easier and more costly efficient to manufacture while maintain similar effects and functions. It can significantly help patients reduce their financial burden. In this study, we focused on the effect of FMOD and its peptide on diabetic wound healing and diabetic fibroblasts activities to discuss the function both *in vivo* and *in vitro*.

Since type 2 diabetes is the majority population among diabetes and affects 6.28% populations worldwide[38], we chose to focus more on type 2 diabetic phenotype in this study. Specially, there are three most commonly used strains of mice to simulate wound healing in diabetic individuals – Akita, db/db and NONcNZO10/LtJ. Akita mouse model has been proven to better simulate type 1 diabetes[39, 40]. And as a commonly used type 2 mouse model, db/db does have been used for certain types of wound healing studies[41-43], however, the pathophysiology of diabetes and obesity phenotypes in this mouse model can potentially limit their applications[44]. In contrast, NONcNZO10/LtJ has been chosen in our study due to its moderate maturity-onset obesity and the characteristics of adult-onset diabetes without extreme insulin or leptin levels[45-47]. It has more similarities with human type 2 diabetes genetically and metabolically. Therefore, in this study, we utilized NONcNZO10/LtJ mouse to simulate human type 2 diabetes and studied the effects of FMOD and its peptide on cutaneous and diabetic wound healing.

2. MATERIALS AND METHODS

2.1 Animal surgery procedures

All experiments were performed under institutionally approved protocols provided by the Chancellor's Animal Research Committee at the University of California, Los Angeles (protocol number: 2008-016).

2.1.1 Diabetic mouse model

To better simulate type 2 diabetes with onset obesity and insulin resistance[46], 10-week-old male NONcNZO10/LtJ (JAX no. 004456) mice were ordered from the Jackson laboratory and fed with high fat diet for 4-6 weeks until glucose level is higher than 300 mg/ml[45, 48]. We tested glucose level twice a day throughout the experiments and only mice with glucose level higher than 300 mg/ml will be included in this study[48].

2.1.2 Secondary closure full thickness wound model

14-week-old male NONcNZO10/LtJ mice were anesthetized by 2% isoflurane and oxygen (1.5 liters/minute) inhalation and maintained in an oxygen flow environment upon waking up[49]. A splinted excisional wound model was made on the back of adult male NONcNZO10/LtJ mice[48, 50], as the clinically relevant model for diabetic wound healing deficiencies. Specifically, two circular 6-mm full-thickness excisional wounds extended through the *panniculus carnosus* were created on the dorsal aspect of the mice with a round biopsy punch[51, 52]. All wounds were separated by at least 1 cm to either side. Circular silicone splints with inner diameter 9 mm and outer diameter 16 mm (fabricated from silicone tape, 3M, St. Paul, MN) were used for secondary wound closure and were secured to the skin beyond the wound margins using a silicone-based adhesive (Vapon Inc., Fairfield, NJ) followed by the placement of six interrupted 6-0 polypropylene

monofilament sutures circumferentially around the splint. The splinted excisional wounds were covered with a semi-permeable dressing (TegaDerm, 3M). 4 mg/ml Fibromodulin[53] and 10 mg/ml peptide were injected at 4 points around the wound edge (25 μl/point) and every 48 hours later until 14 days (100μl/wound, 1 time/2 days, 14 days, 6 mice/group) (Figure 1A).

2.2 Histology

Tissue samples for histology were bisected centrally and perpendicular to the long axis of each wound[53]. After fixation with 4% Paraformaldehyde (PFA) at 4°C overnight, sample skins were dehydrated and paraffin-embedded for hematoxylin and eosin (H&E) staining, picro-Sirius red (PSR) staining, and immunohistochemistry (IHC) staining.

2.2.1 H&E staining

Samples were cut into 5-μm sections for H&E staining. To ensure consistent sampling from the center rather than the periphery of the wound, sections were obtained from the cut surface of the wound where it was previously bisected[54]. H&E staining photographs were captured on an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA) equipped with MicroFire 2.2 digital camera (Optronics, Goleta, CA) using PictureFrame 2.0 software (Optronics) at 40× magnification. Image analysis was performed using the NIH program ImageJ[34].

2.2.2. PSR staining

Samples were cut into 10-μm sections for PSR staining[34]. And then they were stained with Sirius red (0.1% of Sirius red F3B in saturated aqueous picric acid, Sigma-Aldrich, MA) for 30 min for collagen bundle staining[55, 56]. Samples were analyzed and

captured under polarized light with 40x magnification[55] and confocal laser scanning microscope (Leica, TCS SP5, Germany) with 1000x magnification[54].

2.2.3 IHC staining for angiogenesis

After deparaffinization and rehydration of the tissue sections, samples were heat treated for antigen retrieval. Hydrogen peroxide (H2O2) was used to avoid endogenous peroxidase activity and reduce background staining. 3% BSA in TBS were used for 2h at room temperature to block nonspecific sites. The primary anti-von Willebrand factor (vWF) antibody (abcam, MA) antibodies in BSA with 1:400 concentration was applied at 4°C overnight, followed by three rinses in PBS[57]. Secondary antibodies consisting of biotinylated goat anti-rabbit IgG (1:1000 dilution with PBS; abcam, MA) were added for 1 hour at room temperature. After washing, the sections were further incubated with VECTASTAIN ABC reagent (1:50 dilution with PBS, avidin biotin immunoperoxidase labeling (ABC) kit, Vector Laboratories) for 30 minutes. Sections were washed with distilled water, counter-stained with hematoxylin, and cover-slipped[31, 58].

2.3 Confocal imaging

After sections were performed with PSR staining as previously described[55], confocal images were captured at 630x magnification with confocal laser scanning microscope (Leica TCS-SP5, Leica Imaging Systems LTD, Germany) to analyze collagen architecture. Samples were scanned and sectioned in 1-μm increments with the entire section thickness of 10 μm. Images were obtained with a quality of 512 x 512 pixel. Confocal imaging analysis was performed as previously described[54]. Fractal dimension (F_D) and lacunarity (L) value have been used to evaluate collagen formation during scarring and wound healing[54].

2.4 Scar evaluation

Scar formation was evaluated with confocal images with PSR staining instead of H&E staining samples for better boundaries between normal and scar tissues. To avoid the effect of individual difference of dermal thickness when evaluating scar formation, we developed a Scar Index (SI) to normalize scar size analysis[53, 54]. Two dermal thickness were measured 700 μm on both left and right wound edge per sample. On each sample with PSR staining, the fibrotic scar tissue area was outlined and measured to extend from the base of the epidermis to the muscle layer. The scar index was calculated by dividing the scar area by the corresponding average dermal thickness (Figure 1B).

2.5 Cell culture

hTERT-immortalized fibroblasts BJ-5ta (CRL-4001, ATCC, VA) were cultured in DMEM (Thermo Fisher, MA) with 10% inactivated fetal bovine serum (FBS, Thermo Fisher, MA), and 1% penicillin/streptomycin (Life Technologies, NY), and high glucose to simulate hyperglycemia scenario or normal glucose to act as a control group. Human primary diabetic dermal fibroblasts (HDDAFB, passage 3-5, catalog #HD2-6067, Cell Biologics, IL) from skin of a 58-year-old female type II diabetic donor and human primary dermal fibroblasts (passage 3-5, catalog #H-6067, Cell biologics, IL) from skin of a 50-year-old healthy female donor were cultured in complete fibroblast medium (catalog #M2267, Cell biologics, IL) coated with gelatin-based coating solution (catalog #6950, Cell biologics, IL). All cells were cultured in cell incubator at 37°C with 5% CO_2 and 95% humidity for the optimal cell growth condition.

2.5.1 Hyperglycemia induction

Based on previous studies[16-18, 59] , we cultured human fibroblast cell BJ-5ta with high glucose (50mM) and normal glucose (5mM) for 24h, 48h, and 72h, respectively, and exam the proliferation rate change to determine the optimal time for hyperglycemia induction[60-62].

2.6 Cell proliferation

Cell proliferation was determined with EdU assay (abcam, ab219801, MA) as it is a sensitive and robust technique for proliferation rate. Hyperglycemia BJ-5ta pre-treated with high glucose was plated onto a 96-well plate in 200 μl culture media at 60%-70% confluence. After cells were recovered overnight, medium with 200 nM FMOD, 200 nM peptide, 100 pM TGF-β1 are added with EdU solution for 3.5 h under optimum cell growth condition. EdU reaction was performed after 3.5h and cells were stained with DAPI. Cell counts were calculated with Image J and proliferation rate was obtained by dividing cell counts by proliferated cells.

2.7 Scratch assay

Primary human diabetic dermal fibroblast and normal dermal fibroblast from donors from passage 3-5 were grown in 6-well tissue culture plates until confluence. After 12 h serum starvation, the cell monolayer was scraped with a 1000 μl pipette tip to generate a single 1-mm wide gap. After that, cells were cultured in the medium with 200 nM FMOD, 200 nM peptide, and/or 100 pM TGF-β1 for 10 h. After 10 h, photos were captured and wound areas analyzed with ImageJ. Migration rate was quantified by measuring wound gap areas right after scraping and 10 h later. Cell migration (%) = $(Gap_{0h} – Gap10h)/Gap_{0h} \times 100\%$[63].

2.8 Cell contraction

24-well cell contraction assay kit (Cell Biolabs, CBA-201, San Diego, CA) was used to determine fibroblast contraction rate. More specifically, primary human diabetic fibroblasts from donor were seeded on 24-well plate with 3 x 10^5 cells/ml in cold collagen gel working solution (500 μl/well) and incubated for 1 h at 37 °C. After collagen was polymerized, 1 mL cell culture medium was added to each well. Cells with collagen were incubated for two days and treated with 200 nM FMOD, 200 nM peptide, and/or 100 pM TGF-β1. After stress were developed, the collagen matrix was gently released with a sterile spatula to initiate cell contraction. Pictures were captured at different timepoints (0 min, 20 min, 40 min, 60 min, and 80 min) and were analyzed with ImageJ for collagen area size and contraction rate[64, 65].

2.9 Cell invasion

96-well collagen cell invasion assay kit (Cell Biolabs, San Diego, CA) was used to determine fibroblast invasion property in this experiment. 10^6 cells/ml in serum free media were added in the collagen layer of the membrane, and medium with 10% FBS was added in the feeder tray. After incubated for 15 h, invasive cells passed through the matrix layer and cling to the bottom of the membrane while non-invasive cells stay in the chamber tray. Cells on the bottom of the collagen membrane were lysed and quantified using a fluorescent dye. The fluorescence was read with a fluorescence plate reader at 480 nm/520 nm.

2.10 Cell adhesion

Human dermal fibroblast (BJ-5ta) and rat dermal fibroblasts (RDF) were cultured with DMEM with 0.5% FBS for serum starvation overnight. The next day, cells were plated

onto 96-well plates with a concentration of 5 x 10^5 cells/ml (100 μl/well)[66, 67]. Then fibroblasts were treated with full DMEM with control or 200 mM FMOD for 60 min. After medium was gently removed, cells were fixed with 4% PFA and washed three times to remove the unattached cells. Triton X-100 (Sigma-Aldrich, Germany) were used to increase cell membrane permeability and cell were stained with DAPI for cell counts. Pictures were captured after staining and analyzed with ImageJ.

2.11 Western blot

Human dermal fibroblast BJ-5ta were seeded on 10 mm^2 cell culture plate and treated with FMOD at different timepoints for 1 h, 2 h, 4 h, 6 h, 12 h, and 48 h after serum starvation. Nuclear and cytoplasmic proteins were isolated using an NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, MA)[68]. Anti-Erk1/2, JNK, p38 antibodies (abcam, MA) were used with different concentrations for protein detection.

2.12 Statistical analysis

All statistical analyses were conducted per consultation with the UCLA Statistical Biomathematical Consulting Clinic. Initial animal numbers were on the basis of an $\alpha = 0.05$, power = 0.8. Statistical analysis was computed by GraphPad Prism (GraphPad software, San Diego, CA). Data were generally presented as mean ± the standard deviation. $P < 0.05$ was considered statistical significance. One-way ANOVA and two-sample t-test were used to compare results of two groups. Individual comparisons between two groups were determined by the Mann-Whitney test for non-parametric data.

3. RESULTS

3.1 FMOD and its peptide significantly promote diabetic wound healing.

3.1.1 FMOD and its peptide reduced scar formation and accelerated diabetic wound closure

To better simulate type 2 diabetes scenario, NONcNZO10/LtJ mouse model was used in our study to determine the effects of FMOD and its peptide on diabetic wound healing. Due to its unique characteristics, NONcNZO10/LtJ mouse model exhibit an adult-onset insulin resistance phenotype, which largely mimic human type 2 diabetes[46, 47, 69]. To induce diabetes, 10-week-old male NONcNZO10/LtJ mice were fed with high-fat diet for 4-6 weeks until glucose level is over 300mg/dl[46]. After 6 weeks, only mice with diabetes were included in the experiment (Figure 2A). After creating full-thickness excisional wounds, PBS, 4mg/ml FMOD, and 10mg/ml F06-C40 were injected around wound edges. FMOD and its peptide have shown more rapid wound closure from day 2, and continued to show better wound reduction throughout the whole experiment (Figure 2B, C). At day 14, both FMOD and peptide groups showed complete wound closure, and the wound remained unclosed in control group (Figure 2C). Consistently we saw more re-epithelialization from F06-C40 group compared with the control group (Figure 2D). In addition, with PSR staining, the scar tissue formation in F06-C40 was significantly reduced compared with control group (Figure 2E). In order to normalize scar size and avoid inaccuracy from skin thickness difference, in our lab we developed a novel method to determine scar index by dividing scar size by skin thickness (Figure 1B). Control group has significant higher scar index, indicating a larger scar formation compared with peptide

group (Figure 2F). Putting together, FMOD and peptide significantly reduced scar formation and accelerate wound closure in diabetic mice.

3.1.2 FMOD and its peptide promoted angiogenesis and improved collagen architecture in diabetic mice

In order to study the mechanism of FMOD and its peptide on promoting diabetic wound healing, we stained capillaries with vWF to determine the blood vessel formation. 14 days after surgery, diabetic mice samples were stained and capillaries were calculated with image J. We have found that FMOD and its peptide showed more vWF staining, indicating increased capillary formation and improved angiogenesis (Figure 3A-D). With confocal microscope, collagen architectures were precisely captured. Collagen architectures in diabetic mice with no wounds showed a bundled, woven-like structure with thick collagen fibers. And scar tissues in diabetic mice in control group exhibit thin collagen fibers with homogeneous structure. The collagen fibers have been thickened in diabetic mice treated with F06-C40, indicating an improved extracellular matrix formation during wound healing and scar formation. The newly formed collagen fibers have more similarities with non-wound skin in treatment group compared with the control group (Figure 3E). According to our previous studies[54], the improvement of extracellular matrix and collagen architecture can be quantified and calculated with F_D and *L*. F_D provides a measurement of how completely an object fills space and increases with structure density; while *L* provides a measurement of heterogeneity and increases with more heterogeneity. Together, F_D and *L* indicate the complexity and density of collagen fibers. The lower F_D is and the higher *L* is, the more similar the collagen fiber is with non-wound skin tissue[54]. In this study, we see an increased F_D value and a decreased *L* in

scar tissue. However, the changed is altered in F06-C40 treated group (Figure 3F), indicating an improved extracellular matrix formation.

3.2 FMOD and its peptide improved diabetic fibroblast characteristics in vitro.

After confirming FMOD and its peptide accelerate wound healing, reduce scar formation, increase angiogenesis, and improve extracellular matrix formation in diabetic mouse models, we further studied the effect of FMOD and its peptide on diabetic fibroblasts to reveal the mechanism on a cellular level.

3.2.1 Hyperglycemia fibroblasts exhibit a reduced proliferation, and FMOD and its peptide do not alter proliferation rate

Our previous studies have found that FMOD effectively promote normal dermal fibroblast migration/invasion and myofibroblast maturation and function, which are the major contributions to drive wound closure[32]. However, under a diabetic scenario, molecular and cellular activities and behaviors of dermal fibroblasts could be significantly different from normal non-diabetic fibroblasts[15, 18, 70, 71]. In order to simulate hyperglycemia scenario in dermal fibroblasts, human fibroblast BJ-5ta were treated with normal glucose (NG, 5mM) and high glucose (HG, 50mM) in 24h, 48h, and 72h, and cell proliferation of dermal fibroblasts with EdU assay was tested to determine the optimal treatment time for hyperglycemia[17, 59, 61]. After 24h, we could already see a decrease of proliferation in high glucose treated group compared with normal glucose group. The decrease of proliferation showed a time-dependent manner with even lower proliferation rate after 48h and 72h treatment with high glucose (Figure 4A, B). Due to the drastic decrease of fibroblast proliferation rate after 72h of high glucose treatment, we decided to choose 48h high-glucose treatment as hyperglycemia induction time.

Cell proliferation rate was determined after fibroblast hyperglycemia scenario has been induced. After 48h pre-treated with NG or HG, fibroblasts were cultured with cell medium with FMOD or its peptide for 3.5h. The concentrations of FMOD and its peptide were based on previous studies[32]. Then cell proliferation rate was tested with EdU assay. Cell proliferation was decreased after high glucose treatment, however, FMOD and its peptide do not alter dermal fibroblast proliferation in either normal fibroblast or hyperglycemia fibroblasts (Figure 4C, D).

3.2.2 FMOD and its peptide alter TGF-β1 mediated cell migration in primary diabetic fibroblasts

Cell migration is a hallmark of wound repair and skin cells migrate from wound edges into the wound to restore skin integrity in cutaneous wound healing[72, 73]. To further determine the effects of FMOD and its peptide on dermal fibroblast migration during wound healing, we performed scratch assay since this simple method mimics to some extent migration of cells *in vivo*[74]. We cultured primary human normal and diabetic fibroblast from two donors to better study the effect of FMOD and its peptide. When we treated primary human normal and diabetic fibroblasts with FMOD or TGF-β1, we did not detect any significant difference between control group and treatment group (Figure 5), however, when normal primary fibroblasts were treated with FMOD and TGF-β1, there is a significant increase in cell migration after 10h, indicating FMOD alters TGF-β1 mediated cell migration in normal human fibroblasts. In diabetic fibroblasts, although there is no significant difference between FMOD and control groups, we could see a tendency of increase in cell migration after treating with FMOD and TGF-β1. Interestingly, the peptide F06-C40 can independently accelerate cell migration without the treatment of TGF-β1,

and the cell migration rate is further elevated with the presence of both peptide and TGF-β1 (Figure 5), indicating the peptide has the ability to improve cell migration both TGF-β1 mediated and non-TGF-β1 mediated pathways. Therefore, in primary normal fibroblasts we found FMOD accelerate cell migration in the presence of TGF-β1 and F06-C40 promotes cell migration without TGF-β1 and the effect is further elevated with the presence of TGF-β1. While in primary diabetic human fibroblast, we see an increase trend of cell migration with the treatment of FMOD and TGF-β1, although it has no statistical significance. And F06-C40 significantly increase cell migration and the effect is further improved with TGF-β1 with the same pattern as normal fibroblasts.

3.2.3 FMOD and its peptide accelerate cell contraction and invasion in primary human diabetic fibroblasts

Other than cell proliferation and migration, cell contraction/invasion is also an essential healing response that functions to reduce the size of the tissue defect and subsequently decrease the amount of damaged tissue that needs repair, which involves myofibroblasts in existing fibers and surrounding margins of the wound[23, 75, 76]. The impairment of cell contraction in diabetic patients[77, 78] made it harder for wound and extracellular matrix to perform similar as normal skins. That also plays a role in delayed diabetic wound healing. To further elucidate the role of FMOD on primary human diabetic dermal fibroblasts, we seeded cells into collagen gel to simulate extracellular matrix structure and treated cells with FMOD, F06-C40, and/or TGF-β1 for 2 days, and initiated contraction for 80 min and captured images of collagen gel every 20 minutes. We found that TGF-β1 promotes primary diabetic fibroblast contraction (Figure 6A, B) similar as it promotes normal dermal fibroblast contraction as previous studies have shown[79, 80].

FMOD and its peptide both increase primary diabetic fibroblast contraction, however, the increase of contraction rate was not as significant as that induced from TGF-β1 (Figure 6A, B). When diabetic cells were treated with both FMOD and TGF-β1, or F06-C40 and TGF-β1, we could see an even higher elevation of contraction rate (Figure 6A, B). It indicated that both FMOD and F06-C40 increase diabetic fibroblast contraction, and the contraction was further accelerated with the presence of TGF-β1, possibly due to the myofibroblast differentiation induced with TGF-β1.

The migration of fibroblast is an essential and rate-limiting step to repair wound due to its central role in the formation of granulation tissue[81, 82], and it is one of the main steps that delays diabetic wound healing. In order to fully investigate the function of FMOD and F06-C40 during diabetic wound healing, invasion assay of diabetic fibroblasts was performed after primary normal and diabetic fibroblasts were treated with FMOD, F06-C40, and/or TGF-β1. And then fluorescence of cell lysate was read to determine cell counts that successfully went through the invasion membrane. We found that in primary normal fibroblasts FMOD itself did not increase cell invasion, but cell invasion was increase with the presence of both FMOD and TGF-β1 (Figure 6C). Interestingly, peptide itself already promotes cell invasion in the primary normal fibroblasts and the cell invasion was further increased with the presence of TGF-β1. However, in primary diabetic fibroblasts, we did not see a significant increase of cell invasion after FMOD and/or TGF-β1 treatment, although we did see a trend of increase. With F06-C40 treatment, it also did not show an increase of cell invasion in primary diabetic fibroblasts, however, with the presence of TGF-β1, we could see a significant increase of cell invasion in primary

diabetic fibroblasts compared with the control group (Figure 6C). These indicated that in diabetic scenario, the effects on FMOD and its peptide on cell invasion are not as significant as it on normal dermal fibroblasts, however, they have the potential to induce a higher increase of cell invasion in diabetic dermal fibroblasts with the presence of TGF-β1.

3.3 FMOD promotes fibroblast cell adhesion via integrin

During wound healing process, fibroblast cell adhesion plays an essential role[83-86], thus we examined cell adhesion of fibroblast after FMOD treatment. Different concentrations of FMOD were administrated in human fibroblasts and rat dermal fibroblast (RDF), and we found FMOD enhanced fibroblast cell adhesion both in human fibroblast and RDF in a dose-dependent manner (Figure 7A-D). To determine the underlying mechanism of the effect of FMOD on increasing cell adhesion, we used a general integrin antagonist[66], RDG, that broadly bond with integrin ligands on cells to test if the cell adhesion elevation is mediated via integrin. When we treated human and rat dermal fibroblasts with RGD, or negative control (EDTA), fibroblast cell adhesion was significantly diminished (Figure 7E, F), indicating RGD was able to decrease cell adhesion by blocking integrin signaling. To confirm if FMOD mediates fibroblast cell adhesion through integrin, different concentrations of RGD was treated together with FMOD in both human and rat dermal fibroblasts. As a result, RGD successfully diminished FMOD mediated cell adhesion elevation in human fibroblasts with a concentration over 50μm (Figure 7G), and in RDF the effect was diminished from 10μm of RGD (Figure 7H), indicating FMOD may enhance cell adhesion through integrin. We would like to further investigate the specific integrin which FMOD mediated, thus different anti-integrin

antibodies that target varies integrin ligands were used in this study. Antibodies that targeted ligand α1, α5, α6, α9, αv, and ligand β1, β2 successfully reduced FMOD mediated cell adhesion (Figure 7I), indicating FMOD potentially increasing fibroblast cell adhesion through integrins with those ligands.

3.4 FMOD alters TGF-β1 non-canonical signaling pathway

Previous studies have shown FMOD selectively increases Smad2/3 pathway and downregulates AP-1 TGF-β1-mediated pathway. By delicately orchestrating TGF-β1 pathway, FMOD promotes TGF-β1 mediated fibroblast migration and contraction, and reduces TGF-β1 mediated extracellular matrix accumulation and fibrotic scar formation[32]. In addition, in this study we discovered FMOD itself does not alter fibroblast cell migration and invasion, but rather, FMOD with the presence of TGF-β1 increase cell migration and invasion. Therefore, further investigation of the effect of FMOD on TGF-β1 signaling pathway is needed to better elucidate the molecular mechanisms. Since the effect of FMOD on canonical TGF-β1 Smad2/3 pathway has been studies previously[32], in this study, we focused more on the effect of FMOD on non-canonical TGF-β1 MAPK pathway[87-89]. More specifically, TGF-β pathway Erk, JNK and p38 MAPK signaling cascades were studied in this experiment. BJ-5ta fibroblasts were treated with FMOD, TGFβ1, or both FMOD and TGF-β1 on different time points at 1h, 2h, 4h, 6h, 12h, 48h, respectively, to determine changes in MAPK signaling pathway over time. In Erk signaling pathway cascades, FMOD itself does not alter Erk signaling pathway, however the elevation of Erk expression by TGF-b1 was diminished with the presence of FMOD at 2h, 4h, and 12h (Figure 8A, C), and Erk activation from TGF-β1 was also diminished with the

presence of FMOD at 2h, 4h, and 12h, which was indicated by phosphorylated Erk (Figure 8A, D). In JNK pathway, similar pattern was shown with FMOD diminishing the activation of JNK pathway mediated by TGF-β1 at 2h and 4h (Figure 9). And FMOD also decreased the elevation of p38 activation mediated by TGF-β1 at 4h of treatment (Figure 10). Thus TGF-β1 activates downstream MAPK pathways, and with the presence of FMOD, the activation of non-canonical TGF-β1 MAPK pathway was decreased. And FMOD potentially promote wound healing by modulating TGF-β1 pathways.

4. DISCUSSION

Wounds, especially diabetic non-healing wounds have been affecting millions of patients worldwide[3]. Amputation and even death are not uncommon for uncontrolled non-healing diabetic wounds[90], which bring tremendous pain to patients physically, psychologically, and mentally. The alterations in diabetes at a cellular and molecular level, including the imbalanced production of growth factors and extracellular matrix components, insufficient angiogenesis, increased inflammation, made diabetic wounds much harder to heal and treat. Interestingly, FMOD and its peptide have shown to restore extracellular matrix, and cell functions after wounds and promote angiogenesis[31, 33, 53, 63], which is required for diabetic wound healing. In this study, I focused on revealing the effect of FMOD and its peptide on diabetic wound healing, and elucidating the underlying mechanism of FMOD on promoting healing process in both cutaneous and diabetic wounds.

To fully simulate type II diabetes scenario, diabetic NONcNZO10/LtJ mouse model was used and high glucose was induced with high fat diet. *In vivo*, FMOD and its peptide both showed a significant improvement on diabetic NONcNZO10/LtJ mice wound healing and scar size (Figure 2). They significantly reduce scar size, accelerate wound closure and re-epithelialization, promote angiogenesis, and ameliorate extracellular matrix formation and collagen architecture, which are all essential for wound healing process. Interestingly, when we compared the wound size between control and treatment groups, a larger difference in wound area reduction between control and treatment group was observed at the early stage (day 2-6 post operation) than later stage (day 10-14 post operation). As we all know, wound healing generally involves three main stages: acute

inflammation, proliferation and remodeling, resulting in the formation of a scar[91-93]. The larger difference in the earlier stage indicates FMOD and its peptide may have a more significant effect on the inflammation and proliferation stage than remodeling. It is consistent with our observation that diabetic control group shows less capillary formation (Figure 3) and more inflammation on the appearance than FMOD group. The potential effect of FMOD on reducing inflammation is very promising because one of the most important defects in diabetic wound healing is the impairment of inflammatory response and angiogenesis. However, more studies need to be performed to validate the effect of FMOD and its peptide on different stage of healing process. For example, cytokines and growth factors including fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), and neutrophils and macrophages need to be studied to determine the effect of FMOD on the inflammatory stage during diabetic wound healing. In addition, granulation tissue formation and myofibroblasts differentiation during diabetic wound healing need to be determined to validate the effect of FMOD and its peptide on proliferation stage. *In vitro* data shows FMOD and F06-C40 are more capable of improving fibroblasts migration, invasion, and contraction during normal wound healing process than diabetic wound healing process. In diabetic fibroblasts, FMOD shows a tendency of increased migration and contraction, however, not statistically significant, while F06-C40 shows a better performance, and the effect is further improved with the presence of TGF-β1. TGF-β1 is an important molecule for myofibroblast differentiation and fibrosis formation[94, 95]. The further elevation of migration and contraction in primary diabetic fibroblasts with the presence of TGF-β1 may suggest myofibroblast differentiation may play a role in the improvement of diabetic wound healing mediated by

FMOD and its peptide. In addition, the difference of efficacy of FMOD between normal and diabetic wound healing may be due to the impairment of healing process in diabetic scenario. In this study, primary dermal fibroblast from only two donors were studied. The small sample size is not able to rule out the individual difference. Dermal fibroblasts from more donors and varies ages, races, genders of donors need to be included for a more consistent result. Furthermore, the effect of FMOD and its peptide on diabetic keratinocytes and endothelial cells migration and proliferation is indispensable in order for us to fully study its re-epithelialization and angiogenesis in diabetic scenario.

TGF-β1 plays an essential role in wound healing process and scar formation. During wound healing process, TGF-β1 expression promotes migration and activation of inflammatory cells, re-epithelialization and angiogenesis, thus accelerates inflammation and proliferation phase during healing process[96]. During remodeling stage, TGF-β1 promotes fibroblasts to myofibroblast transition, which leads to scar formation[96]. The high expression of TGF-β1 is correlated with better re-epithelialization and angiogenesis, but also create excessive scar formation and fibrosis. Therefore, the double-edge sword effect of TGF-β1 during wound healing makes it important to fully study TGF-β1 mediated pathways and elucidate the mechanisms underlying. FMOD has been proven to be a TGF-β modulator and it does not just simply blockade TGF-β1 signaling pathway during skin wound regeneration, but rather delicately modulates TGF-β isoforms and their activities[53]. For example, FMOD promotes TGF-β1-induced Smad pathway during wound healing for desirable activities such as cell migration, while blocks undesirable activities such as excessive scar tissue formation [31, 63, 97]. However, the effect of FMOD on noncanonical TGF-β1 pathway remains unknown. In this study I focused on

determine the effect of FMOD on TGF-β1-induced noncanonical MAPK pathways, including Erk, p38 and JNK. Erk is required for disassembly of cell adherens and in cell culture systems, phosphorylation of Smads by Erk inhibits Smad activity[87, 88]. While JNK/p38 pathway is activated by TGF-β1 independently of Smads, TRAF6-TAK1-JNK/p38 pathway plays an important role in TGF-β1 induced apoptosis in conjunction with Smad-dependent pathway[88]. In our study, FMOD downregulated TGF-β1-induced MAPK pathways at the early stage in Erk, p38, and JNK signaling pathways, indicating FMOD may reduce inhibition of Smad activity mediated by Erk and mediate cell apoptosis during wound healing. It is consistent with previous studies and the effect of FMOD on wound healing, although further investigation is needed to validate the function of FMOD on TGF-β1 noncanonical pathways.

The healing of skin and oral mucosa shows similar processes during tissue repair, however, human gingiva and oral mucosa often exhibit a scarless wound healing[98] similar to fetal healing[99], whereas cutaneous wound healing shows more scarring compared with gingival wound healing. It shows an improved angiogenesis, reduced inflammation, Interestingly, FMOD is essential for scarless wound healing process and the lack of FMOD altered scarless wound healing in fetus. In addition, FMOD showed similar characteristics of improved angiogenesis and reduced inflammation. Therefore, FMOD expression and function during oral mucosa healing may be studied as a future direction.

In summary, FMOD and its peptide promotes diabetic wound healing, reduces diabetic scar formation, improves extracellular matrix formation and angiogenesis in diabetic mice. In vitro, FMOD and its peptide promotes diabetic fibroblast migration,

invasion and contraction. During cutaneous wound healing process, FMOD promotes cell adhesion via integrin, and delicately modulates TGF-β1-induced signaling pathways. In future study, the effect of FMOD during early stage of wound healing such as inflammation and proliferation needs to be further investigated to fully elucidate the underlying mechanism of diabetic wound healing.

5. FIGURES AND TABLES

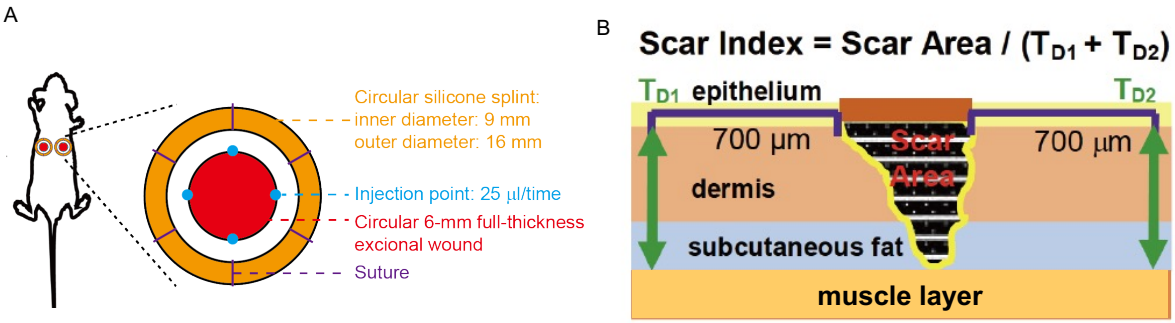


Figure 1. Schematic diagram.

A. Full thickness excisional wound model. The diagram of the splinted excisional wound model on the back of adult male NONcNZO10/LtJ mice. Two circular 6-mm full-thickness excisional wounds were performed on the back of each adult mice. Each wound was fastened with double layers of circular silicone splint with the inner diameter as 9-mm and outer diameter as 16-mm. Fibromodulin and its peptide was injected at 4 points around the wound edge, 25μl/point. The injection was repeated every two days for 14 days in total.

B. Scar Index of scar area. Images were captured from polarized microscope after PSR staining. Two dermal thickness measurements were taken 700 μm away from the left and right wound edge per sample. And the fibrotic scar tissue area was outlined and measured to extend from the base of the epidermis to the muscle layer. The Scar index was calculated by dividing the scar area by the corresponding average dermal thickness.

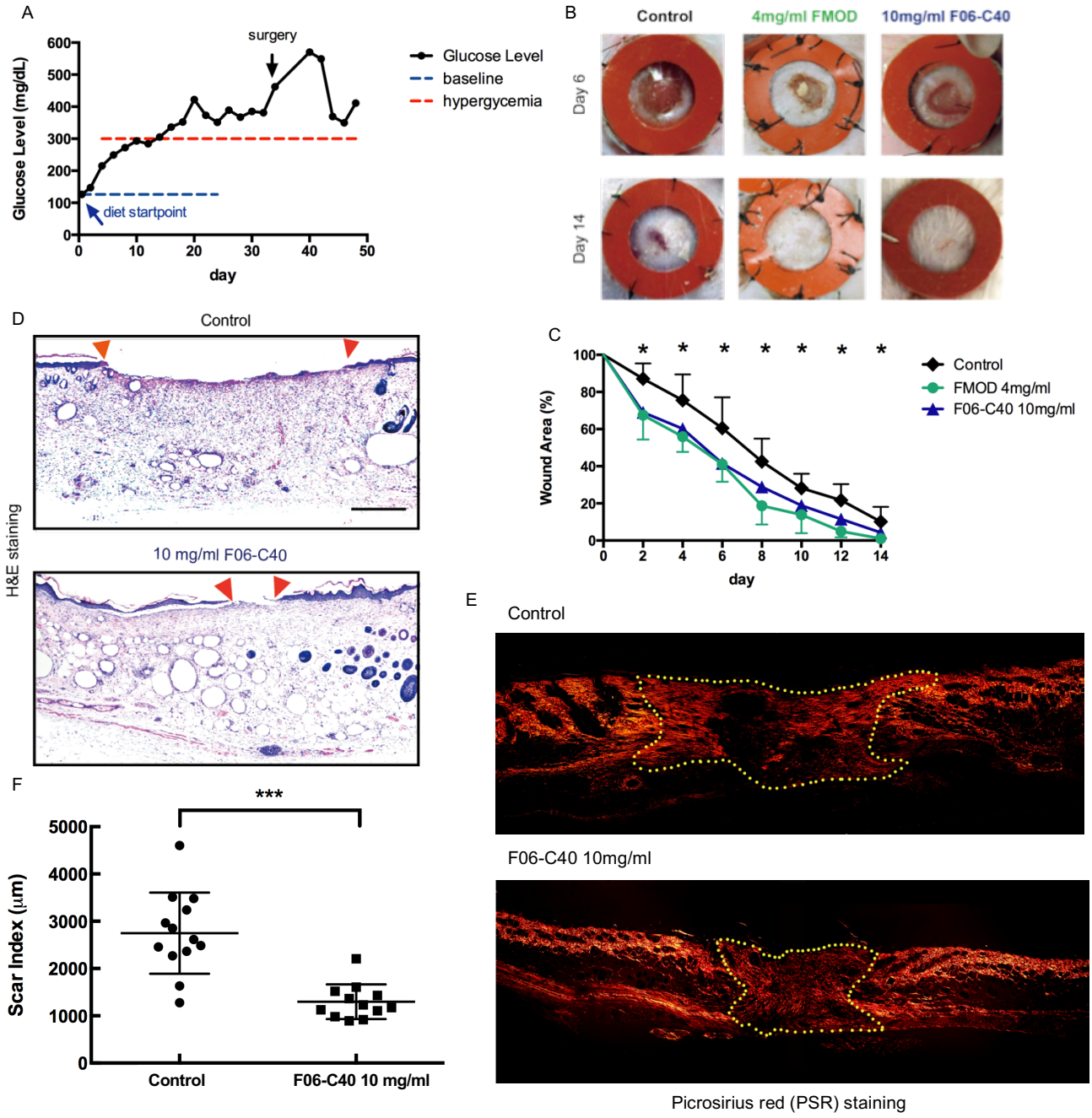


Figure 2. FMOD and its peptide promote wound closure and reduce scar formation.

A. Glucose level for diabetic mice throughout the experiment. Glucose level for diabetic mice were measured twice every day throughout the whole experiments. High fat diet started 4-6 weeks before surgery.

The blue dot line is the baseline for diabetic mice before diabetes induction with high fat diet; all mice included in the experiment have glucose level over 300 mg/dl (red dot line); B. Gross view of the wounds of diabetic mice 6 days and 14 days post-op after FMOD (4mg/ml) and its peptide (10mg/ml) administration; C. Statistical analysis of wound areas quantified at day 0, 2, 4, 6, 8, 10, 12, and 14. Expressed as a percentage of the initial wound size. Results given as mean +/- SD. *, $P < 0.05$; D. H&E staining for re-epithelization after peptide injection. Red arrows show the re-epithelialization and wound edge; E. PSR staining shows scar formation of diabetic mice after peptide injection, red dot line circled scar area; F. Statistical analysis of scar index after F06-C40 administration.

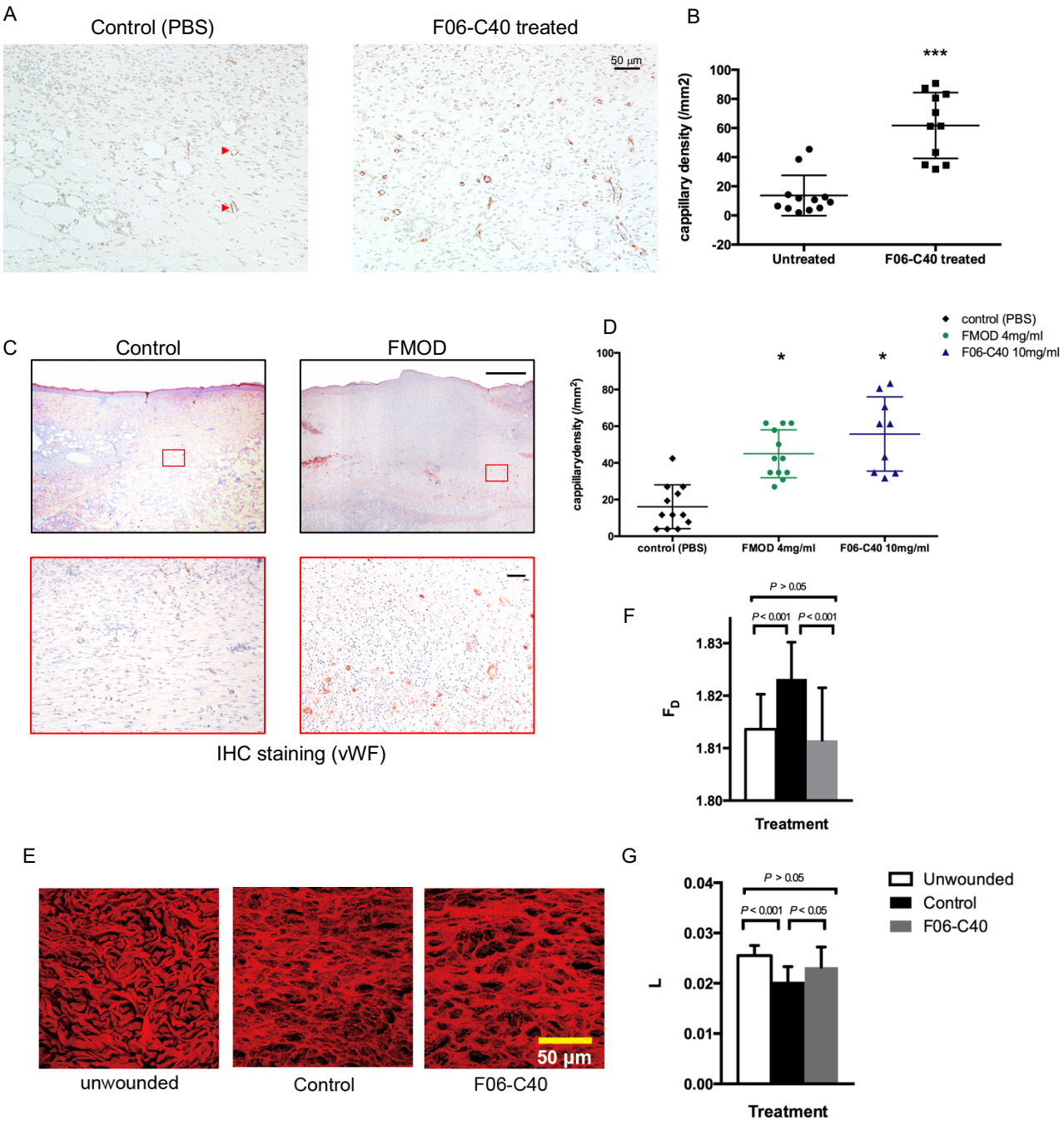


Figure 3. FMOD and its peptide promote angiogenesis and improve extracellular matrix formation.

A. IHC staining for vWF with 200x magnification in diabetic NONcNZO10/LtJ mice 14 days post-op. Red arrows showed new capillary formation in control group; B. Quantification analysis for capillary density 14 days post op in normal and diabetic after NONcNZO10/LtJ mice; C. IHC vWF staining in diabetic NONcNZO10/LtJ mice 14 days post-op. Images on the top with black square showed the 40x magnification,

the area circled with red square on the top images were captured in 200x magnification and showed as images below; D. Quantification analysis for capillary density, green dots are FMOD group and blue triangles are peptide group; E. Confocal images (630x magnification) for collagen architecture after PSR staining; F. F_D analysis for confocal image scar area. F_D value ranges from 1 to 2 and shows the structure density and how complete the objects fill in the image. The lower F_D is, the more similar it is to normal collagen architecture; G. *L* analysis for confocal image scar area. *L* value ranges from 0 to 1 and shows the heterogeneity of the image. The higher *L* is, the more similar it is to normal collagen fibers.

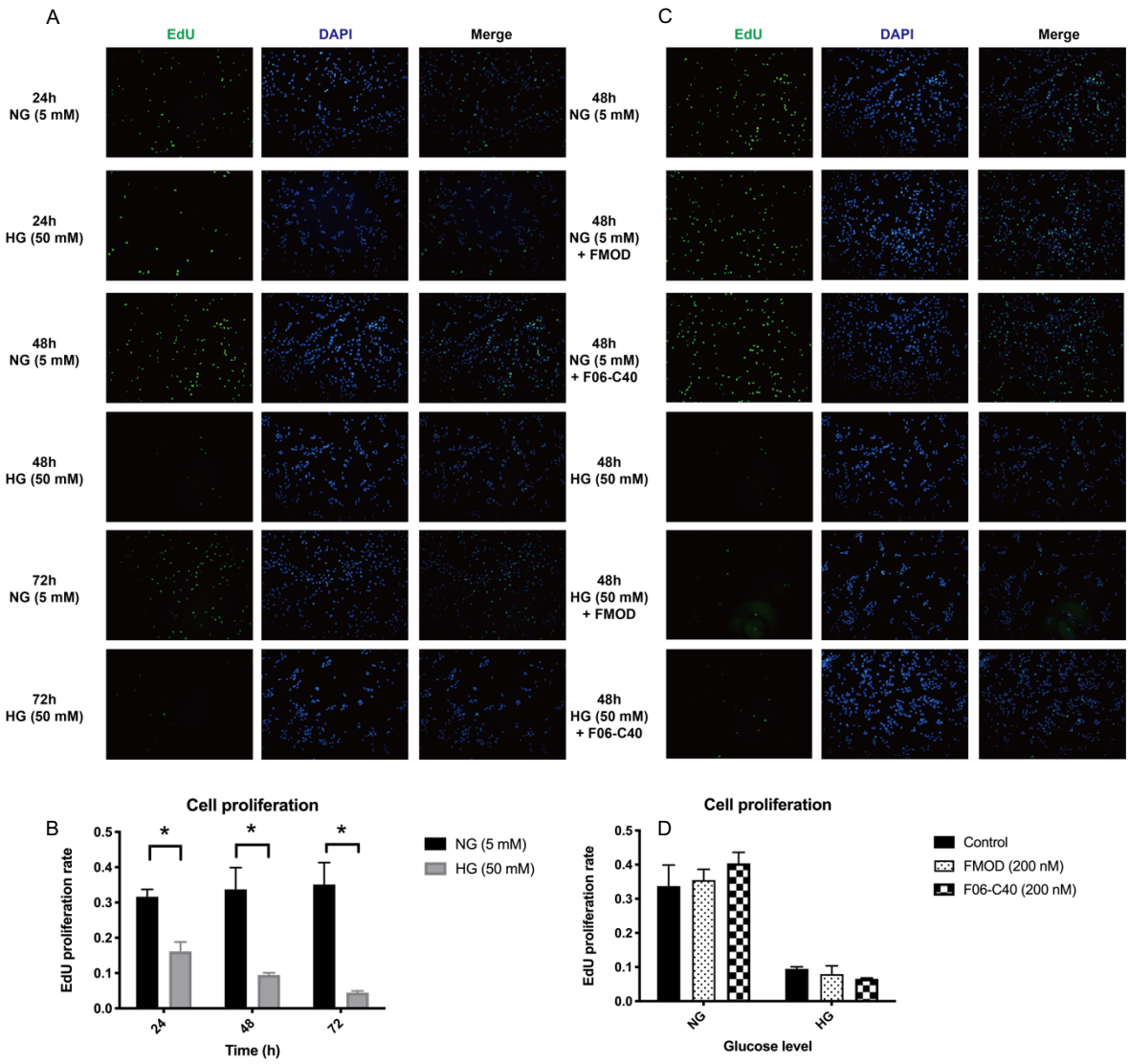


Figure 4. Cell proliferation in hyperglycemia human fibroblast BJ-5ta.

A. Hyperglycemia induction. Human fibroblast BJ-5ta was treated with high glucose (50 mM) and normal glucose (5 mM) for 24 h, 48 h, and 72 h, respectively. EdU assay was performed to determine proliferation rate. Proliferated cells (green) were stained with iFluor 488 azide dye. And cell counts were determined with DAPI (blue). Merge images show the proliferation rate; B. Quantification analysis for cell proliferation after NG and HG treatment at different time points. *: $p < 0.05$; C. EdU assay for proliferation rate with treatment of control, FMOD, and peptide for 3.5 h. BJ-5ta cells were pre-treated with NG/HG for 48 h for hyperglycemia induction; D. Quantification analysis for proliferation rate.

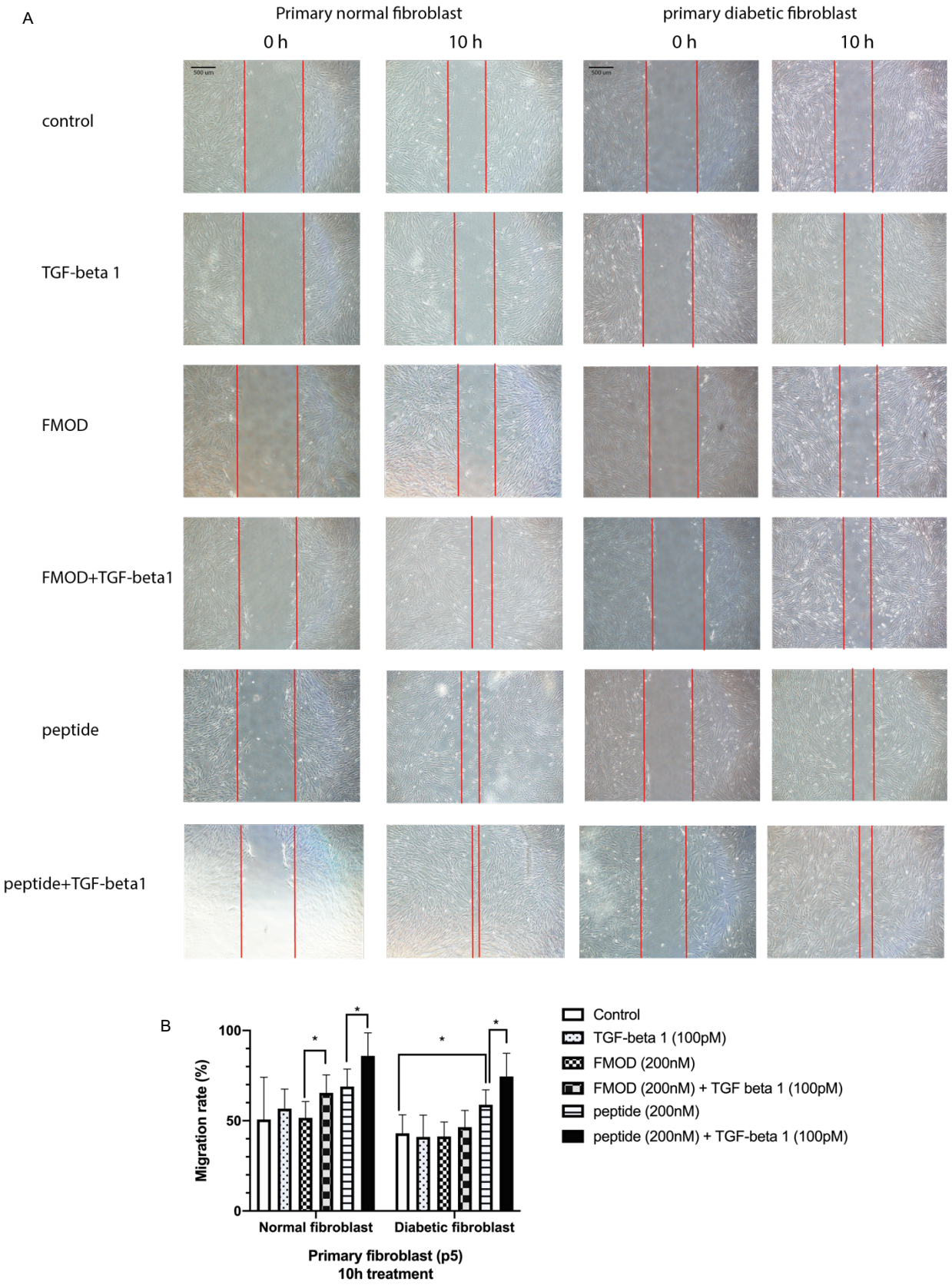

A
Primary normal fibroblast
primary diabetic fibroblast
0 h
10 h
0 h
10 h
control
TGF-beta 1
FMOD
FMOD+TGF-beta1
peptide
peptide+TGF-beta1
B
Migration rate (%)
100
50
0
Normal fibroblast
Diabetic fibroblast
Primary fibroblast (p5)
10h treatment
Control
TGF-beta 1 (100pM)
FMOD (200nM)
FMOD (200nM) + TGF beta 1 (100pM)
peptide (200nM)
peptide (200nM) + TGF-beta 1 (100pM)

Figure 5. Scratch assay for both normal and primary diabetic dermal fibroblasts.

A. Scratch assay. Wound area was generated with 1000 ml pipette tip for 1 mm gap. Primary human normal and diabetic fibroblasts (passage 5) were treated with FMOD, F06-C40, and/or TGF-β1 for 10 h. Red line shows the wound area before (0 h) and after (10 h) treatment; B. Quantification analysis for scratch assay.

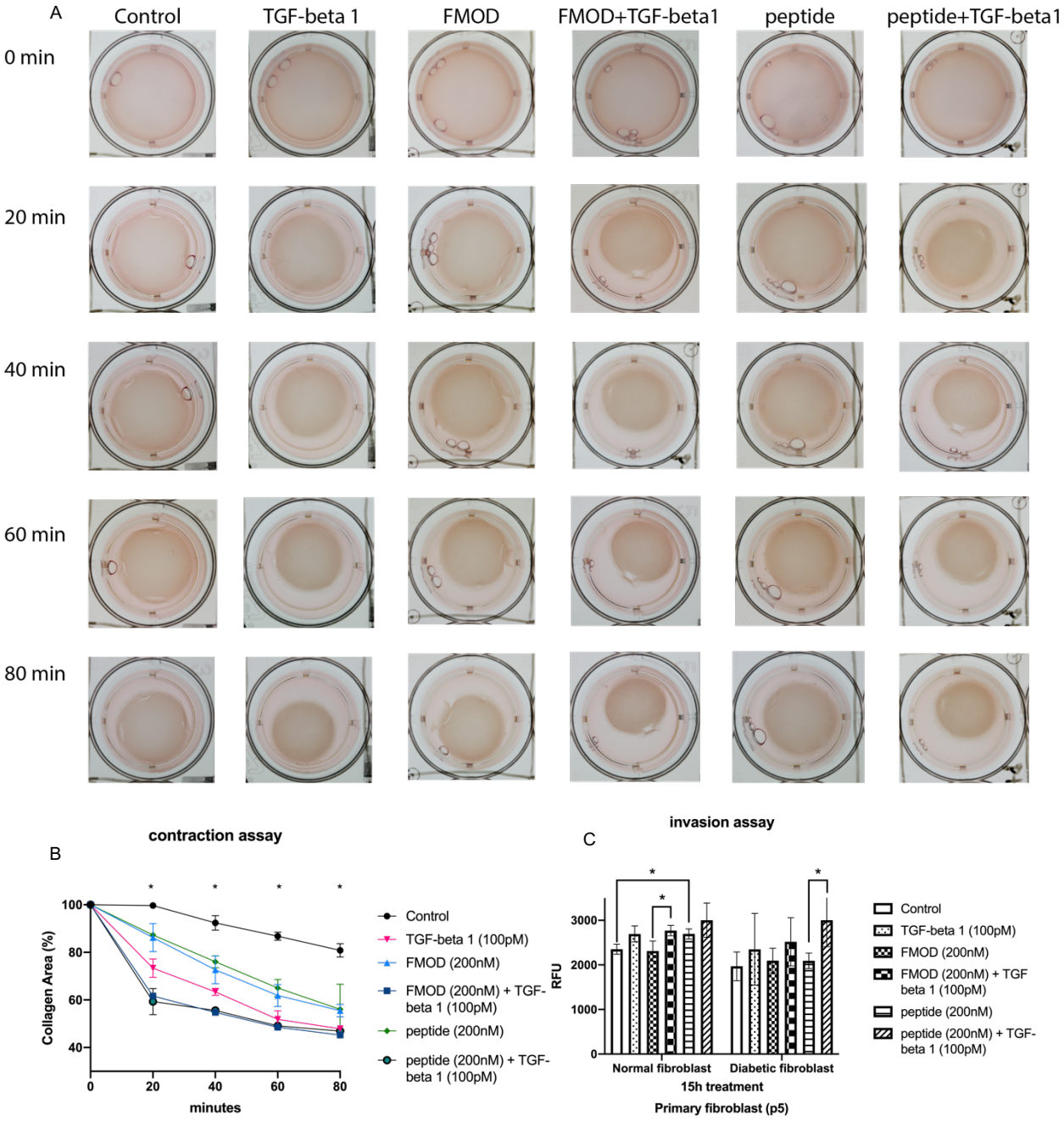
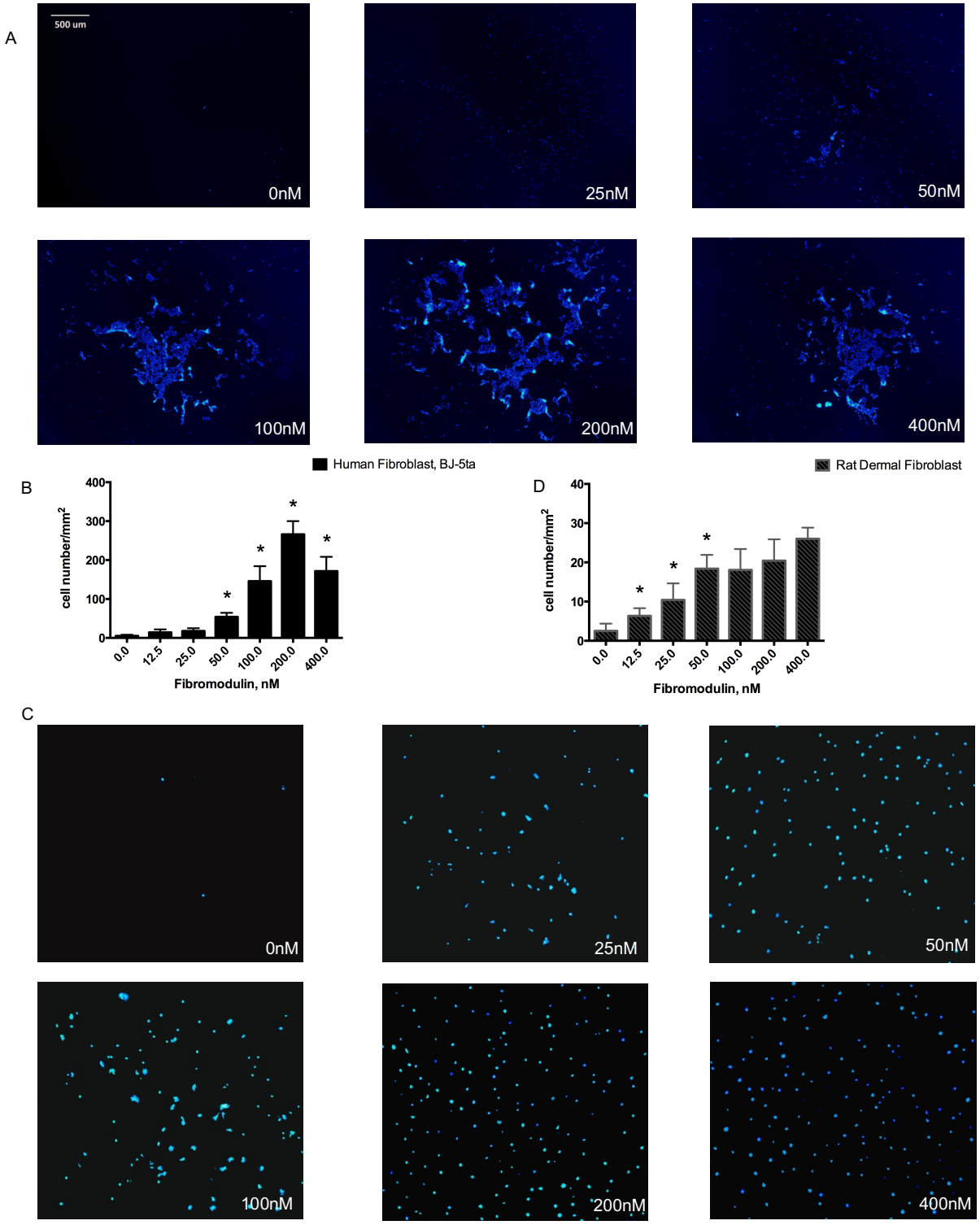


Figure 6. Cell contraction and invasion assay for diabetic fibroblasts.

A. Contraction assay for primary human diabetic fibroblasts. Human primary diabetic fibroblasts (passage 4) were seeded onto contraction collagen gel, and were treated with FMOD, peptide, and/or TGF-β1 for 80 min. Pictures were captured at 0 min, 20 min, 40 min, 60 min, and 80 min. Collagen gel area was determined and calculated with ImageJ; B. Quantification analysis for collagen area (%). Collagen area (%) was

calculated with $Area_{target}/Area_{0min}$ x 100%. *: $p < 0.05$; C. Invasion assay. Quantification analysis of invasion rate of primary human normal and diabetic fibroblasts (passage 5) after treated with FMOD, peptide, and/or TGF-β1 for 15 h. Invasion rate was shown with the fluorescence intensity of cell lysate. *: $p < 0.05$.


A
500 um
0nM
25nM
50nM
100nM
200nM
400nM
B
Human Fibroblast, BJ-5ta
cell number/mm2
Fibromodulin, nM
0.0
12.5
25.0
50.0
100.0
200.0
400.0
D
Rat Dermal Fibroblast
C

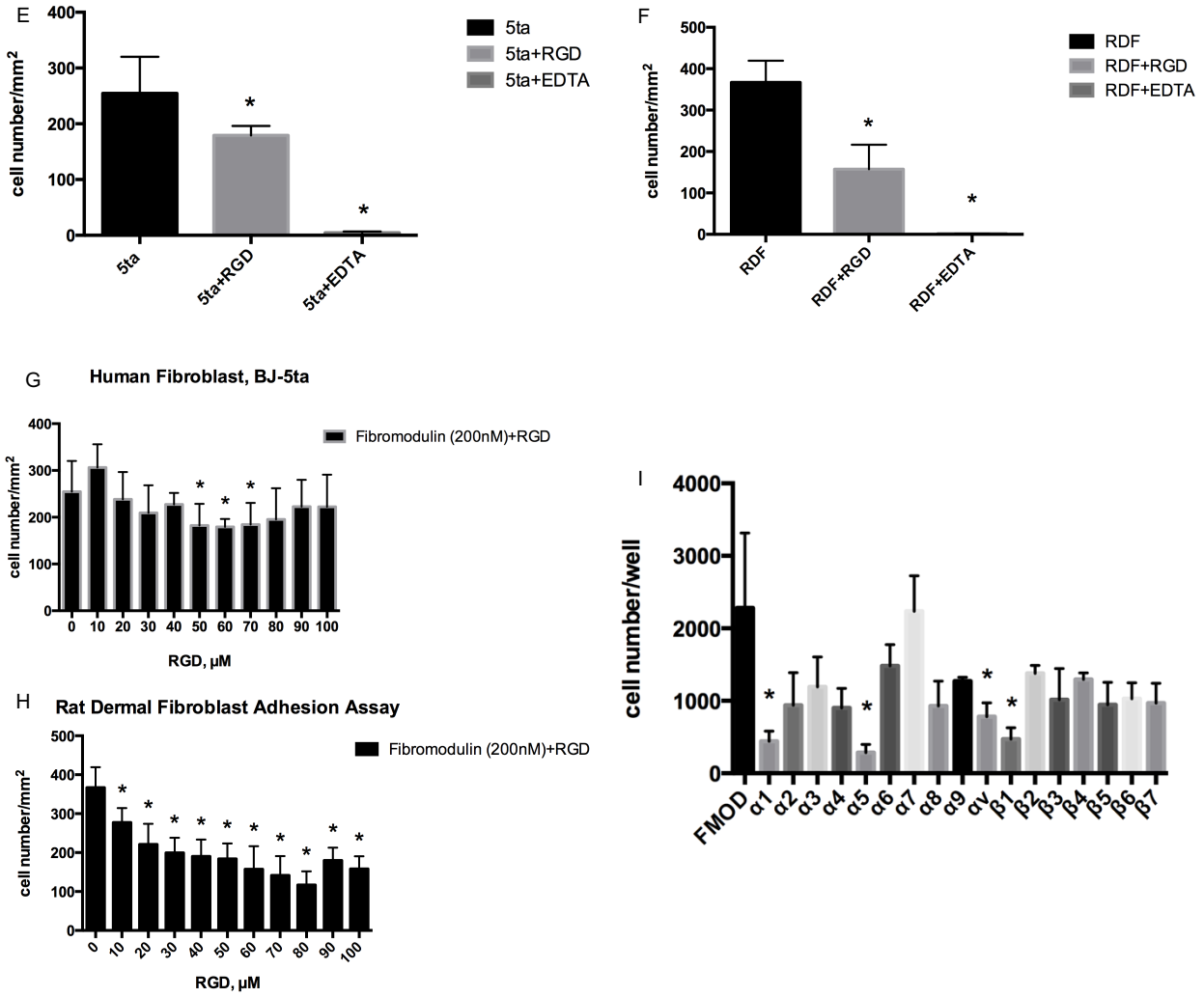


Figure 7. Cell adhesion of both human fibroblasts and rat dermal fibroblast (RDF).

A. Adhesion assay for human dermal fibroblasts BJ-5ta after treated with FMOD with a concentration of 0 mM, 25 mM, 50 mM, 100 mM, 200 mM, and 400 mM, respectively. Cell counts were shown with DAPI (blue) and calculated with ImageJ; B. Quantification analysis for adhesion assay for human dermal fibroblasts; C. Adhesion assay for rat dermal fibroblasts after treated with FMOD with a concentration of 0 mM, 25 mM, 100 mM, 200 mM, and 400 mM, respectively. Cell counts were shown with DAPI (blue) and calculated with ImageJ; D. Quantification analysis for adhesion assay for rat dermal fibroblasts; E. Cell adhesion of human fibroblast BJ-5ta after treated with integrin antagonist RGD, and negative control EDTA, cell adhesion was determined with cell density (cell counts per mm^2); F. Cell adhesion of rat dermal fibroblasts after treated with integrin antagonist RGD, and negative control EDTA, cell adhesion was determined with cell density (cell counts per mm^2); G. Cell adhesion of human dermal fibroblast BJ-5ta after treated with FMOD and

different concentration of integrin antagonist RGD to determine whether the elevation of cell adhesion from FMOD can be diminished by integrin antagonist RGD and the optimal dose of RGD to diminish the effects of FMOD, *: $p < 0.05$; H. Cell adhesion of rat dermal fibroblasts after treated with FMOD and different concentration of RGD to determine whether RGD can diminish the effect of FMOD on RDF, *: $p < 0.05$; I. Cell adhesion of human dermal fibroblast BJ-5ta after treated with FMOD and different antibodies against integrin ligands to determine which ligands might play a role in the elevation of cell adhesion mediated by FMOD.

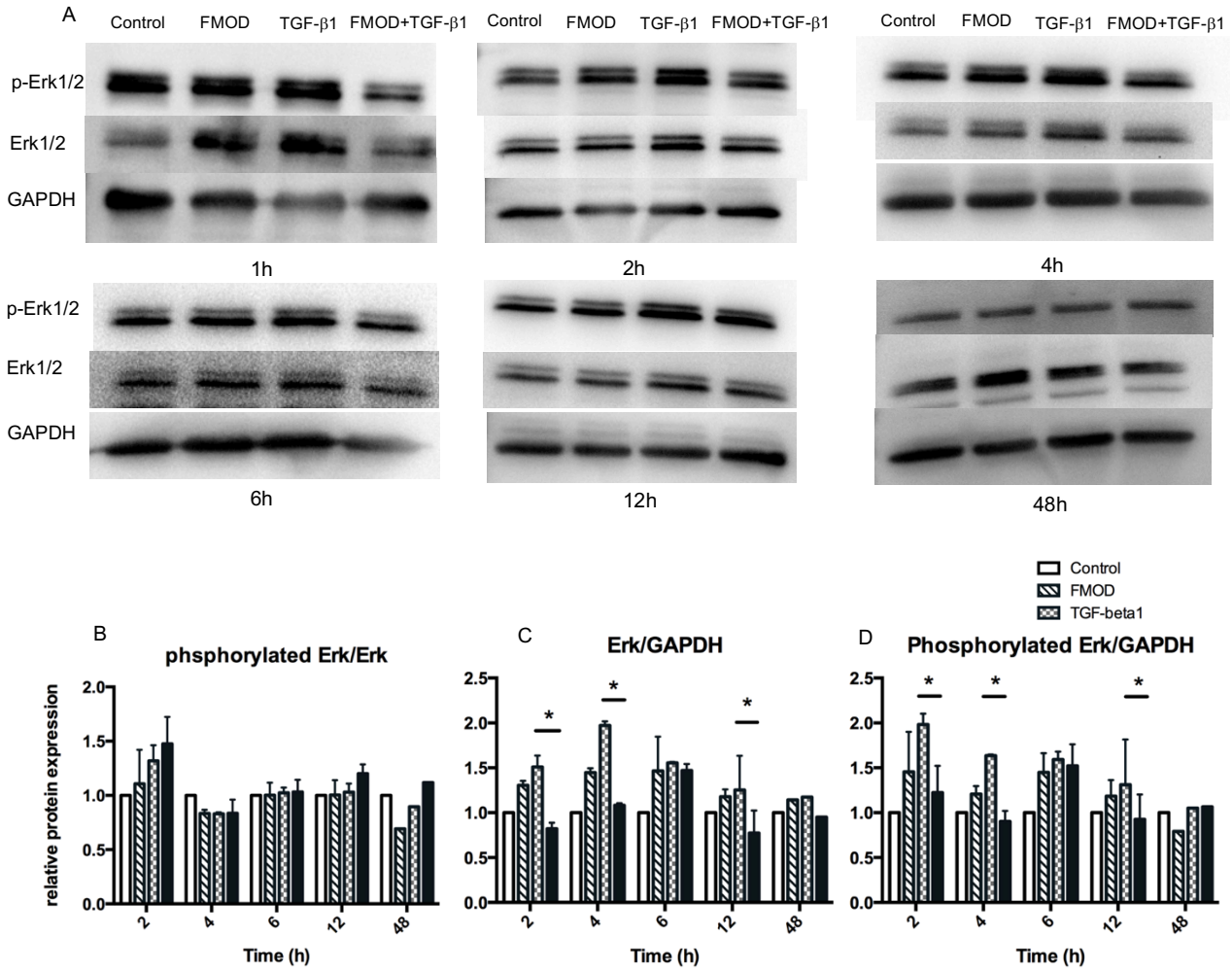


Figure 8. Western blot for Erk ½ signaling pathway.

A. Western blot analysis for Erk ½ pathway after treated with FMOD for 1 h, 2 h, 4 h, 6 h, 12 h, and 48 h, respectively. GAPDH as control; B. Quantification analysis for protein expression of Erk activation (phosphorylated Erk) relative to total Erk protein; C. Quantification analysis for total Erk protein expression; D. Quantification analysis for activated Erk (phosphorylated Erk).

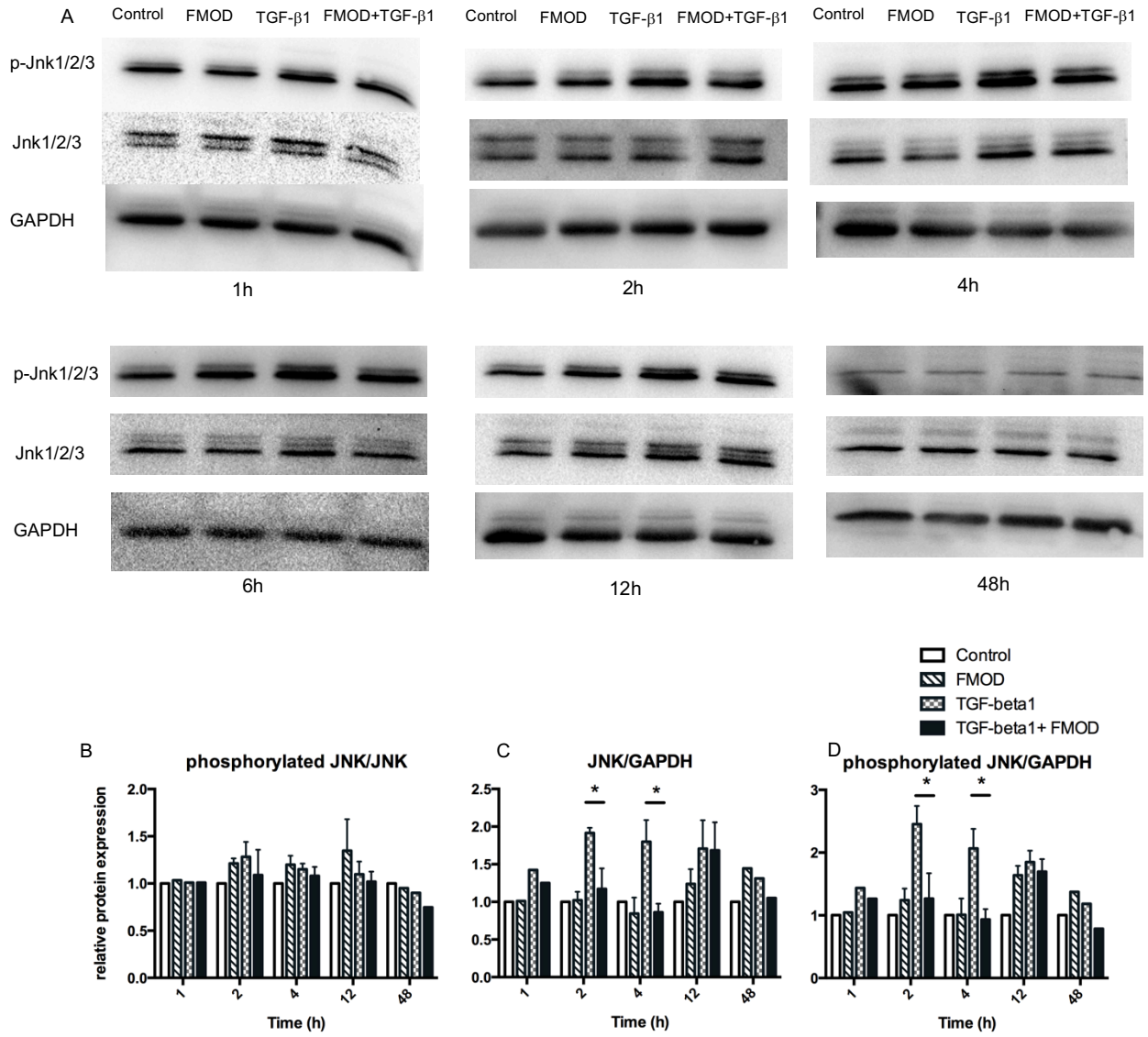


Figure 9. Western blot for JNK1/2/3 pathway.

A. Western blot analysis for JNK 1/2/3 pathway after treated with FMOD for 1 h, 2 h, 4 h, 6 h, 12 h, and 48 h, respectively. GAPDH as control; B. Quantification analysis for protein expression of JNK activation (phosphorylated JNK) relative to total JNK protein; C. Quantification analysis for total JNK protein expression; D. Quantification analysis for activated JNK (phosphorylated JNK).

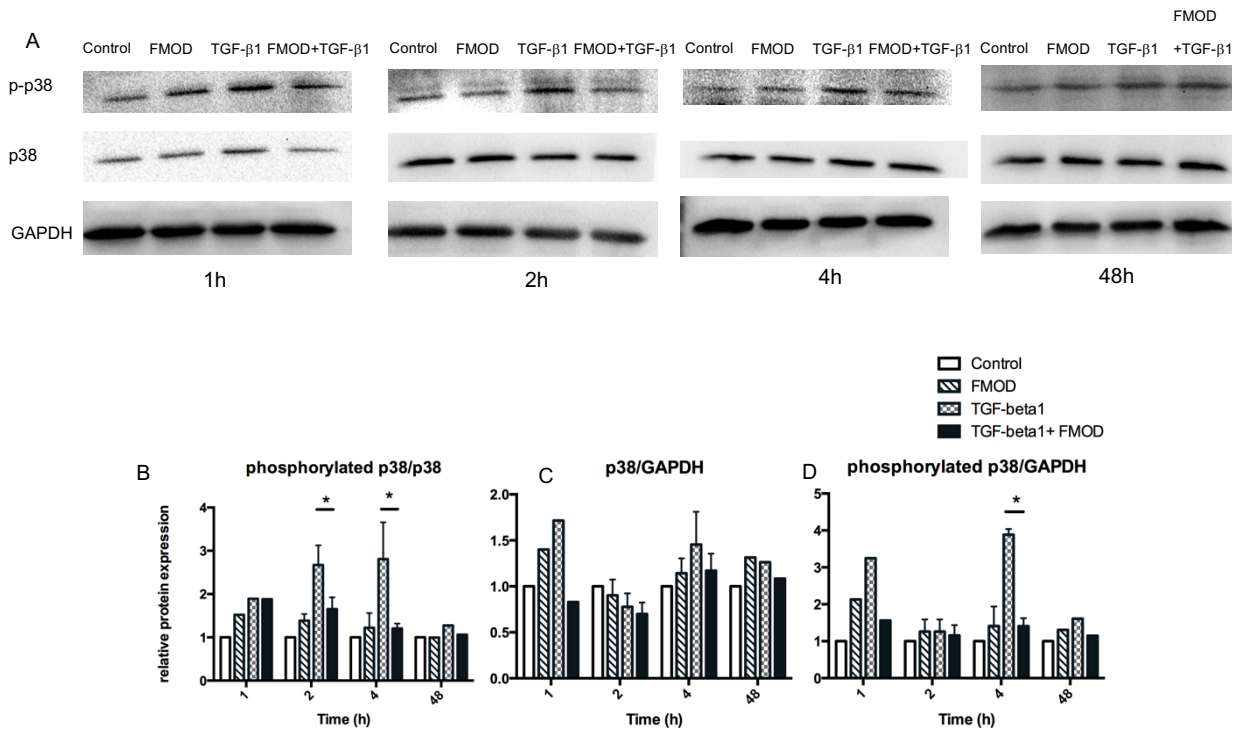


Figure 10. FMOD effect on p38 pathway.

A. Western blot analysis for p38 pathway after treated with FMOD for 1 h, 2 h, 4 h, 6 h, 12 h, and 48 h, respectively. GAPDH as control; B. Quantification analysis for protein expression of p38 activation (phosphorylated p38) relative to total p38 protein; C. Quantification analysis for total p38 protein expression; D. Quantification analysis for activated p38 (phosphorylated p38).

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