Original Paper

Growth Differentiation Factor-9 Promotes Fibroblast Proliferation and Migration in Keloids through the Smad2/3 Pathway

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Key Words

Keloids • Fibroblast • Growth differentiation factor-9 • Smad pathway

Abstract

Background: Keloids are fibroproliferative scars that develop as a result of a dysregulated wound healing process; however, the molecular mechanisms of keloid pathogenesis remain unclear. Keloids are characterized by the ability to spread beyond the original boundary of the wound, and they represent a significant clinical challenge. Previous work from our group suggested that growth differentiation factor (GDF)-9 plays a role in the invasive behavior of keloids. Here, we examined the involvement of GDF-9 in keloid formation and spread and elucidated a potential underlying mechanism. **Methods:** The expression of GDF-9, cyclooxygenase (COX)-2, vascular epidermal growth factor (VEGF)-C, matrix metalloproteinase (MMP)-2, MMP-9, transforming growth factor (TGF)- β 1, and the related signaling pathway components in human keloid tissues or keloid fibroblasts (kFBs) was monitored by qRT-PCR and western blot. A series of overexpression and silencing experiments in normal and keloid fibroblasts were used to modify the expression of GDF-9. The effects of GDF-9 on kFB proliferation and migration were assessed using the CCK-8, cell cycle and scratch wound healing assays. **Results:** GDF-9 promotes fibroblast proliferation and migration. GDF-9 silencing in kFBs decreased cell proliferation, blocked cell cycle progression, downregulated the angiogenic markers COX-2 and VEGF-C, and downregulated MMP-2 and MMP-9 expression, whereas it had no effect on the levels of TGF- β 1. GDF-9 silencing significantly inhibited Smad2 and Smad3 phosphorylation in kFBs. **Conclusions:** GDF-9 promotes the proliferation and migration of kFBs via a mechanism involving the Smad2/3 pathway.

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Introduction

Keloids represent a dysregulated cutaneous wound healing process and are characterized by the abnormal proliferation of fibroblasts and the excessive deposition of collagen [1]. Keloids are fibroproliferative scars that can form after burns or other skin injuries. Although keloids share several features with hypertrophic scars, such as excessive collagen deposition, they differ in that keloids can extend beyond the borders of the wound and they rarely regress over time [2]. The mechanisms underlying the formation and progression of keloids remain unclear, and despite the availability of a variety of treatments, the recurrence rate of keloids remains high [3].

Surgical excision of keloids is aimed at promoting the occurrence of a new healing process and requires complete removal of abnormal tissue [4]. However, surgical treatment often results in the recurrence of keloids, which can be bigger than the original lesion. Radiation, which is currently used as postoperative adjuvant therapy and has shown significant efficacy in reducing recurrence, is associated with an increased risk of skin cancer [5]. Other treatments including laser therapy, cryotherapy, and occlusive dressings have shown limited efficacy and significant adverse effects [4]. It is shown that early into wound healing, subcutaneous fibroblasts are essential [6]. Corticosteroid injections, which are applied intralesionally and reduce scar volume by decreasing inflammation, increasing vasoconstriction, and inhibiting fibroblast proliferation, are associated with significant side effects. The development of steroid tapes and plasters has significantly improved the effect of steroid injections when used in combination [7, 8]. Besides, stem cell based treatment such as mesenchymal stem cells, hematopoietic stem cells, and skin-derived precursors in wound healing is progressively being valued, but more protracted studies are needed to determine whether the observed effects are clinically significant [9-11]. Despite the availability of many therapeutic options, an optimal treatment modality has not been identified to date, underscoring the need to improve our understanding of the mechanisms of keloid formation.

The mechanisms underlying the pathogenesis of keloids are poorly understood, and the lack of a reliable animal model has hindered research efforts [4]. A number of genetic, systemic, and local factors involved in the formation of keloids have been identified [8, 12]. A genome-wide association study identified four single nucleotide polymorphism loci in three chromosomal regions that are significantly associated with keloid development [13]. Sex hormones such as estrogen and androgen, which have vasodilatory effects, have been suggested to affect the inflammatory response in keloids, as reflected by the higher risk of keloid development during adolescence and pregnancy [14]. Another factor associated with keloid development is hypertension, which could be attributed to the effect of hypertension on increasing inflammation by damaging blood vessels [15, 16]. Mechanical forces play an important role in keloid development, and several surgical techniques are being developed to reduce the mechanical forces around keloids [8].

The transforming growth factor (TGF)- β /Smad signaling pathway plays an important role in a variety of processes including cell growth, proliferation, differentiation and apoptosis, and TGF- β is involved in many fibrotic diseases including keloids through its role in promoting extracellular matrix (ECM) production and tissue fibrosis [17, 18]. TGF- β is a member of a family of secreted cytokines that binds to and induces the dimerization of TGF- β type II receptors and the subsequent phosphorylation of TGF- β type I receptors, which phosphorylate Smad2/3 leading to the translocation of Smad4 to the nucleus and the activation of the expression of target genes [19]. TGF- β receptors and Smad proteins are overexpressed in keloids compared with normal skin, and increased expression of the phosphorylated forms of Smad2/3 has been reported in keloid fibroblasts (kFBs) [20, 21]. Growth differentiation factor (GDF)-9, a member of the TGF- β family that is mainly expressed in oocytes and ovarian follicles, induces Smad2 activation in human granulosa-luteal cells through a pathway that is activated by TGF- β and activin [22]. In previous work from our group, we showed that GDF-9 is upregulated in fibroblasts derived from the peripheral area of keloids, suggesting that it plays a role in the invasive behavior of keloids [23]. In

the present study, we further investigated the role of GDF-9 in keloids and explored the underlying mechanisms.

Materials and Methods

Human subjects

Surgically excised keloids (n=6), hypertrophic scar tissues (n=6), and discarded normal skin (n=6) were donated from patients who received plastic surgery without previous treatment for their scarring. The handling of human tissue was approved by the Ethics Committee of Shanghai Ninth People's Hospital. Detailed information of patients, lesion locations and harvested areas is provided in Table 1.

Primary cell isolation and in vitro culture

The kFBs and normal fibroblasts (nFBs) were isolated from surgically excised keloids and normal skin as previously described [3]. The epidermis and subcutaneous connective tissues of specimens were removed, specimens were cut into 1–3 mm³ pieces and digested in 0.15% collagenase (Roche Applied Science, Indianapolis, IN, USA) in DMEM medium (Gibco, Gaithersburg, MD, USA) at 37°C on a shaker for 3 h. The digested suspension was filtered through a nylon mesh, and the filtrate was centrifuged at 1500 rpm for 5 min. The fibroblast pellets were resuspended and cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in 10 cm dish in an incubator at 37°C and 5% CO₂. After reaching confluence, cells were passaged and subcultured at a ratio of 1:3 and the first passage cells were harvested for analysis.

Cell transfection and plasmid construction

The coding sequence of GDF-9 was amplified by RT-PCR with the following primers: forward, 5'-TCC TGA GGA GCG TCA ATG AC-3'; reverse, 5'-ACC GCA CCA TCA TTC AGT GG-3'. The PCR fragments and the pGC-FU plasmid were digested with Age I and ligated with T4 DNA ligase to produce pGC-FU-GDF-9. To generate the recombinant lentivirus LV-GDF-9, 293T cells were co-transfected with the pGC-FU plasmid with a cDNA encoding GDF-9, pHelper 1.0 plasmid, and pHelper 2.0 plasmid using Lipofectamine 2000 (Invitrogen). After 48 h, the supernatant was harvested and the viral titer was calculated by transducing 293T cells.

Table 1. Characteristics of patients in this study

Patient	Gender	Age (years)	Origin	Duration (years)
Normal				
N1	Female	19	Chest	
N2	Male	34	Arm	
N3	Male	22	Face	
N4	Male	46	Shoulder	
N5	Female	25	Arm	
N6	Male	26	Face	
Hypertrophic scar				
H1	Male	22	Shoulder	1 years
H2	Male	40	Arm	2.5 years
H3	Female	44	Face	2 years
H4	Female	37	Neck	1 years
H5	Male	31	Chest	2 years
H6	Male	26	Face	1.5 years
Keloid				
K1	Female	31	Arm	8 months
K2	Female	38	Chest	1 years
K3	Male	21	Face	1.5 years
K4	Female	28	Shoulder	1.5 year
K5	Male	42	Chest	1 year
K6	Male	10	Arm	1 years

Short interfering RNA (siRNA) and transfection

siRNA transfection was used to downregulate GDF-9 expression, and all the siRNAs were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The GDF-9 targeted siRNA (siGDF-9) and control siRNA (siCtrl) were dissolved separately in OptiMem I (Invitrogen). After 10 min of equilibration at room temperature, each RNA solution was combined with the respective volume of the Lipofectamine™ 2000 solution, mixed gently, and allowed to form siRNA liposomes for 20 min. The kFBs were transfected with the transfection mixture in antibiotic-free cell culture medium.

Scratch wound healing assay

Scratch wound healing assay was performed as described previously with

certain modifications [24]. Briefly, fibroblasts were seeded into 6-well plates coated with 3.85 mg/mL of rat tail collagen (BD Biosciences, San Diego, CA, USA) and the surface was scratched with a pipette tip at specific areas. Cell migration was assessed after 48 h using a microscope (Carl Zeiss, Jena, Germany) and the percent of migrated cells was calculated.

Cell cycle analysis

The kFBs or nFBs were harvested, washed and fixed in chilled 70 % ethanol at 4 °C for 30 min. The cells were stained with 100 µg/mL propidium iodide and treated with 1 mg/mL of RNase-A (Sigma, St. Louis, MO, USA) for 30 min at 37°C. The DNA content of 10000 cells was determined with a FACScan flow cytometer (BD Biosciences). The data were analyzed with Modfit LT 3.0 software (Verity Software House, Topsham, ME, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues and cultured cells at the indicated time points and was subsequently reverse-transcribed using the AMV Reverse Transcription System according to the manufacturer's instructions (Takara, Shiga, Japan). qRT-PCR was performed using SYBR Green PCR mix on an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). Thermocycler conditions included 2-minute incubation at 50°C, then 95°C for 10 minutes; this was followed by a 2-step PCR program, as follows: 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. β-actin was used as an internal control to normalize differences in the amount of total RNA in each sample. Primers are listed in Table 2.

Western blot analysis

Protein was extracted from human keloid tissues, nFBs, and kFBs using a protein lysis buffer. Approximately 50 µg of total protein from each sample were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat dried milk plus 0.1% Tween 20 for 2 hours at room temperature, incubated with the primary antibodies diluted 1,000-fold that recognized GDF-9, vascular epidermal growth factor (VEGF)-C, cyclooxygenase (COX)-2, matrix metalloprotease (MMP)-2, MMP-9, TGF-β1, p-Smad2/3, Smad2/3 or β-actin (Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with the corresponding horseradish-peroxidase conjugated secondary antibodies for 1 h at 37°C. Bands were detected using FluorChem E system (Alpha Innotech Corp, Santa Clara, CA, USA). The semiquantitative analysis (AlphaView Software 3.3; ProteinSimple, San Jose, CA, USA) results were expressed as the optical volume densities (OD × mm²) normalized to β-actin.

Cell proliferation assays

Cell proliferation was determined with the CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, kFBs or nFBs were seeded into 96-well plates at an initial density of 5000 cells per well. Transfected cells were plated at day 1 after transfection. Ten microliters of the kit reagent were added to each well at 12, 24, 36, 48, 60 or 72 h after seeding, and all plates were scanned by a microplate reader (Thermo Scientific, Waltham, MA, USA) after a further 2 h. Cell proliferation was evaluated by absorbance at 450 nm.

Statistical analysis

All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Table 2. Quantitative real-time PCR primers

Name	Sequence
GDF-9 forward	5'-CAGAAGTGACCGTAGTCCACCC-3'
GDF-9 reverse	5'-CACCTCGCCCAACAGATAGAAC-3'
VEGF-C forward	5'-GCCCCA AACCAGTAACAATC-3'
VEGF-C reverse	5'-GCTGGCAGGGAACGTCTAAT-3'
COX-2 forward	5'-CAGGGTTGCTGGTGGTAGGA-3'
COX-2 reverse	5'-GCATAAAGCGTTTGCGGTAC-3'
MMP-2 forward	5'-CTTCCAAGTCTGGAGCGATGT-3'
MMP-2 reverse	5'-TACCGTCAAAGGGGTATCCAT-3'
MMP-9 forward	5'-TGCCCGGACCAAGGATACAGTTT-3'
MMP-9 reverse	5'-GTTTCAGGGCGAGGACCATAGAGG-3'
TGF-β1 forward	5'-CTAATGGTGGACCGCAACAAC-3'
TGF-β1 reverse	5'-CGGTTTCATGTCATGGATGGTG-3'
β-actin forward	5'-ATCATGTTTGAGACCTTCAA-3'
β-actin reverse	5'-CATCTCTTGCTCGAAGTCCA-3'

Differences between 2 groups were calculated using Student's t-tests. ANOVA (analysis of variance) was applied for comparison of the means of multiple groups, in which the Student-Newman-Keuls (SNK) test was further used for the comparison of 2 groups. A p value <0.05 was defined as significant. All values were presented as mean±S.E.M.

Results

GDF-9 is upregulated in keloid tissues and kFBs

The expression of GDF-9 was assessed in tissue samples from surgically excised keloids and hypertrophic scars and compared with that in normal skin tissues by qRT-PCR. The results showed approximately 800-fold higher levels of GDF-9 mRNA in keloids than in normal and hypertrophic scar tissues (Fig. 1A). Western blot analysis confirmed these results, showing higher expression of the GDF-9 protein in keloids than in extracts from normal and hypertrophic scar tissues (Fig. 1B). Then, we determined the GDF-9 mRNA and protein levels in nFBs isolated from normal skin tissues and kFBs isolated from keloids. Similarly, GDF-9 mRNA (Fig. 1C) and protein (Fig. 1D) levels in kFBs were significantly higher than those in nFBs, suggesting that GDF-9 is essential for the development of keloids.

Fig. 1. Expression of GDF-9 in human keloids and keloid fibroblasts (kFBs). qRT-PCR (A) and western blot analysis (B) were used to detect the relative mRNA expression and protein levels in human keloids, hypertrophic scar specimens, and normal tissues derived from 6 patients for each group, respectively. For qRT-PCR analysis, data were normalized to the expression level in normal tissues. For western blot analysis, representative images are shown on the left and summarized results are shown on the right. (C and D) Normal fibroblasts (nFBs) and kFBs were isolated from human normal and keloid tissues derived from 6 patients for each group, respectively. The relative mRNA expression and protein levels of GDF-9 were measured by qRT-PCR (C) and western blot analysis (D). For qRT-PCR analysis, data were normalized to the expression level in nFBs. For western blot analysis, representative images are shown on the left and summarized results are shown on the right. All experiments were repeated three times and data were presented as mean±S.E.M. Differences in (A) and (B) were performed by ANOVA (SNK test). Differences in (C) and (D) were performed by Student's t-tests. *, p<0.05, **, p<0.01.

Effects of GDF-9 on the proliferation of kFBs

The role of GDF-9 in keloids was further examined by assessing the effect of GDF-9 overexpression or silencing on the proliferation of nFBs and kFBs. Figure 2A shows the effective overexpression of GDF-9 in nFBs, with an approximately 3-fold increase in GDF-9 mRNA in GDF-9 lentivirus transfected cells compared with that in vector control transfected cells. Western blot analysis confirmed the overexpression of GDF-9 in nFBs at the protein level (Fig. 2B). SiRNA mediated silencing of GDF-9 resulted in an approximately 6-fold reduction in the level of GDF-9 mRNA compared with that in the controls (Fig. 2C), and the effect of siGDF-9 was confirmed at the protein level (Fig. 2D). Assessment of cell proliferation by the CCK-8 assay showed that GDF-9 overexpression significantly increased the proliferation of nFBs, whereas GDF-9 silencing significantly inhibited the proliferation of kFBs compared with normal and vector or siCtrl transfected cells over a period of 72 h (Fig. 3A). Cell cycle analysis by flow cytometry indicated that GDF-9 overexpression increased the G2/M and S cell population and decreased the G1 population in nFBs, whereas GDF-9 silencing increased the percentage of cells in the G1 phase of the cycle in kFBs compared with negative controls (Fig. 3B).

Because GDF-9 induces the synthesis of cyclooxygenase-2 (COX-2) in granulosa cells [25] and COX-2 is upregulated in many cancers and is associated with increased VEGF production and angiogenesis [26], we assessed the effects of GDF-9 on the levels of VEGF-C and COX-2 in nFBs and kFBs. The results of qRT-PCR (Fig. 4A) and western blot analysis (Fig. 4B and C) showed that GDF-9 overexpression significantly upregulated the expression of VEGF-C and COX-2 in nFBs, whereas GDF-9 silencing significantly downregulated these factors in kFBs at the mRNA and protein levels (Fig. 4). Taken together, these results indicated that GDF-9 promotes proliferation and upregulates angiogenic markers in kFBs.

Fig. 2. Overexpression or silencing of GDF-9 in nFBs or kFBs. nFBs and kFBs were isolated and cultured *in vitro* and GDF-9 was overexpressed by lentiviral vector or silenced by siGDF-9. Validation of GDF-9 overexpression in nFBs at the mRNA and protein levels were measured by qRT-PCR (A) and western blot analysis (B). For qRT-PCR analysis, data were normalized to the expression level in the vector control (n=3). For western blot analysis, representative images are shown on the left and summarized results are shown on the right. Validation of GDF-9 knockdown in kFBs at the mRNA and protein levels were measured by qRT-PCR (C) and western blot analysis (D). For qRT-PCR analysis, data were normalized to the expression level in siCtrl cells (n=3). All the data were presented as mean±S.E.M. Differences in this figure were performed by Student's t-tests. *, p<0.05, **, p<0.01.

Fig. 3. Effect of GDF-9 on the proliferation of nFBs and kFBs. nFBs and kFBs were isolated and cultured *in vitro* and GDF-9 was overexpressed by lentiviral vector or silenced by siGDF-9. (A) Assessment of cell proliferation by CCK-8 assay in nFBs and kFBs at the indicated times after seeding (n=3). (B) Assessment of cell cycle progression by flow cytometry in nFBs and kFBs (n=3). All the data were presented as mean±S.E.M. Differences in this figure were performed by ANOVA (SNK test). *, p<0.05.

Fig. 4. Effect of GDF-9 on the expression of angiogenic markers (VEGF-C and COX2) in nFBs and kFBs. nFBs and kFBs were isolated and cultured *in vitro* and GDF-9 was overexpressed by lentiviral vector or silenced by siGDF-9. qRT-PCR (A) and western blot (B and C) assessment of the expression of VEGF-C and COX2 in nFBs and kFBs at the mRNA and protein levels. For qRT-PCR analysis, data were normalized to the expression level in normal untransfected cells (n=3). For western blot analysis, representative images (B) and summarized results (C) are shown. All the data were presented as mean±S.E.M. Differences in this figure were performed by ANOVA (SNK test). *, p<0.05.

GDF-9 promotes the migration of fibroblasts

The effect of GDF-9 on the migration of fibroblasts was assessed by performing a scratch wound healing assay, which showed that GDF-9 overexpression significantly promoted nFB

Fig. 5. Effect of GDF-9 on the migration of nFBs and kFBs. nFBs and kFBs were isolated and cultured *in vitro* and GDF-9 was overexpressed by lentiviral vector or silenced by siGDF-9. Cell migration was detected by scratch wound healing assay. Representative images are shown on the left (A) and summarized results of the migration percentage are shown on the right (B). qRT-PCR (C) and western blot (D-F) assessment of the expression of migration related factors (MMP-2, MMP-9 and TGF-β1) in nFBs and kFBs at the mRNA and protein levels. For qRT-PCR analysis, data were normalized to the expression level in normal untransfected cells (n=3). For western blot analysis, representative images (D) and summarized results (E and F) are shown. All the experiments were repeated three times and data were presented as mean±S.E.M. Differences in this figure were performed by ANOVA (SNK test). *, p<0.05; **, p<0.01.

migration, whereas GDF-9 knockdown significantly inhibited kFB migration compared with normal and vector control cells (Fig. 5A and B). These results were confirmed by analyzing the expression of MMP-2, MMP-9 and TGF-β1 by qRT-PCR (Fig. 5C) and western blot (Fig. 5D-F). GDF-9 overexpression significantly upregulated MMP-2 and MMP-9 in nFBs, whereas GDF-9 silencing significantly downregulated MMP-2 and MMP-9 at the mRNA and protein levels (Fig. 5C-F). However, GDF-9 overexpression or silencing had no effect on the expression of TGF-β1 in both cell types. Taken together, our results suggest that GDF-9 acts on fibroblast migration via a TGF-β independent pathway.

The effects of GDF-9 on fibroblasts are mediated by the Smad2/3 signaling pathway

To examine the mechanisms underlying the role of GDF-9 in keloids, the activity of members of the Smad pathway was examined in nFBs and kFBs based on previous studies showing the involvement of Smad signaling in the pathogenesis of keloids [27]. Our results showed that GDF-9 overexpression promoted Smad2/3 phosphorylation, significantly increasing the phospho-Smad2 (pSmad2)/Smad2 and pSmad3/Smad3 ratios in nFBs, whereas GDF-9 silencing significantly inhibited Smad2 and Smad3 phosphorylation in kFBs compared with untransfected and vector control cells (Fig. 6A and B). Taken together,

Fig. 6. Involvement of the Smad signaling pathway in the effect of GDF-9. nFBs and kFBs were transfected with a lentiviral GDF-9 overexpression or siGDF-9 silencing vector. Expression of Smad2/p-Smad2 (A) and Smad3/p-Smad3 (B) was assessed by western blot analysis. Representative images are shown on the top and summarized results of the ratio of p-Smad2/3 to Smad2/3 were calculated and plotted on the bottom. All experiments were repeated three times and data were presented as mean $\pm$ S.E.M. Differences in this figure were performed by ANOVA (SNK test). *, $p < 0.05$.

these results indicated that the effects of GDF-9 on kFBs may be mediated by the Smad2/3 signaling pathway.

Discussion

Keloids, whose name is derived from the Greek word *cheloides*, which means “crab’s claw”, are the result of a pathological wound healing process and their clinical management remains challenging [2]. Keloids have a high recurrence rate, and despite several treatment options, no effective curative therapies are currently available. Furthermore, the mechanisms underlying keloid formation and progression remain unclear. In previous work from our group, we identified GDF-9 as a factor potentially involved in the invasive behavior of keloids [23]. In the present study, we further investigated the role of GDF-9 in keloid formation and progression and explored the underlying mechanisms.

GDF-9 is a growth factor that has been studied for its role during the early stages of follicular development; however, its role in other processes has not been investigated in detail. Similar to other TGF- β family members, GDF-9 signals through type I and II single transmembrane serine/threonine kinase receptors and the Smad pathway [28]. GDF-9 interacts with and signals through bone morphogenetic protein receptor II (BMPR2) and TGF- β R1, leading to the phosphorylation of receptor regulated Smads [29]. GDF-9 has been investigated for its role in cancer, although studies have shown conflicting results. GDF-9 plays a tumor suppressor role in breast cancer: its expression is correlated with good prognosis and it inhibits breast cancer cell invasiveness [30]. By contrast, GDF-9 expression is correlated with the severity of oral squamous cell carcinoma and it plays a tumorigenic role in lung adenocarcinoma [31, 32]. In prostate cancer, GDF-9 increased the motility and adhesive capacity of prostate cancer cells via a mechanism involving the Smad pathway [33]. The results of the present study showed that GDF-9 is overexpressed in keloid tissues and cultured kFBs. GDF-9 silencing decreased cell proliferation, blocked cell cycle progression, and downregulated the angiogenic markers COX-2 and VEGF-C. A scratch wound healing assay showed that GDF-9 silencing inhibited keloid fibroblast migration and downregulated MMP-2, which catalyzes the degradation of the ECM and is therefore involved in the invasiveness of tumor cells. However, whether GDF-9 acts directly on MMP-2 or indirectly via its effect

on COX-2 remains unclear, as COX-2 upregulates matrix metalloproteinases and plays a role in cancer cell invasiveness [34, 35]. Taken together, our results supported the role of GDF-9 in promoting kFB proliferation and migration. However, GDF-9 overexpression or silencing had no effect on TGF- β expression, suggesting that GDF-9 acts via a different pathway.

A cDNA microarray analysis of differentially expressed genes identified 402 genes showing different expression levels between keloids and normal skin among 8400 human genes analyzed [36]. Among the upregulated genes, the study focused on NGF and TGF- β , which is involved in several fibrotic disorders such as pulmonary fibrosis, systemic sclerosis, fibrotic diseases of the kidney and liver, and in keloids and hypertrophic scars [37]. The profibrotic activity of TGF- β is thought to be related to its ability to induce the production of ECM proteins and upregulate protease inhibitors, leading to the inhibition of the enzymatic breakdown of the ECM [38]. TGF- β , in particular the TGF- β 1 and β 2 isoforms, have been implicated in the pathogenesis of keloids and keloid fibroblasts were shown to produce up to 12 times more collagen than normal fibroblasts in response to exogenous TGF- β [39, 40]. In the present study, we showed that the effect of GDF-9 on keloid fibroblast proliferation and migration was independent of TGF- β expression; however, GDF-9 silencing inhibited Smad2/3 phosphorylation, suggesting that GDF-9 functions via a Smad dependent pathway in keloid fibroblasts. Previous studies demonstrated the involvement of TGF- β associated signaling pathways, in particular the Smad pathway, in the pathogenesis of keloids. The upregulation of TGF- β receptors and increased phosphorylation of Smad3 were identified in keloid fibroblasts compared with normal fibroblasts, suggesting a role for TGF- β signaling in keloid disease [20]. Phan et al. suggested that the activation of Smad signaling is a factor in the pathogenesis of keloids, as indicated by the upregulation of TGF- β receptors, Smad2/3, and the phosphorylated form of Smad2 in kFBs [21]. Collagen synthesis in keloid fibroblasts was inhibited by receptor for activated C-kinase via the inhibition of TGF- β 1/Smad signaling, supporting the role of Smads in keloid disease [27]. The role of the TGF- β 1/Smad signaling pathway in keloid formation was shown to be mediated by the MAPK pathway, as demonstrated by the effect of specific inhibitors of MAPK signaling in cells stimulated with exogenous TGF- β 1 [41]. A recent study analyzed keloid and hypertrophic scar samples and suggested that crosstalk between the TGF- β /Smad and Wnt/ β -catenin pathways plays a role in pathological scar formation [42]. These studies support the present results suggesting that GDF-9 promotes keloid fibroblast proliferation and migration via a Smad2/3 dependent pathway.

In conclusion, the present study confirmed the involvement of GDF-9 in keloid disease and elucidated a potential underlying mechanism by which GDF-9 promotes the proliferation and motility of keloid fibroblasts through a mechanism involving the modulation of Smad2/3 activity. These results suggest potential novel targets for the treatment of keloids.

Abbreviations

COX, (cyclooxygenase); ECM, (extracellular matrix); GDF, (growth differentiation factor); kFBs, (keloid fibroblasts); MMP, (matrix metalloprotease); nFBs, (normal fibroblasts); qRT-PCR, (quantitative real-time PCR); siRNA, (short interfering RNA); TGF, (transforming growth factor); VEGF, (vascular epidermal growth factor).

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Disclosure Statement

The authors declare no conflict of interest.

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