

Apigenin inhibits growth and migration of fibroblasts by suppressing FAK signaling

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ABSTRACT

The naturally occurring compound apigenin has many biological effects, including anti-inflammatory, antioxidative and anticancer effects. Although hypertrophic scar formation is a common surgical complication, there is still no good treatment for it. In the present study, we examined the effect of apigenin on hypertrophic scar. After isolating fibroblasts from human hypertrophic scars, we assess the effects of apigenin on fibroblast cell survival, apoptosis and migration. The results showed that apigenin dose-dependently inhibited the growth and migration of hypertrophic scar fibroblasts. By inhibiting FAK kinase activity and FAK phosphorylation, apigenin also inhibited activation of the FAK signaling pathway. Apigenin thus appears to inhibit the growth and migration of hypertrophic scar fibroblasts by inhibiting FAK signaling. This suggests apigenin could potentially provide a new option for the treatment of hypertrophic scars.

INTRODUCTION

The clinical manifestations of hypertrophic scars share many of the characteristics of tumors, including invasion of the undamaged dermis, no clear dividing line between the scar and normal skin, infiltrative growth, long-term sustainability without spontaneous disappearance, and a high recurrence rate after surgery [1]. Some tumors are familial and manifested through autosomal dominant or recessive inheritance. Likewise, hypertrophic scars also exhibit a certain familial genetic tendency and clustering, which is consistent with autosomal dominant hereditary diseases. Although often treated surgically, there are a wide variety of non-operative treatments for hypertrophic scars, which can be divided into physical therapies and drug treatments [2, 3]. Drugs for the treatment of scars are mainly adrenal cortical hormones and antitumor drugs [4]. However, because these drugs have obvious adverse side effects, their clinical application is limited. Consequently, there is a need for an effective drug treatment for hypertrophic scar that has fewer adverse effects.

Apigenin is a common flavonoid found in a variety of fruits and vegetables, including parsley, onions, apples, and certain seasonings. Apigenin has gained increasing attention due to its anti-inflammatory, antioxidant, sedative, antihypertensive and antitumor effects [5, 6]. Apigenin has also been shown to suppress cell survival of a wide variety of tumor cells, including prostate cancer, breast cancer, melanoma and leukemia cells [7–10]. Several mechanisms for its antitumor effect have been elucidated, including cell cycle arrest with dysregulation of cell cycle proteins, induction of apoptosis, inhibition of fatty acid synthesis, production of reactive oxygen species, and inactivation of survival signaling [11]. However there have been no reports examining the effect of apigenin on hypertrophic scars.

Like tumors, hypertrophic scars are cell survival-based ailments, which may be the result of abnormal expression of oncogenes and/or tumor suppressor genes [12]. Tumor-related genes mediate the development of hypertrophic scars in two ways: through mutation of tumor suppressor genes (e.g., P53,

Fas, P27, Rb, exon27 and P16) leading to loss of inhibition of fibroblast cell survival and through expression of oncogenes promoting fibroblast cell survival and apoptosis resistance (e.g., c-myc, c-fos, Bcl-2, Tenascin-C) [13–15]. These two kinds of regulation make the fibroblasts proliferate too fast, decrease the apoptosis, and then form hypertrophic scar. In addition, FAK, VEGF, FGF, PDGF, TGF and IGF proteins are all overexpressed in hypertrophic scar fibroblasts (HSFs) and play key roles in the formation and development of the scars [16]. FAK (focal adhesion kinase) acts as a free signaling molecule in the cytoplasm of fibroblasts and plays an important role in TGF- β -mediated intracellular signal transduction. FAK also reportedly promotes the cell survival and contracture of hypertrophic scars [17, 18].

In the present study, we first evaluated the activity of apigenin in HSFs. We found that apigenin effectively suppresses the growth and migration of HSFs by suppressing the phosphorylation of FAK (Tyr397). We propose that apigenin could be utilized to both treat and prevent hypertrophic scar.

RESULTS

Apigenin inhibits the cell survival and migration of HSFs

Cell clone formation and MTT assays were performed to assess the inhibitory effect of apigenin on the growth of HSFs and human keloid fibroblasts (HKFs). The results showed that apigenin inhibited the cell survival of both HSFs and HKFs in a concentration-dependent manner (Figure 1A–1B). The respective IC_{50} 's were 36.27 μ M and 47.51 μ M (Figure 1C). To investigate the mechanism by which apigenin inhibited HSF and HKF growth, cells were exposed to selected concentrations of apigenin for 24 h, after which cell cycle analysis was performed. Apigenin-treated cells were arrested at G1 phase while control cells were unaffected (Figure 1D–1E). Transwell migration assays (with or without Matrigel) showed that apigenin also dose-dependently suppressed migration of both HSFs and HKFs (Figure 1F–1I).

Apigenin suppresses the kinase activity of FAK and inhibits the FAK^{p-Tyr397}

Earlier studies indicate that FAK and Collagen I may be abnormally expressed in hypertrophic scars [19, 20]. By comparing the FAK content in normal skin tissue, normal scar tissue and hypertrophic scar tissue, we found that the FAK expression was highest in hypertrophic scars (Figure 2A–2B). Kinase Glo luminescence assays showed that apigenin

significantly and dose-dependently inhibits the kinase activity of FAK (Figure 2C). Moreover, using HSFs transfected with wild-type FAK (FAK-WT) or a FAK Y397A mutant (FAK-KM) and then treated for 24 h with different concentrations of apigenin, we found that apigenin dose-dependently inhibited FAK-WT kinase activity but had little effect on the kinase activity of FAK-KM (Figure 2D). In another set of experiments, HSFs transfected siRNA targeting FAK (siFAK) or control (si-NC) and then treated for 24 h with different concentrations of apigenin. Subsequent Kinase Glo luminescence assays showed that apigenin had little effect on kinase activity after FAK was silenced (Figure 2E). Western blot and real-time PCR showed that after treatment with 10 or 20 μ M apigenin for 24 h, the level of FAK phosphorylated at Tyr397 (FAK^{p-Tyr397}) was dramatically decreased, while there was little change of total FAK (Figure 2F–2I). Western blotting and real-time PCR also showed that when FAK was overexpressed, the inhibitory effect of apigenin on FAK^{p-Tyr397} was significantly increased (Figure 2J–2K). By contrast, after silencing FAK, apigenin had little inhibitory effect (Figure 2L–2M).

Apigenin inhibits HSF cell survival by suppressing FAK^{p-Tyr397}

We next assessed the effect of apigenin on HSF cell survival after overexpression of FAK or its silencing. The results show that FAK promoted HSF cell survival and that the inhibitory effect of apigenin on HSF cell survival was enhanced when FAK was overexpressed, (Figure 3A). Conversely, apigenin had almost no inhibitory effect on the cell survival of HSFs in which FAK was silenced (Figure 3B). In cells overexpressing FAK, apigenin also exerted an inhibitory effect on cyclin D1, cell survival-related mediator acting downstream of FAK. This inhibitory effect of apigenin on cyclin D1 was absent after silencing FAK (Figure 3C–3F).

Apigenin inhibits the HSF migration by suppressing FAK^{p-Tyr397}

Finally, we examined the effect of apigenin on the migration of HSFs in which FAK was overexpressed or silenced. The results show that FAK promoted HSF migration and that, as with its effect on cell survival, the inhibitory effect of apigenin on HSF migration was enhanced when FAK was overexpressed (Figure 4A) but was lost when FAK was silenced (Figure 4B). Moreover, in cells overexpressing FAK, apigenin also exerted an inhibitory effect on the migration related enzyme MMP2, downstream of FAK. This inhibitory effect was absent after silencing FAK (Figure 4C–4F).

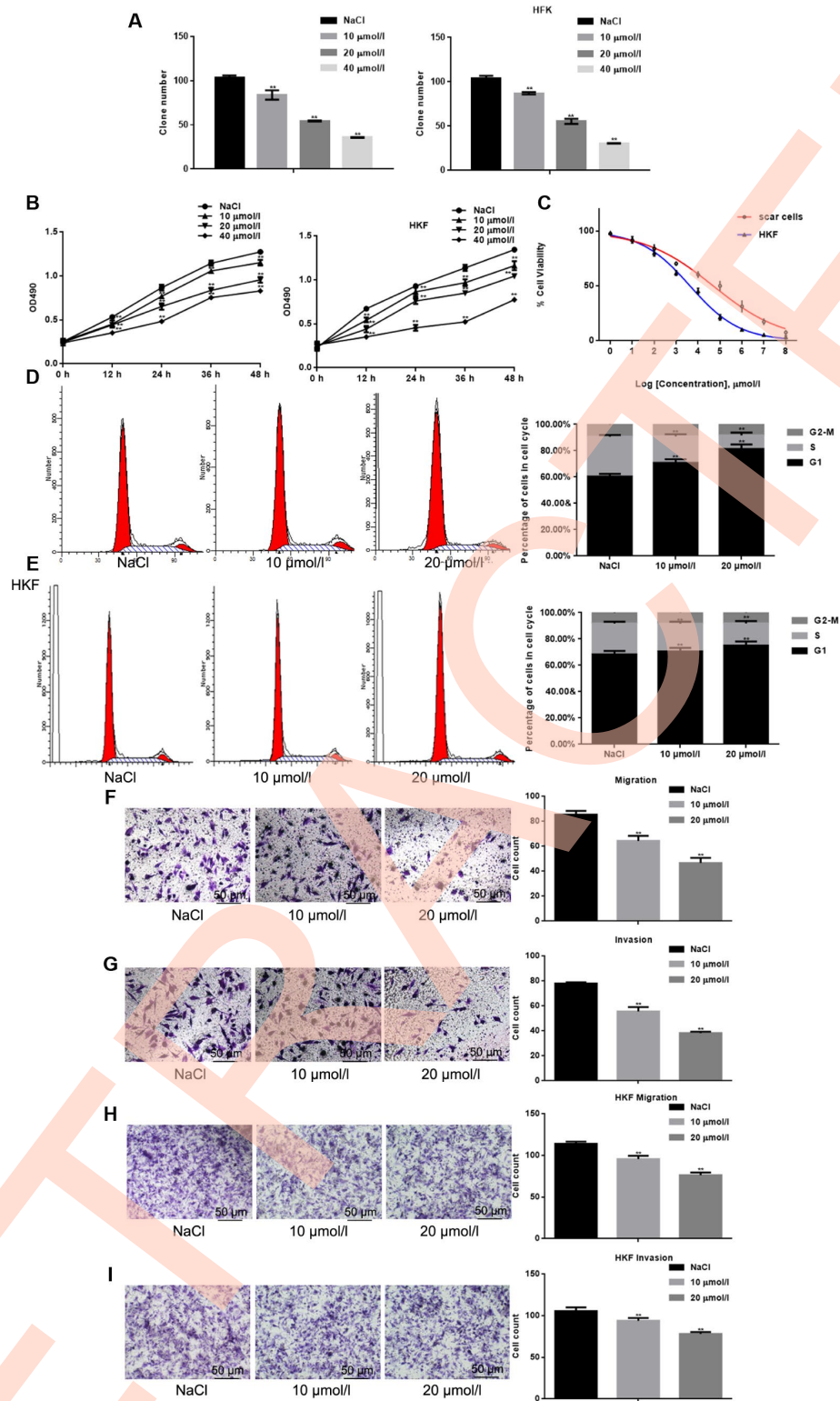


Figure 1. Apigenin inhibits the cell survival and migration of HSFs. (A) Cell clone formation assays. The results shown depict the mean±SD of three experiments done in triplicate. ****P < 0.05 vs. NaCl.** (B) MTT assays of HSFs and HKFs cultured with indicated concentrations of apigenin. Results depict the mean±SD of three experiments done in triplicate. ****P < 0.05 vs. NaCl.** (C) IC₅₀ values for the inhibitory effects of apigenin on HSFs and HKFs were calculated from the linear curve fitted to the MTT data. (D, E) FACS analysis of HSFs and HKFs pre-treated with the indicated concentrations of apigenin. The experiment was repeated three times. ****P < 0.05 vs. NaCl.** (F–I) Transwell assays showing the effects of pretreatment with apigenin for 24 h on HSF and HKF migration, with and without Matrigel. Migrated cells were counted, and the results depict the mean±SD of three experiments. ****P < 0.05 vs. NaCl.**

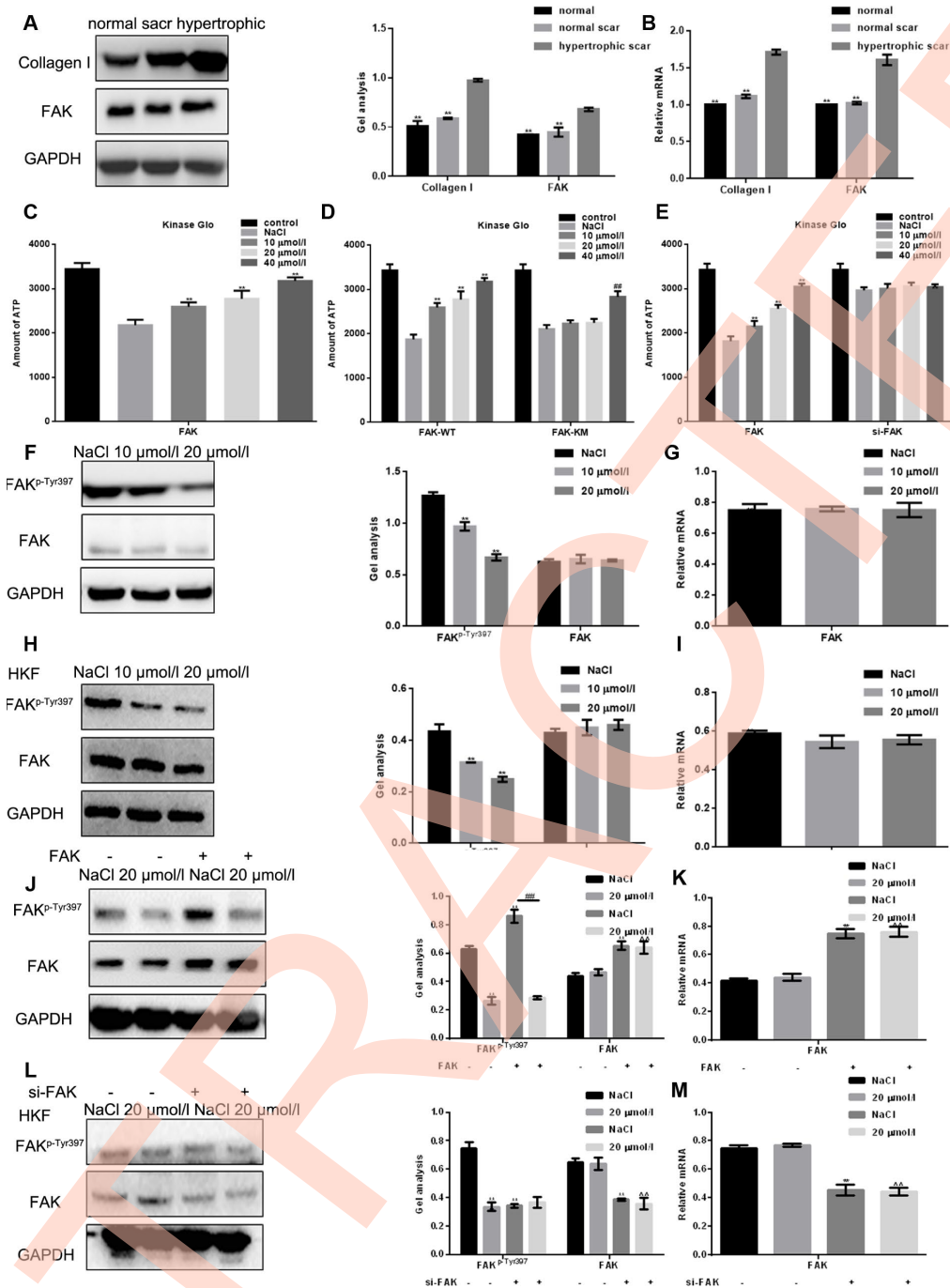


Figure 2. Apigenin suppresses the kinase activity of FAK and inhibits FAK phosphorylation. (A, B) Western blot and real-time PCR analyses showing the Collagen I and FAK content in normal skin tissue, normal scar tissue and hypertrophic scar tissue. $** P < 0.05$ vs. hypertrophic scar tissue. (C) Kinase Glo luminescence assays showing the inhibitory effect of apigenin on the kinase activity of FAK; 2 μ M ALX was used as an activated control. Data are shown as the mean \pm SD. $** P < 0.05$ vs. NaCl-treated FAK group. (D) Kinase Glo luminescence assays comparing the inhibitory effects of apigenin on the kinase activities of FAK-WT and FAK-KM(Y397A); 2 μ M ALX used as an activated control. Data are shown as the mean \pm SD. $** P < 0.05$ vs. NaCl-treated FAK-WT group; $### P < 0.05$ vs. NaCl-treated FAK-KM (Y397A) group. (E) Kinase Glo luminescence assays examining the inhibitory effects of apigenin on the kinase activities of FAK in HSFs transfected with si-FAK; 2 μ M ALX used as an activated control. Data are shown as the mean \pm SD. $** P < 0.05$ vs. NaCl-treated FAK group. (F–I) Western blot and real-time PCR analyses of FAK^{p-Tyr397} and FAK levels in HSFs and HKFs treated for 24 h with NaCl or the indicated concentration of apigenin. $*** P < 0.05$ vs. NaCl. (J, K) Western blot and real-time PCR analyses of the effect of 20 μ M apigenin FAK^{p-Tyr397} and FAK levels in HSFs overexpressing empty vector or FAK. $** P < 0.05$ vs. NaCl group; $### P < 0.05$ vs. NaCl with FAK-overexpressed group. (L, M) Western blot and real-time PCR analyses of the effect of 20 μ M apigenin on FAK^{p-Tyr397} and FAK levels in HSFs transfected with si-FAK or si-NC (negative control). $** P < 0.05$ vs. NaCl group.

DISCUSSION

The application of traditional Chinese medicines and their derivatives to treat hypertrophic scar has been widely studied in recent years [21, 22]. This is in large part because traditional Chinese medicines have the advantages of being inexpensive with few side effects and able to be used for long periods [23].

Apigenin has anti-tumor properties and functions against some chronic diseases. In vitro studies indicate that apigenin can inhibit the migration of fibroblasts through the TGF-beta 1 pathway and thus play a role in

the treatment of asthma and chronic sinusitis. However, there are few studies on hypertrophic scars [24, 25]. We first explored the mechanism of apigenin in hypertrophic scar, through in vivo and in vitro experiments.

Because the clinical manifestation as well as the underlying genetic and biochemical processes in hypertrophic scars are similar to those of tumors, some of the drugs and treatment methods used for cancers have been effectively applied to hypertrophic scars [26]. However, these treatments can have severely adverse side effects. Newer drugs isolated from plants are able

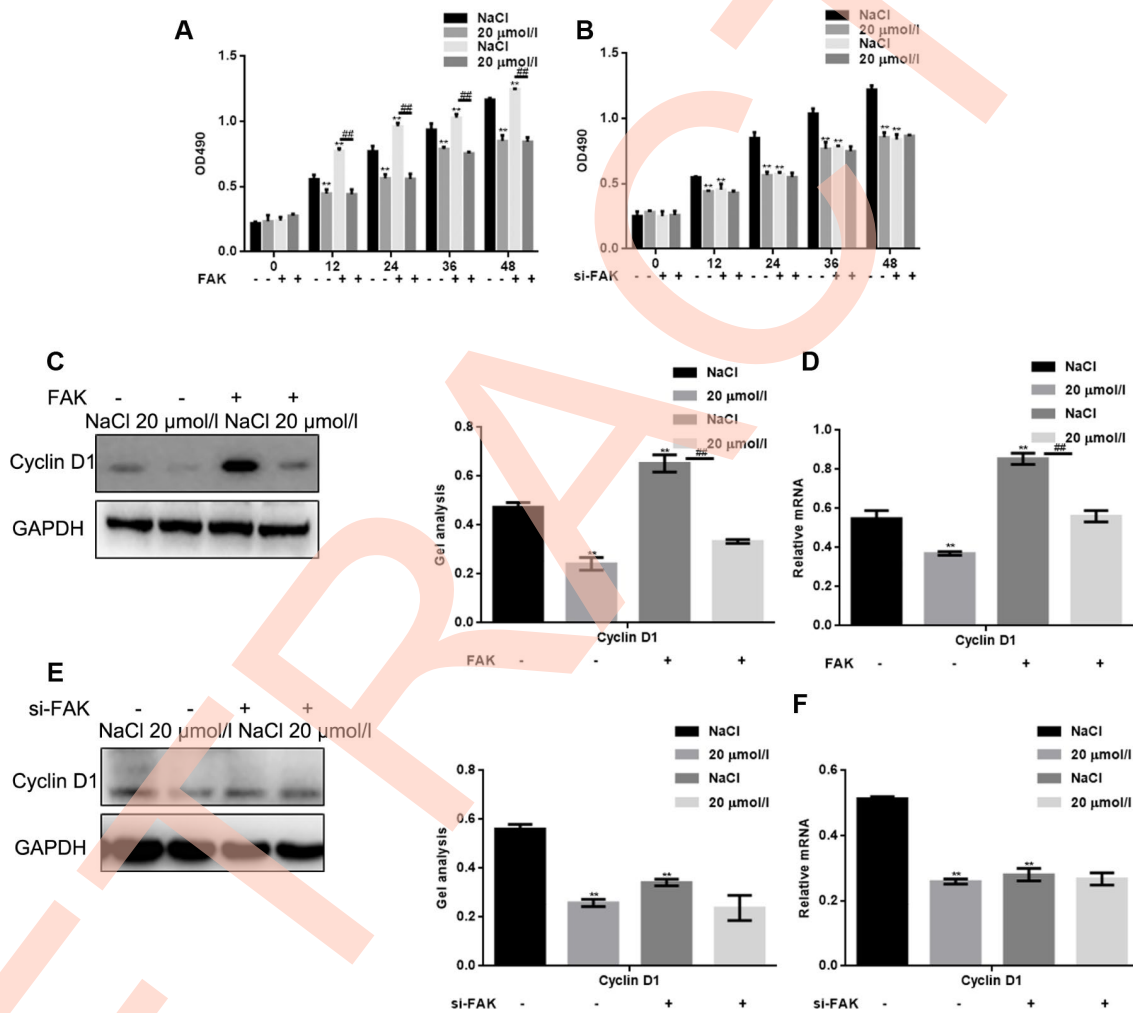


Figure 3. Apigenin inhibits HSF cell survival by suppressing FAK phosphorylation. (A) HSFs overexpressing vector or FAK were treated with 20 μ M apigenin, after which MTT assays were performed at the indicated times. Results depict the mean \pm SD of three experiments done in triplicate. ** $P < 0.05$ vs. NaCl group; ### $P < 0.05$ vs. NaCl with FAK overexpression group. (B) HSFs transfected with si-FAK or si-NC (negative control) were treated with 20 μ M apigenin, after which MTT assays was performed at the indicated times. Results depict the mean \pm SD of three experiments done in triplicate. ** $P < 0.05$ vs. NaCl group. (C, D) HSFs overexpressing empty vector or FAK were treated with 20 μ M apigenin, after which western blot and real-time PCR assays were performed to detect levels of cyclin D1 expression. ** $P < 0.05$ vs. NaCl group; ## $P < 0.05$ vs. NaCl with FAK overexpression group. (E, F) HSFs transfected with si-FAK or si-NC (negative control) were treated with 20 μ M apigenin, after which western blot and real-time PCR assays were performed to detect levels of Cyclin D1 expression. ** $P < 0.05$ vs. NaCl group.

to suppress the occurrence and development of hypertrophic scars by regulating fibroblast growth, apoptosis and migration, as well as the angiogenesis supporting the growth. Here, we showed for the first time that apigenin inhibits the cell survival of

fibroblasts by blocking progression of the fibroblast cell cycle from G1 to S phase. Apigenin also inhibits invasion and migration by fibroblasts. Apigenin may thus be an effective agent for the treatment and prevention of hypertrophic scar.

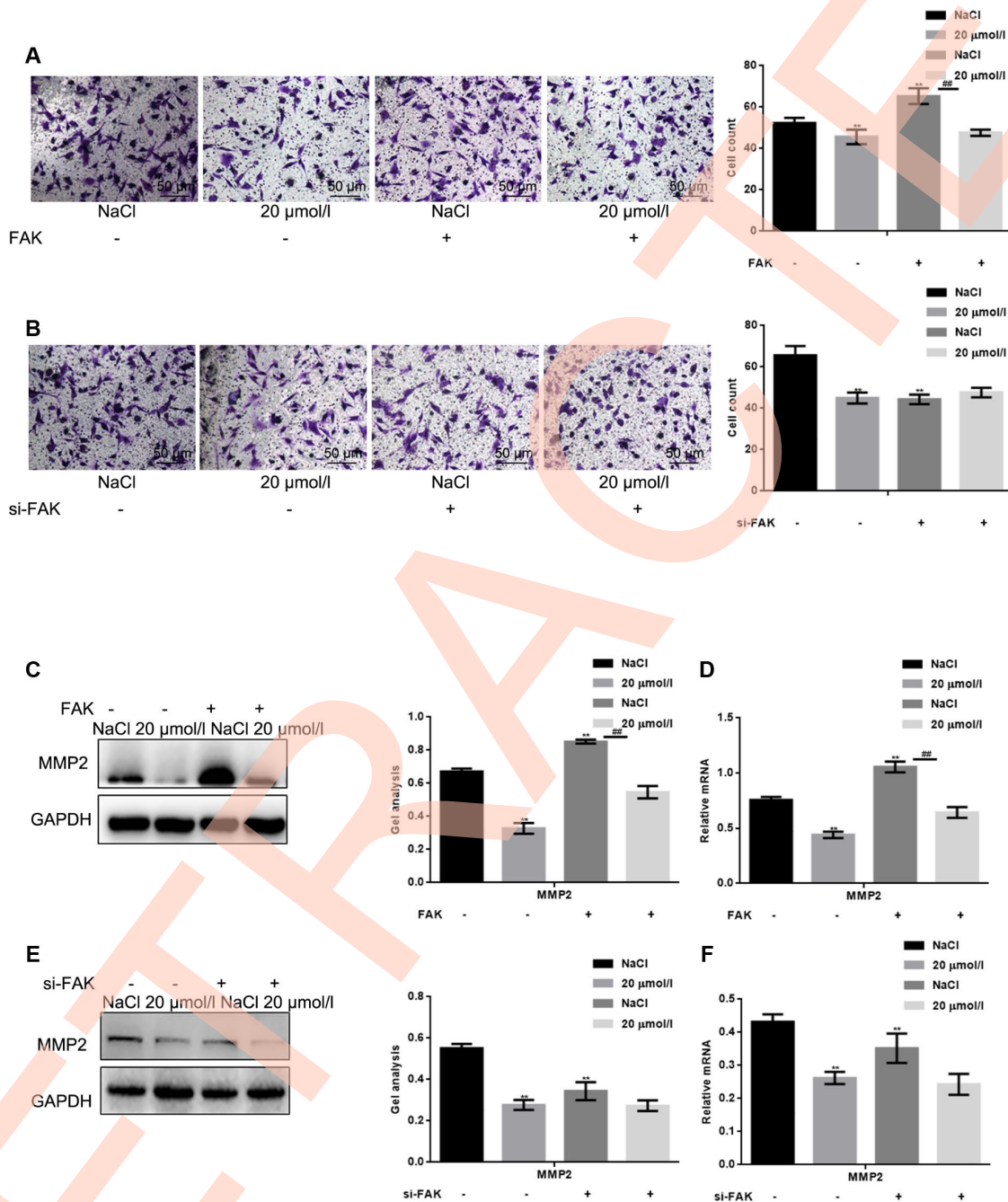


Figure 4. Apigenin inhibits HSF migration by suppressing FAK phosphorylation. (A) Transwell assays (without Matrigel) with HSFs overexpressing vector or FAK and treated with 20 μM apigenin. $**P < 0.05$ vs. NaCl group. $##P < 0.05$ vs. NaCl with FAK overexpression group. (B) Transwell assays (without Matrigel) with HSFs transfected with si-FAK or si-NC (negative control) and treated with 20 μM apigenin. $**P < 0.05$ vs. NaCl group. (C, D) HSFs overexpressing vector or FAK were treated with 20 μM apigenin, after which western blot and real-time PCR assays were performed to detect levels of MMP2 expression. $**P < 0.05$ vs. NaCl group. $##P < 0.05$ vs. NaCl with FAK overexpression group. (E, F) HSFs transfected with si-FAK or si-NC (negative control) were treated with 20 μM apigenin, after which western blot and real-time PCR assays were performed to detect levels of MMP2 expression. $**P < 0.05$ vs. NaCl group.

Table 1. Basic information of patients.

Group	Normal skin	Normal scar	Hypertrophic scar
sex			
Male	1	2	2
female	2	1	6
Average age Position	40.19±7.27	43.52±5.79	37.98±11.23
Chest	3	2	5
Shoulder	0	1	2
back	0	0	1

Integrin- and TGF- β receptor-mediated transduction pathways both play important roles in the abnormal biological behavior of HSFs, and their point of convergence is FAK. It has been reported that FAK is excessively activated within hypertrophic scars and that inhibition of FAK inhibits the development of hypertrophic scars [19, 20]. It was suggested in these studies that FAK could serve as a potential molecular target for hypertrophic scar therapy [19, 20]. Apigenin reduced integrin protein levels and inhibited the phosphorylation of FAK and extracellular signal-regulated kinase (ERK1/2), which induce the inhibition of melanoma and pancreatic cancer [27, 28]. However, there they have not been confirmed by in vivo studies. Using Kinase Glo assays to assess the effect of apigenin on the activity of FAK kinase, we found that apigenin significantly inhibited FAK kinase activity, which resulted in a significant decrease in the level of FAK phosphorylation (activation). This apigenin-induced decrease in FAK activation within HSFs led in turn to decreases in HSF cell survival and migration. Although it has been previously reported that apigenin inhibits the migration and transformation of fibroblasts [24, 29, 30] and that it also inhibits FAK activity [28, 31, 32], this is the first report on the effects of apigenin on primary human fibroblasts and the first examining those effects in the context of hypertrophic scarring.

In summary, apigenin was shown to be capable of inhibiting FAK kinase activity and suppressing FAK-mediated pathogenic HSF behaviors including excessive growth and migration.

METHODS

Tissues samples

The use of human samples (scar tissues) was conducted under a protocol (No: 2012087) reviewed and approved by General Hospital of the Northern Theater, PLA, and all experiments were carried out in accordance with the approved guidelines.

Hypertrophic scar tissues were obtained from eight patients undergoing plastic surgery between June 2014

and June 2016 in the Department of Plastic Surgery of General Hospital of the Northern Theater, PLA (Shenyang, China). Normal skin tissue was obtained from the remaining skin tissue of three patients undergoing skin flap surgery. Normal scar tissue was obtained from the normal scars of three patients who underwent a second operation. None of the patients from whom tissue samples were collected had a systemic disease. In addition, no patients had been exposed to any hormonal drugs within 3 months prior to the operation, and none received any local medication or laser treatments for their scars. Diagnoses of all patients were confirmed clinically and pathologically. Basic information of patients was shown in Table 1.

Before the experiment, all patients were informed about the purpose and procedures of the study and voluntarily agreed to provide tissues. Written consent was obtained from all participants, and all protocols were approved by the Ethics Committee of General Hospital of the Northern Theater, PLA. Patients received no treatment before skin excision.

The dermal portion was minced and incubated in a solution of 0.1 mg/ml collagenase type I (Beyotime Biotechnology, Shanghai, China) for 3 h at 37 C, and then cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) medium containing 10% fetal bovine serum in a culture bottle. Normal fibroblasts and normal scar fibroblasts began to grow around 9-10 d later. The hypertrophic scar fibroblasts expanded to the surrounding area and fused locally after 7-8 d. All cells have clear boundaries, large bodies, prismatic or irregular triangles, and there is no significant difference in cell shape and size. From 14 to 18 d, the brush-like tissue began to grow around the tissue mass in a radial shape with clear edges. Compared with normal fibroblasts and normal scar fibroblasts after cell fusion, hypertrophic scar fibroblasts have disordered arrangement. Fibroblasts were used at the third to fifth passage in all experiments in this study.

Cell culture

HKFs were purchased from Bioleaf Corporation (Shanghai, China). Human hypertrophic scar cells and

HKFs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA) at 37°C in incubator with a humidified atmosphere of 5% CO₂ and 95% air.

Formation of cell clones

Cells in the logarithmic growth phase were digested with 0.25% trypsin, dispersed into single cells, and suspended in DMEM containing 10% FBS. The cells were then inoculated into dishes containing 10 ml of medium at 37°C to a density of 50 cells per dish. The dishes were then rotated gently to evenly distribute the cells. After incubation for 24 h, the supernatant was discarded, and the cells were carefully washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and stained Giemsa stain (Beyotime Biotechnology, Shanghai, China) for 30 min. The dye solution was then slowly removed with running water, and the cells were air dried. Finally, the dish was inverted and overlaid with transparent film with a grid, after which the numbers of clones were counted under a microscope.

MTT assays

Cells were plated to a density of 1×10⁴ cells/well in 96-well plates and incubated for 24 h in 100 µl of DMEM containing 10% FBS. Thereafter, the indicated treatments were added to the cells, which were then incubated for an additional 0, 12, 24, 36 or 48h. At the end of the incubation, cellular cell survival was measured using a modified tetrazolium salt-3-(4-5 dimethylthiozol-2yl)-2-5diphenyl-tetrazolium bromide (MTT) assay. For this, 10 µl of MTT solution (5 mg/ml) were added to the cells, which were then for 4 h, after which 150 µl of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formed formazan crystals. The optical density was then measured at 490 nm using a microplate reader (Bio-Rad, CA, USA). NaCl solution was the solvent of Apigenin. NaCl was used as a control.

Flow cytometry

Cells were incubated with the indicated concentrations of apigenin for 24 h. Thereafter, the cells were collected, washed with cold PBS and fixed in 70% ethanol on ice for at least 1 h. The cell pellet was washed three times with cold PBS and stained with 50 µg/ml PI for 15 min at 37°C. The cells were then analyzed using a FACS Vantage flow cytometer with the Cell Quest acquisition and analysis software program (Becton Dickinson and Co., San Jose, CA).

Transwell assays

The migration capacities of cells were determined using transwell chambers (8 pm pore size, Corning, NY, USA)

with or without Matrigel. Cells were plated to a density of 1×10⁴ cells in the upper part of each chamber, which contained 100 µl of serum-free DMEM. The lower compartments were filled with 600 µl of DMEM containing 10% FBS. After incubation for 8 h at 37°C, non-invading cells were removed from the upper surface, and the invaded cells on the lower surface of the filter were fixed, stained with 0.1% trypan blue (Beyotime Biotechnology, Shanghai, China), photographed and counted under high-power magnification.

Real-time PCR

Following treatment various processing factors for 24 h, total cellular RNA was extracted from HSFs using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. Thereafter, 1 mg of total RNA was reverse transcribed to cDNA using a RT reaction kit (Promega, USA). Real-time PCR was performed according to the manufacturer's instructions using SYBR Premix Ex Taq (TaKaRa, Japan) as a DNA-specific fluorescent dye. Gene expression levels were calculated relative to the expression level of GAPDH. The primers used are listed in Table 2.

Western blot analyses

Proteins were extracted from cells using RIPA buffer, separated by SDS-PAGE and electro-transferred onto the PVDF membranes. The membrane were blocked with 5% nonfat dry milk in TBST for 1 h at room temperature, after which the proteins were probed with specific antibodies against Collagen I, cyclin D1, MMP9, FAK, phospho-FAK(Tyr397) and GAPDH (1:1,000 dilution; BOSTER, Wuhan, China). Relative expression of detected proteins was quantified and normalized to GAPDH using Image J software (National Institutes of Health, Bethesda, MD, USA).

Kinase Glo Luminescence assays

Kinase Glo Luminescence assays [20] were in run in black 384-well plates. Aliquots containing the indicated concentration of apigenin and 1 µl of FAK kinase (Invitrogen, USA) were added to kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂ and 0.2 mM DTT) to a final volume of 20 µl. The mixture was incubated for 3 h at 37°C and then mixed with 20 µl of Kinase Glo Luminescent (Promega, USA) reaction solution. After allowing the mixture to stand for 10 min, luminescence was measured.

HSFs were transfected with FAK-WT/FAK-KM (Y397A) (an unphosphorylated mutant) or FAK/si-FAK. After 24 h, the cells were washed with ice-cold PBS and suspended in cold lysis buffer, and the

Table 2. Primers for real-time PCR.

Name	Forward primer(5'→3')	Reverse primer(5'→3')
Cyclin D1	CCGAGGAGCTGCTGCAAATGGAGCT	TGAAATCGTGCGGGGTCATTGCGGC
MMP2	CGCATCTGGGGCTTTAAACAT	TCAGCACAAACAGGTTGCAG
FAK	CAACCACCTGGGCCAGTATTATCAG	ATGGTTAGGGATGGTGCC
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

supernatants were pre-adsorbed onto agarose beads (Sigma-Aldrich, USA) for 2 h at 4 °C. The beads were then washed with cold PBS and mixed with kinase buffer to a volume of 20 µl. Kinase Glo Luminescence assays were then run as described above.

Cell transfection

Upon reaching 60–70% confluence, hypertrophic scar fibroblasts were transfected with 2µg of vector/FAK-WT/FAK-KM(Y397A) (Shenggong, Shanghai, China) by using Lipofectamine 2000 reagent (Kangchen Biotech Inc., Shanghai, China) according to the manufacturer's instructions.

FAK silencing

HSFs diluted in complete medium without antibiotics were plated to a density of 3×10^5 cells/well in 12-well plates. The cells were then transfected for 48 h with si-FAK plasmid or empty vector using Lipofectamine 2000 according to the manufacturer's instructions. Transfectants were selected in medium containing puromycin (1.5 µg/ml).

Statistical analysis

Results are represented by the mean ± standard deviation (SD) of at least three independent experiments. Two-tailed Student's t test was used to compare group means. Differences were considered significant when $P < 0.05$.

Ethics approval

Research involving human subjects, human material, or human data, was performed in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of General Hospital of Northern Theater. PLA (R20111762).

AUTHOR CONTRIBUTIONS

Hongyi Wang: conceived of the study, carried out the molecular studies. Bingyu Guo: carried out the molecular studies. Shixiu Lin: participated in its design and coordination and helped to draft the manuscript.

Peng Chang: conceived of the study. Kai Tao: helped to write the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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