Distribution of fibroblast growth factors and their roles in skin fibroblast cell migration

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Abstract. Fibroblast growth factor (FGF)2/basic FGF is a member of the fibroblast growth factor family. Its function in skin wound healing has been well-characterized. However, the function of other FGFs in skin tissues remains to be elucidated. In the present study, *FGF* expression patterns in heart, liver, skin and kidney tissues were analyzed. Notably, in contrast to other tissues, only four *FGFs*, *FGF2*, *7*, *10* and *21*, were dominant in the skin. To examine FGF function in the wound healing process, mouse NIH3T3 fibroblast cells were treated with FGF2, FGF10 and FGF21, and cell migration was monitored. The results revealed that FGF treatment promoted cell migration, which is an important step in wound healing. In addition, FGF treatment enhanced the activity of c-Jun N-terminal kinase (JNK), a key regulator of fibroblast cell migration. To analyze its role in cell migration, *FGF7* was overexpressed in fibroblast cells via a lentivirus system; however, this did not change cell migration speed. *FGF2*, *7*, *10* and *21* were highly expressed in skin tissue, and all except FGF7 regulated fibroblast cell migration and activated JNK. The results of the present study increase our understanding of the role of FGFs in skin wound healing.

Introduction

Mammalian genomes contain 23 members of the fibroblast growth factor (FGF) family (1), which are essential for metabolism and development. FGFs have been identified to be involved in the processes of embryogenesis, gastrulation, somitogenesis, body plan formation, organogenesis and skin wound healing (2-7). FGF21 is the most studied family member, and has been reported to be preferentially expressed in the liver early in development (8). However, recent studies have identified that FGF21 production is inducible by starvation or drug administration, and revealed its diverse functions in glucose homeostasis and protection of the liver and heart from injury (9-11). FGF19, 21 and 23 belong to the FGF19 subfamily. FGF21 primarily binds to the FGF receptor 1c isoform, the activation of which requires the presence of the cofactor β-klotho (12,13). A recent study reported that FGF1 regulated insulin sensitivity in order to maintain blood sugar homeostasis (14).

Skin wound repair requires the cooperation of various cell types, including keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. Fibroblast cell proliferation and migration, collagen deposition and remodeling, wound contraction and angiogenesis are important steps of this process (15,16). Extracellular matrix (ECM) forms the largest component of the dermal skin layer; therefore, repair of ECM is key to wound healing (15). Fibroblasts form a critical cell layer that participates in the production and remodeling of the ECM, and their proliferation and migration is important for the formation of granulation tissue and skin repair (16). FGF2/basic (b)FGF is well-known for its efficacy in skin wound healing, via effects on cell proliferation and migration (16). However, the role of other FGFs in this process remains to be elucidated.

In the present study, mouse heart, liver, skin and kidney tissues were analyzed to determine the expression of *FGFs*. Numerous *FGFs* were relatively highly expressed in tissues; however, in skin only four *FGFs* (*FGF2*, *7*, *10* and *21*) were identified. In addition, the function of four FGFs in fibroblast cell migration was analyzed and possible roles of FGFs in skin wound healing were identified.

Materials and methods

Fibroblast cell culture. The mouse NIH3T3 fibroblast cell line (Nanjing Branch Bai Biological Technology Co., Ltd.,

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Nanjing, China) was placed into 25-cm^2 flasks pretreated with fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated horizontally for 1 h and then vertically for 3 h in an atmosphere of 5% CO_2 at 37˚C. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose, 10% FBS and 1% penicillin-streptomycin. Medium was replaced every three days. The cultured cells were digested and passaged with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) when cell confluence reached ~80%. Cells passaged 3-6 times were used in the following experiments.

Overexpression of FGF7 in fibroblast cells. To overexpress *FGF7* in NIH3T3 cells, *FGF7* open reading frame sequences (NM_008008.4, NCBI) were synthesized and N-terminally fused with FLAG-tag coding sequences (Sangon Biotech Co., Ltd., Shanghai, China). To transduce NIH3T3 cells with lentivirus (Beijing Omega Bio-Technology Co., Ltd, Beijing, China), NIH3T3 cells were seeded at $2x10^5$/well into 24-well plates. Following overnight culture, 3, 9 or 12 μl lentivirus (10^8/ml) was added to the wells in the presence of 4 mg/ml Polybrene® (Sigma-Aldrich, St. Louis, MO, USA). The plates were then centrifuged at 800 x g at room temperature for 1 h and returned to culture in DMEM. Transduced cells, and mock-treated NIH3T3 cells, were analyzed 24 h later by a confocal microscope (Olympus, Japan) to detect expression of the reporter green fluorescent protein (GFP), and then >20 single transfected cells expressing GFP were collected and maintained for 5-6 generations to increased the cell number. The dose of 12 μl lentivirus was selected for subsequent experiments.

Wound healing assay. Cell migration was determined using the wound healing scratch assay. Cells were seeded into 6-well plates (10^3/plate) and grown overnight at 37˚C. Confluent cells were cultured in DMEM containing 0.5% FBS and 5 μg/ml mitomycin-C for 24 h at 37˚C, and then wounded by a 1-mm linear scratch from a sterile pipette tip. Images of the wounded cell monolayers were captured at 0, 12 and 24 h following wounding using an inverted light microscope (model IX70; Olympus Corporation, Tokyo, Japan) equipped with a charge-coupled device camera (CoolSNAP HQ; Nippon Roper K.K., Chiba, Japan) and controlled by MetaMorph® software version 7 (Universal Imaging, Inc., Bedford Hills, NY, USA). All experiments were performed in the presence of 5 mg/ml of mitomycin-C to inhibit cell proliferation. Cells were treated prior to wound healing with 100 ng/ml FGF2, FGF10 or FGF21 (Sigma-Aldrich) for 1 h, or transduced to overexpress *FGF7* as previously described, and wound healing was measured at 12 and 24 h. A total of 20 cells/experiment at the edge of wound region were randomly selected from the wound area. At 12 and 24 h following wounding, the distance between the 20 selected cells and the wound edge at 0 h was measured using Image J Fiji software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. The cells were lysed in an ice-cold lysis solution [7 M urea, 2 M thiourea, 2% 3-([3-Cholamidopropyl] dimethylammonio)-1-propanesulfonate, 40 mM Trizma® base, 40 mM dithiothreitol, 1% protease inhibitor] for 10 min. Following centrifugation at 15,000 x g for 15 min at 4˚C, the protein concentration in the supernatant was measured by the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. The proteins (20 μg) were loaded onto a 10% SDS-PAGE gel, separated by electrophoresis at 100 V for 2 h and transferred onto Immobilon-P Transfer Membranes (Merck Millipore, Tokyo, Japan). The membranes were blocked with Tris-buffered saline containing 5% skim milk and 0.05% Tween-20 for 1 h and then probed with primary antibodies at 4˚C overnight. Anti-GAPDH (mouse monoclonal; dilution, 1:2,500; catalog no., mAbcam 9484; Abcam, Cambridge, UK), anti-phospho-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) phosphorylated at Thr183/Tyr185 (rabbit monoclonal; dilution, 1:1,000; catalog no., 4668; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-JNK (rabbit monoclonal; dilution, 1:1,000; catalog no., ab179461) and anti-FLAG (mouse monoclonal; dilution, 1:2,000; catalog no., ab49763; Abcam) were used as primary antibodies. The membranes were then incubated for 1 h with an anti-mouse (dilution, 1:2,000; ab131368; Abcam) or anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution, 1:2,000; catalog no., 7074; Cell Signaling Technology, Inc.), and visualized using an electrochemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK). Images of western blots were captured using an ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences).

Total RNA extraction, complementary DNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Male C57/BL6J mice, aged 3 months and weighing 2835 g, were obtained from the Laboratory Animal Centre of Wenzhou Medical University (Wenzhou, China). The mice were housed at 22˚C and 50% humidity, with a 12 h light/dark cycle. Mice had free access to food and water. Approval was given for the use of mice in the present study by the Ethics Committee of Wenzhou Medical University (Wenzhou, China). All mice were anesthetized via intraperitoneal injection of 3% sodium pentobarbital (45 mg/kg) and their dorsal areas were completely depilated using sodium sulfide (8.0%; w/v; both SigmaAldrich) prior to tissue extraction during surgery. Total RNA was extracted from liver, heart, kidney and skin tissues of 9 mice, which were randomly divided into three groups (3 mice/group) for biological duplication (the nature of each group was the same, and simply served as a replication), using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse-transcribed using a GoScript Reverse Transcription kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. PCR was performed using the SapphireAmp® Fast PCR Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) on a T100 thermal cycler (BioRad Laboratories, Inc.), with the following cycling conditions: 95˚C for 5 min, followed by 35 cycles at 94˚C for 30 sec and 58˚C for 30 sec, 72˚C for 30 sec, elongation at 72˚C for 5 min and maintenance at 10˚C. Gene expression levels were quantified by the ΔΔCq method as described previously (17). mRNA levels were normalized against those of GAPDH using Image J2x version 2.1.4.7 software and the $2^{-\Delta\Delta Cq}$ method (18). Primers used for RT-qPCR are listed in Table I.

Table I. Primer sequences.

Primer	Forward sequence	Reverse sequence
FGF1	TGCTCTACTGCAGCAACG	CTAGTCAGAAGACACCGG
FGF2	CAAGAACGGCGGCTTCTTC	GGAAGAAACAGTATGGCCT
FGF3	CAAGCTCTACTGCGCTACC	GTCCACCTGTATGCAGCT
FGF4	TACTGCAACGTGGGCATC	GGAAGTGGGTTACCTTCA
FGF5	GAAGTAGCGCGACGTTTTC	GGCTTAACACACTGGCTTC
FGF6	CTCTACTGCAACGTGGGC	GGAAGTGAGTGACAGTCA
FGF7	AGACTGTTCTGTCGCACC	CCGCTGTGTGTCCATTTAG
FGF8	ACCTACCAGCTCTACAGCC	GGCGGGTAGTTGAGGAACT
FGF9	CTGCAGGACTGGATTTCATTT	GTTCAGGTACTTTGTCAGGG
FGF10	TGTCCGCTGGAGAAGGCTGTTC	CTATGTTTGGATCGTCATGG
FGF11	ATCGTCACCAAACTGTTCTG	CAGGAACACTGTGGAGAGAA
FGF12	TCAGCCAGCAGGGATATTTC	CACGACTTTGCCTCCATTCA
FGF13	TAACCTCATCCCTGTGGG	GAGAACTCCGTGAGATCG
FGF14	CAACCTCATCCCAGTGGGA	GGGACTGTTTCACCAACATC
FGF15	ACTCCGCTGGTCCCTATGTC	CTACATCCTCCACCATCCT
FGF16	GCTTCCACCTTGAGATCTTC	GAGATCTCTGGACATGGAG
FGF17	CCAGCTCTACAGCCGGAC	GGGGCGGAGCCCACAAAT
FGF18	CCAGCTCTATAGCAGGAC	GCTTGGTGACTGTGGTGT
FGF19	AACTTTATCCCCATATTTCACC	GAAGCTGGGACTCTTCACT
FGF20	TCAGAGAAATTGACTTCTG	GTGTACATCAGTAGGTCTT
FGF21	GATGACGACCAAGACACTG	CGGCCCTGTAAAGGCTCT
FGF22	GCCTCTTCTCCTCCACTC	CGAGACCAAGACTGGCAG
FGF23	ACAGCCAGGACCAGCTATC	CTCGCGAGAGCAGGATACA
GAPDH	GCCAAGGTCATCCATGACAACT	GAGGGGCCATCCACAGTCTT

FGF, fibroblast growth factor.

Phylogenetic analysis. FGF sequences used for similarity searches were collected from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/gene/?term=FGF). ClustalW (http://align.genome.jp) was used to perform a homologous sequence alignment of the amino acid sequences of FGF family proteins using default settings. Based on the results of sequence alignment, an unrooted phylogenetic tree of the FGF gene family was constructed using Mega software version 6.0 (http://www.megasoftware.net). The neighbor-joining method was applied to construct a phylogenetic tree (19), in which Poisson correction, pairwise deletion and bootstrapping (1000 replicates; random seeds) served as default values to evaluate the reliability of the tree.

Statistical analysis. Statistical analyses were performed in GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error. Comparisons between groups were performed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FGF expression patterns in mouse tissues. The expression patterns of 23 FGF members in mouse tissues was dissected by analyzing their transcript levels in liver, heart, kidney and skin tissue samples from 9 C57/BL6J male mice. RT-qPCR results revealed that *FGF1*, *6*, *7*, *9*, *10*, *12*, *13*, *16* and *18* were highly expressed in the heart (Fig. 1A). In the liver, expression of *FGF1*, *5*, *6*, *7*, *8*, *11*, *12*, *18* and *21* were relatively high, with FGF1 having the greatest expression (Fig. 1B). Notably, the expression of *FGFs* was relatively less complex in skin compared with other tissues. A total of four *FGFs*, *FGF2*, *7*, *10* and *21* were significantly highly expressed in the skin (Fig. 1C). In the kidney, levels of *FGF1*, *5*, *7*, *8*, *9*, *10*, *11*, *12*, *16* and *18* were relatively high (Fig. 1D). FGF2/bFGF is widely known for its efficacy in wound healing (7,20), and as the profile of *FGFs* is relatively simple in skin tissue, a phylogenetic tree was generated to understand the associations between the four FGF proteins. The data revealed that FGF7 and FGF10 belong to the same sub-group, while FGF2 shares a sub-group with FGF1. FGF21 is on a separate branch of the tree (Fig. 2), indicating that the four FGFs are not highly correlated.

FGF2, 10 and 21 promote fibroblast cell migration. *FGF2*, *7*, *10* and *21* are highly expressed in skin tissue (Fig. 1C). Therefore, the function of these FGFs in wound healing was examined. Wound healing involves multiple steps, including cell proliferation and migration. To investigate the effect of

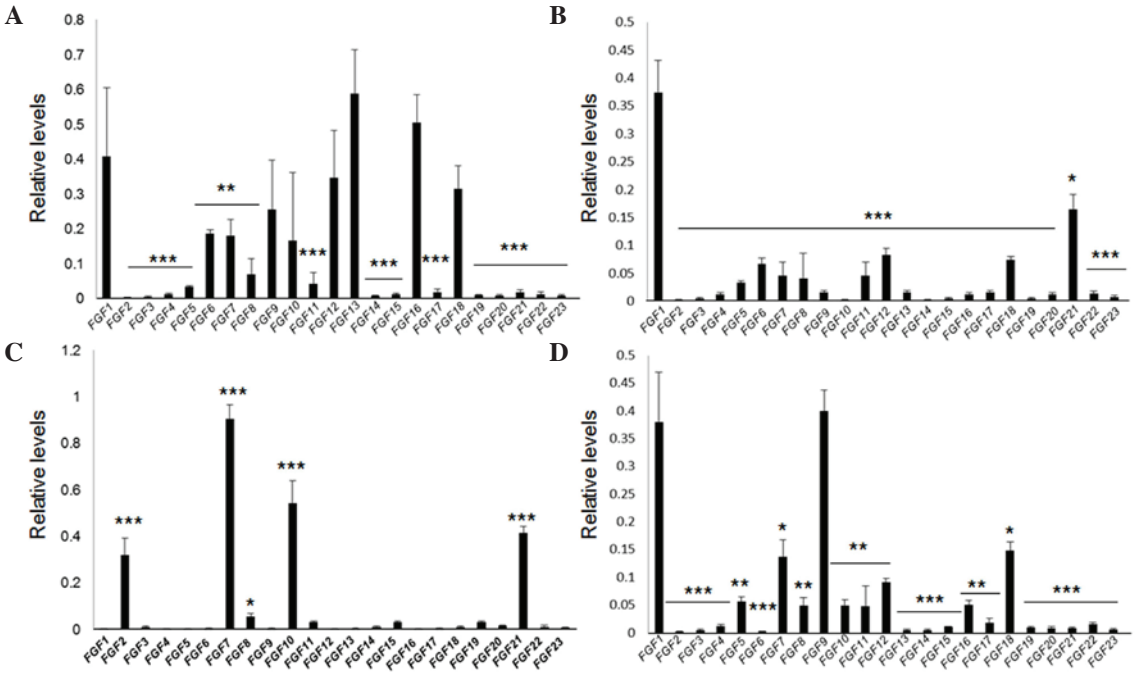

Figure 1. *FGF* expression patterns in various mouse tissues. Expression patterns of *FGFs* were analyzed in (A) heart, (B) liver, (C) skin and (D) kidney tissue by reverse transcription-quantitative polymerase chain reaction. Expression levels of *FGFs* were normalized against those of *GAPDH*. The experiments were repeated at least three times and data are presented as the mean ± standard error of three replicates. FGF, fibroblast growth factor. Significant differences between expression of *FGF1* and other *FGFs* were compared (*P<0.05, **P<0.01 and ***P<0.0001).

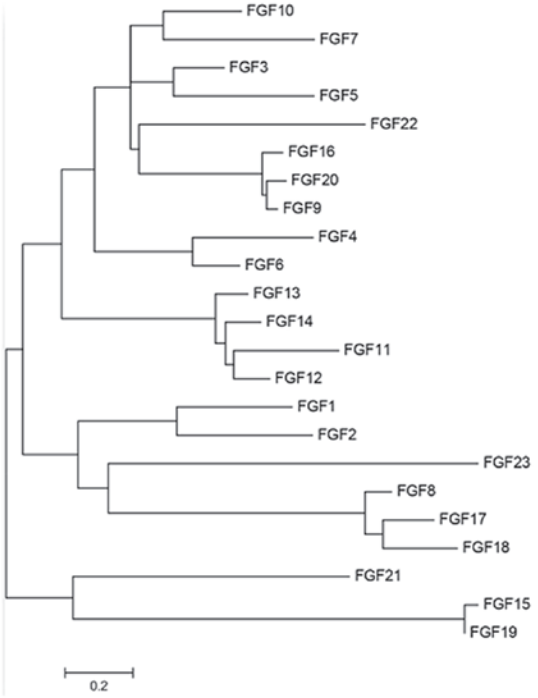

Figure 2. A phylogenetic tree of the 23 FGF proteins from the mouse. Protein sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) using FGF protein fasta sequences, and the phylogenetic tree was constructed using molecular evolutionary genetics analysis software version 6. FGF, fibroblast growth factor. Scale bar indicates genetic distance (substitution per site) between different FGF family proteins. 0.2 above the scale bar indicates a 20% change observed between two sequences.

FGFs on the cell migration process, mouse NIH3T3 foreskin fibroblast cells were grown in a low glucose medium (5.5 mM) containing 5 mg/ml of mitomycin-C, which prevents cell proliferation, for 24 h at 37˚C prior to treatment with FGFs. As presented in Fig. 3, the cells treated with commercially purified FGF2 (P=0.023), 10 (P=0.027) or 21 (P=0.018) at 100 ng/ml exhibited accelerated cell migration 24 h following scratching as compared with control cells. JNK is activated via phosphorylation, and is important for cell migration in wound healing (16); therefore, JNK-phosphorylation was examined following FGF-treatment of NIH3T3 cells for 1 h. The data revealed that treatment with the three FGFs (FGF2, 10 and 21) increased p-JNK levels, while the total JNK level remained unchanged (Fig. 4).

As commercially produced FGF7 is not available, FGF7-FLAG fusion protein was overexpressed in NIH3T3 cells via a lentivirus system. Transduction efficiency was analyzed by monitoring GFP expression, and FGF7 expression was evaluated by western blotting using an anti-FLAG antibody (Fig. 5). In addition, *FGF7* transcripts were analyzed in transduced and non-transduced NIH3T3 cells. RT-qPCR revealed that FGF7 levels were much higher in transduced, (FGF7 OX) compared with non-transduced, cells (Fig. 6A). Furthermore, cell migration was evaluated in FGF7 OX and non-transduced cells. However, *FGF7* overexpression did not significantly alter the speed of cell migration (Fig. 6B and C; P=0.094). These results indicate that FGF2, 10 and 21 are highly expressed in skin tissue, and are involved in the fibroblast cell migration process.

Discussion

Skin wound healing is a complex process that requires keratinocytes, fibroblasts, endothelial cells, macrophages and

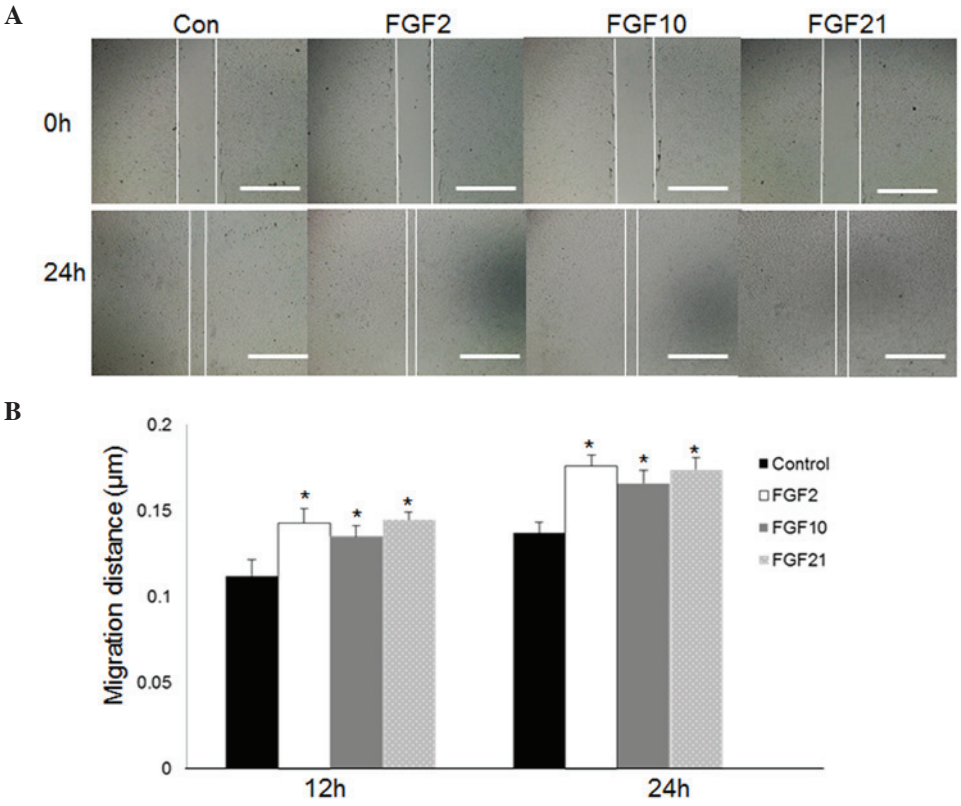

Figure 3. FGF-mediated acceleration of fibroblast cell migration. (A) A wound healing assay was performed to analyze the effects of FGF2, 10 and 21 (100 ng/ml) in mouse NIH3T3 fibroblast cells. (B) Cell migration distance was analyzed by observing the distance between 20 selected cells and the wound edge at 0 h, compared with 24 h. Scale bar=1 μm. Cells treated with commercially purified FGF2, 10 or 21 exhibited accelerated cell migration at 12 and 24 h following scratching. Data are presented as the mean ± standard error of 10 replicates. *P<0.05 vs. the control, as analyzed by Student's t-test. All experiments were performed following treatment of cells with 5 mg/ml mitomycin-C (a cell proliferation inhibitor) for 24 h. FGF, fibroblast growth factor; Con, control cells.

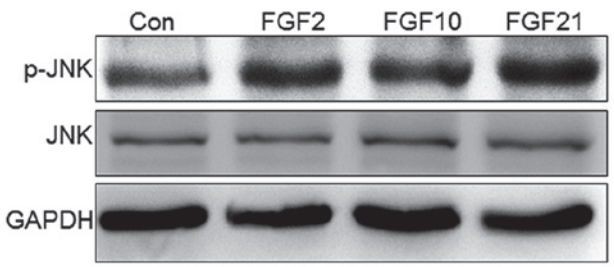

Figure 4. Effect of FGF treatment on JNK expression. Western blot analysis was performed to analyze the protein expression levels of p-JNK and total JNK following treatment of mouse NIH3T3 fibroblast cells with FGF2, 10 and 21 (100 ng/ml) for 1 h. FGF2, 10 and 21 treatment increased p-JNK levels, while total JNK levels remained unchanged. GAPDH served as a loading control. FGF, fibroblast growth factor; JNK, c-Jun N-terminal kinase; p-JNK, phosphorylated JNK; Con, control cells.

platelets. These cells undergo multiple steps including proliferation and migration to rebuild the skin (21). Fibroblasts are critical in wound contraction. Fibroblast cell migration, considered to be a fundamental step in wound healing, involves a multi-step cyclic process, including extension of a protrusion, stable attachment close to the leading edge of the protrusion, forward movement of the cell body, and release of adhesions and retraction at the rear of the cell (22).

FGF2/bFGF is a member of FGF family, and its efficacy in the promotion of fibroblast cell migration is well-understood (7). In the present study, it was observed that besides *FGF2*, *FGF7*, *10* and *21* are highly expressed in skin tissue. Previously, the role of FGF21 in glucose homeostasis has been well-characterized, and its production is induced by stress in the liver and heart (9-11). However, in the present study, *FGF21* expression was revealed to be greatest in the skin, where expression was even higher than in the liver (data not shown). In addition, FGF21 was demonstrated to accelerate the migration of mouse fibroblast cells, similar to FGF2. Furthermore, FGF10 was identified as predominant in skin, and also accelerated cell migration. FGF2 has previously been revealed to accelerate fibroblast cell migration via activation of the phosphoinositide 3-kinase-Ras-related C3 botulinum toxin substrate 1-JNK signaling pathway (7). In the present study, FGF2, 10 and 21 treatment increased JNK phosphorylation levels. *FGF7* has the greatest expression of all FGFs in skin; however, overexpression of *FGF7* in fibroblast cells did not alter cell migration speed, implying that FGF7 may have alternative roles in skin tissue. A previous study demonstrated that induction of *FGF7* expression in the dermal papilla cells of adenosine-stimulated hair, and treatment with exogenous FGF7, stimulated hair fiber elongation in human scalp hair follicle organ cultures (23).

Numerous FGFs were relatively highly expressed in the heart, liver and kidney, suggesting potential, although as yet uncharacterized, roles of these FGFs in various tissues. Notably, *FGF1* was one of the predominant FGFs in all the tissues examined except the skin, and was dominant in the

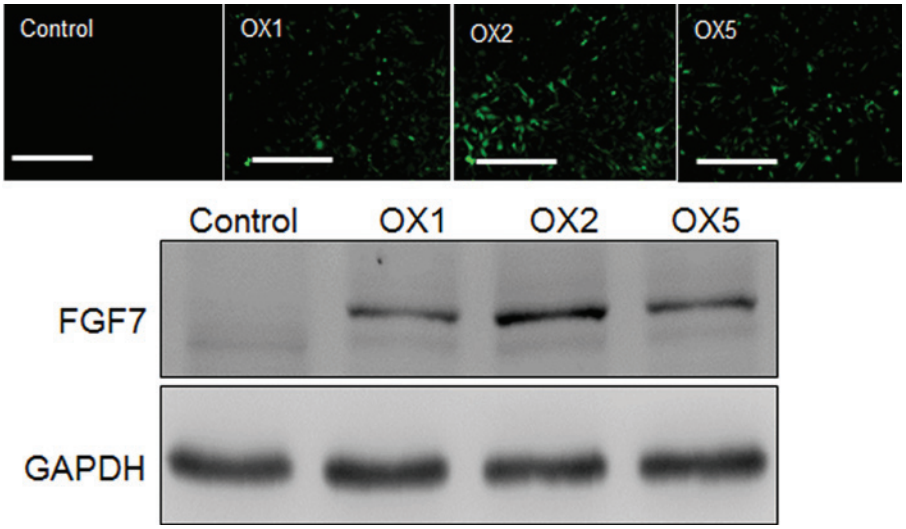

Figure 5. FGF7-FLAG fusion protein expression in NIH3T3 cells. (A) Lenti-FGF7 transformed mouse NIH3T3 fibroblast cells were analyzed for the expression of the reporter gene green fluorescent protein together with parental (non-transduced) NIH3T3 cells. Scale bar=1 μm. (B) Single colonies from lines OX1, 2 and 5 were amplified and FGF7 levels were monitored by western blotting with an anti-FLAG antibody. The dose of 12 μl lentivirus (10^8/ml) was selected for subsequent experiments. FGF, fibroblast growth factor; OX1, cells transduced with 3 μl lentivirus; OX2, cells transduced with 9 μl lentivirus; OX5, cells transduced with 12 μl lentivirus.

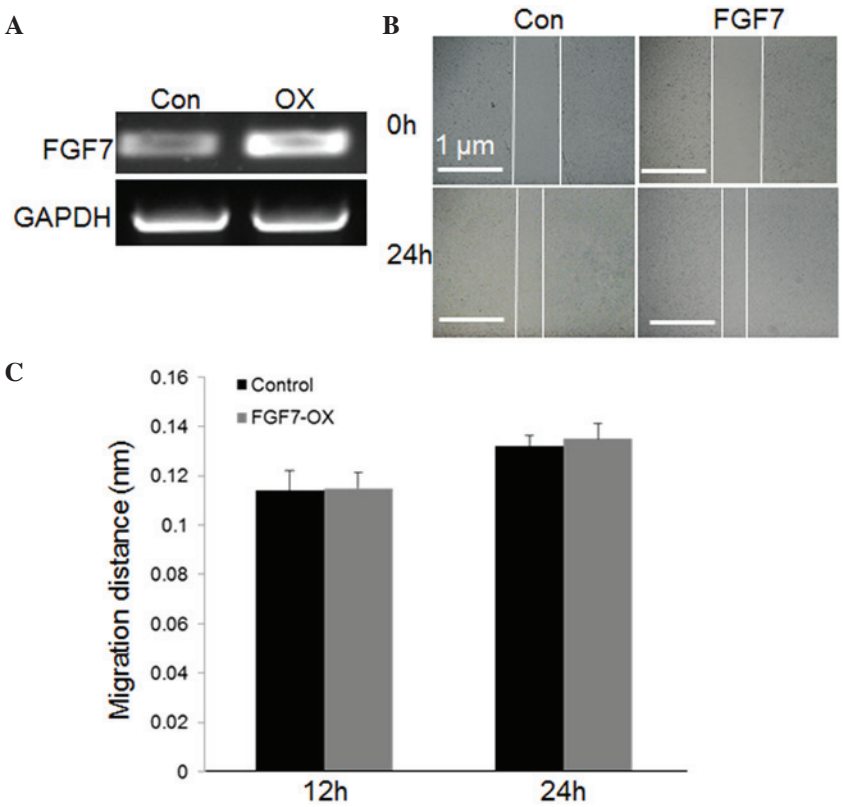

Figure 6. Effects of *FGF7* overexpression on fibroblast cell migration. (A) *FGF7* expression level was analyzed in transduced (12 μl lentivirus; 10^8/ml) and non-transduced cells by reverse transcription-quantitative polymerase chain reaction. *GAPDH* served as a loading control. (B) A wound healing assay was performed to analyze the effects of *FGF7* overexpression on fibroblast cell migration. Scale bar=1 μm. (C) Cell migration distance was analyzed by observing the distance between 20 selected cells and the wound edge at 0 h, compared with 24 h. *FGF7* overexpression did not alter the speed of cell migration. Data are presented as the mean ± standard error of 10 replicates. FGF, fibroblast growth factor; Con, control cells; OX, cells transduced with 12 μl lentivirus (10^8/ml).

liver. Recently, FGF1 was reported to regulate insulin sensitivity (14), therefore, it may be beneficial to analyze the role of FGF1, particularly in the liver. In conclusion, the findings of the present study indicate that FGF family members FGF2, 10 and 21 coordinate to activate the FGF signaling pathway, which is important in the promotion of wound repair. In addition, this experimental approach may provide a basis for isolating and analyzing FGF functions in various tissues.

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