Original Article
Emodin inhibits formation of hypertrophic scars through regulating the p38/MAPK signaling pathway

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Abstract: Hypertrophic scars (HS) often result from overproduction of hypertrophic scar fibroblasts (HSFs) and excessive deposition of collagen. The purpose of this study was to evaluate the pharmacological properties of emodin in HS management. Our results demonstrate that emodin treatment remarkably inhibits HSF proliferation, induces apoptosis, and represses Col I, Col III, and α-SMA expression in in vitro and ex vivo in an HS model. Western blot analysis further revealed that emodin significantly attenuates p38 phosphorylation in a dose-dependent manner. Taken together, these findings indicate that emodin can exert anti-fibrotic activity mainly through interfering with the p38/MAPK signaling pathway. Emodin may be a potential chemopreventive or therapeutic agent for the treatment of human HS.

Keywords: Hypertrophic scars, emodin, p38/MAPK signaling, Col I, Col III

Introduction

Hypertrophic scars (HS) are features of a serious fibrotic skin disorder following burns, trauma wounds, and surgical procedures [1]. Patients with HS often report pain, itching, stiffness, loss of sensation, loss of joint mobility, and anatomical deformities [2, 3]. Currently there are multiple therapies for hypertrophic scars, including surgery, corticosteroids, compression, laser, and cryotherapy. However, none of these treatments has been verified to be effective in fully avoiding excessive HS formation and regenerating healthy dermal tissue with few side effects [4].

Dysregulation of the wound healing process is a critical prerequisite for HS formation [5]. During wound healing, fibroblasts from the border of the wound migrate to the center and transdifferentiate into myofibroblasts that abundantly yield extracellular matrix (ECM) proteins, especially collagen, thus leading to HS formation [6]. HS formation is modulated by several cellular signaling pathways, including the p38/Mitogen-activated protein kinase (MAPK) signaling pathway, which functions through the mitochondrial death pathway [7] and cell transdifferentiation [8].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a natural anthraquinone present in traditional Chinese herbal medicines, especially from Giant Knotweed Rhizome [9]. Several studies have revealed that emodin exerts various potent biological effects, including antimicrobial, anti-inflammatory, and anti-tumorigenic properties [10, 11]. Recently, Tao et al. indicated that emodin gel treatment can suppress HS formation in a rabbit ear scar model [12].

Emodin is reported to exert its therapeutic effects through regulating p38/MAPK signaling in a wide variety of diseases [13-15], however the effects of emodin on p38/MAPK signaling in HS formation remain to be further determined. Thus, the main objective of this work was to investigate the anti-fibrotic properties of emodin during HS development and progression.

Materials and methods

Patient specimens

Human HS tissues were collected from 13 patients (8 men and 5 women, age ranges from 24- to 51-year old) who underwent plastic sur-

Table 1. HS patient information.

ID	Sex	Age	Smoking status	Localization	Surgery date
1	Female	36	No	Face	2017/03/07
2	Male	29	No	Chest	2017/01/14
3	Male	31	Yes	Face	2016/12/25
4	Male	39	No	Arm	2017/04/11
5	Female	42	Yes	Chest	2016/10/16
6	Male	24	No	Arm	2017/04/26
7	Female	28	No	Face	2017/05/05
8	Male	44	No	Face	2016/12/16
9	Male	32	Yes	Arm	2017/03/17
10	Female	36	No	Face	2017/05/28
11	Female	51	No	Arm	2017/02/07
12	Male	39	Yes	Chest	2017/06/20
13	Male	43	Yes	Face	2016/09/22

Table 2. PCR primer sequences.

Gene name	Primer sequences
α-SMA	
Forward	5'-GACAGCTACGTGGGTGACGAA-3'
Reverse	5'-CGGGTACTTCAGGGTCAGGAT-3'
Col I	
Forward	5'-GAGGGCAACAGCAGGTTCACTTA-3'
Reverse	5'-TCAGCACCACCGATGTCCA-3'
Col III	
Forward	5'-CCACGGAAACACTGGTGGAC-3'
Reverse	5'-GCCAGCTGCACATCAAGGAC-3'
GAPDH	
Forward	5'-GTCAACGGATTTGGTCTGTATT-3'
Reverse	5'-AGTCTTCTGGGTGGCAGTGAT-3'

gery in Ningxiang People's Hospital (Hunan, China). The presence of HS was verified through pathological examination. Six hypertrophic scars were located on the face, four on the upper arm, and three on the anterior portion of the chest. Patients did not receive any drugs prior to skin excision. All experimental protocols were approved by the Ethics Committee of Ningxiang People's Hospital, and before the experiments were performed, written consent was obtained from all patients or their relatives. The patient information is listed in **Table 1**.

Cell culture and treatment

The dermal pieces from HS were minced into small pieces and incubated in a solution of collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) at 0.1 mg/ml for 4 hours at 37°C on a rotator to isolate fibroblasts (HSFs). HSFs were

then cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Primary HSFs of passage numbers 6-8 were used for further investigation.

Emodin was purchased from Sigma-Aldrich, dissolved in DMSO and used at different doses (0, 10, 20, 50 µM) to treat either HSFs or tissues.

Cell proliferation assay

Cell proliferation assay was performed using cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). HSFs (1×10^5) were seeded into flat-bottomed 96-well plates with 100 µL of growth medium per well and allowed to attach and grow overnight. The medium was then replaced with 100 µL of growth medium containing different doses (0, 10, 20, 50 µM) of emodin. After 96 hours of incubation, 10 µL of CCK-8 solution was added and the plates were incubated at 37°C for additional 4 hours. The optical density (OD) of each plate was measured at 450 nm in a microplate reader.

Flow cytometric analysis of cell apoptosis

HSFs were incubated on the 6-well plates in medium with different doses (0, 10, 20, 50 µM) of emodin. After 96 hours of incubation, the HSFs were collected, washed twice with cold PBS, resuspended and stained with Annexin V-PE and propidium iodide (PI) using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) for 20 minutes at room temperature in the dark. Quantitative analysis was performed using a flow cytometer (BD Bioscience) with CellQuestPro software (BD Biosciences).

qRT-PCR analysis

Total RNA was extracted from the tissues and fibroblasts using the TRIzol reagent (Invitrogen). Reverse transcription of the total RNA into complementary DNA (cDNA) was performed by using PrimeScript RT Master Mix (TaKaRa, Dalian, China). qPCR analysis was performed using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) by a 7900 Fast

Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). All primers were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). GAPDH was used as the internal reference gene. Relative quantification of individual genes was determined by using the $2^{-\Delta\Delta Ct}$ method [16]. The sequences of primers used in this study are shown in **Table 2**.

Western blot analysis

Protein was extracted from the tissues and fibroblasts using RIPA protein extraction reagent (Beyotime, Shanghai, China) with a protease inhibitor cocktail (Roche, Pleasanton, CA, USA). Equal amounts of protein were separated by 10% SDS-PAGE gel electrophoresis followed by transference onto nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were then blocked with 2.5% nonfat dry milk for 1 hour, followed by incubation with specific primary antibodies at 4°C overnight. After incubation with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies, the immunoreactive protein bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). GAPDH was used as the internal reference gene.

Cultured HS tissues ex vivo

Ex vivo culture of human HS tissues were performed as described previously [17]. The HS tissues were cut into 5×5 mm sections and cultured in DMEM containing different doses (0, 10, 20, 50 μM) of emodin. The medium was changed every 3 days. After 7 days of incubation, the HS tissues were fixed in 4% paraformaldehyde.

Histological analysis

The HS tissues were embedded in paraffin blocks and cut into 4-μm thick tissue sections, which were then subjected to hematoxylin and eosin (H&E) staining. The histologic characteristics of the collagen fibers in the samples were visualized under an optical microscope.

Statistical analysis

All quantitative data are depicted as mean ± standard deviation (SD) from at least three separate experiments. All statistical analyses were performed using GraphPad Prism version 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The differences between groups were compared using one-way ANOVA followed by Tukey's post hoc tests. P<0.05 was considered statistically significant.

Results

Emodin represses proliferation of HSFs

To investigate the possible impact of emodin on growth of HSFs, proliferation activities of HSFs were determined by CCK-8 assay after treatment with indicated doses (0, 10, 20, 50 μM) of emodin for 96 hours. Our data demonstrated that emodin inhibited HSFs proliferation in a dose-dependent manner (**Figure 1A**). Moreover, Western blot analysis showed that the cell proliferation markers Ki67 and PCNA were decreased in a dose-dependent manner in emodin-treated HSFs (**Figure 1B**).

Emodin promotes the apoptosis of HSFs

The effect of emodin on apoptosis of HSFs was next investigated by Annexin-V FITC/PI FACS analysis. As shown in **Figure 2A**, following treatment with different doses of emodin for 96 hours, the percentage of HSFs undergoing apoptosis was significantly increased in a dose-dependent manner. Apoptosis-related genes such as Bcl-2 could also mediate apoptosis in HS [18]. Expression of Bcl-2 was found to be significantly decreased in emodin-treated HSFs (**Figure 2B**).

Emodin decreased the expression of Col I and Col III in HSFs

Through qRT-PCR analysis, it was observed that mRNA expression levels of Col I, Col III, and α-SMA in HSFs were inhibited by emodin treatment in a dose-dependent manner (**Figure 3A**). This dose-dependent effect of emodin on collagen expression was further confirmed by western blot analysis. As depicted in **Figure 3B**, the protein expression levels of Col I, Col III, and α-SMA in HSFs were gradually decreased with increasing doses of emodin. These results suggest that emodin could repress fibrotic-related protein expression to decrease deposition of collagen in HSFs.

Emodin inhibits expression of Col I and Col III in cultured HS tissues

The potential anti-fibrotic effects of emodin were then determined in cultured HS tissues. Treatment with emodin for 7 days revealed
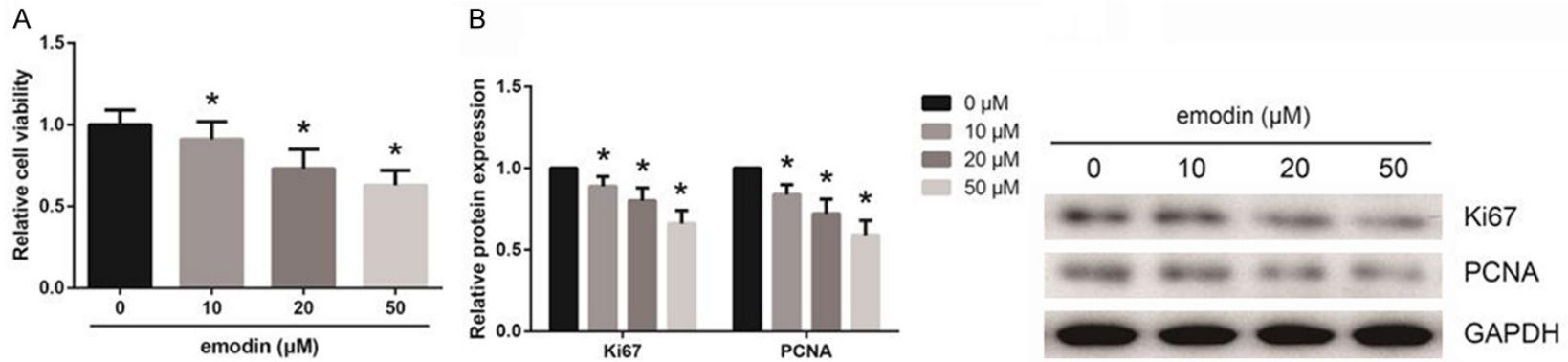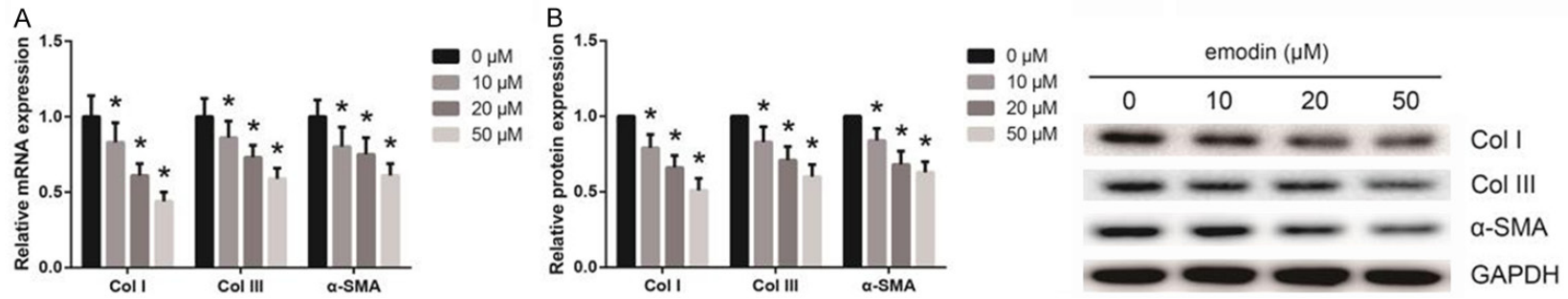
Figure 1. Emodin represses proliferation of HSFs. A. CCK-8 assay was performed to evaluate the proliferation of HSFs. B. Western blot analysis was performed to evaluate the protein expression of Ki67 and PCNA in HSFs. All values are expressed as mean ± SD. *P<0.05 versus HSFs treated with 0 µM emodin.
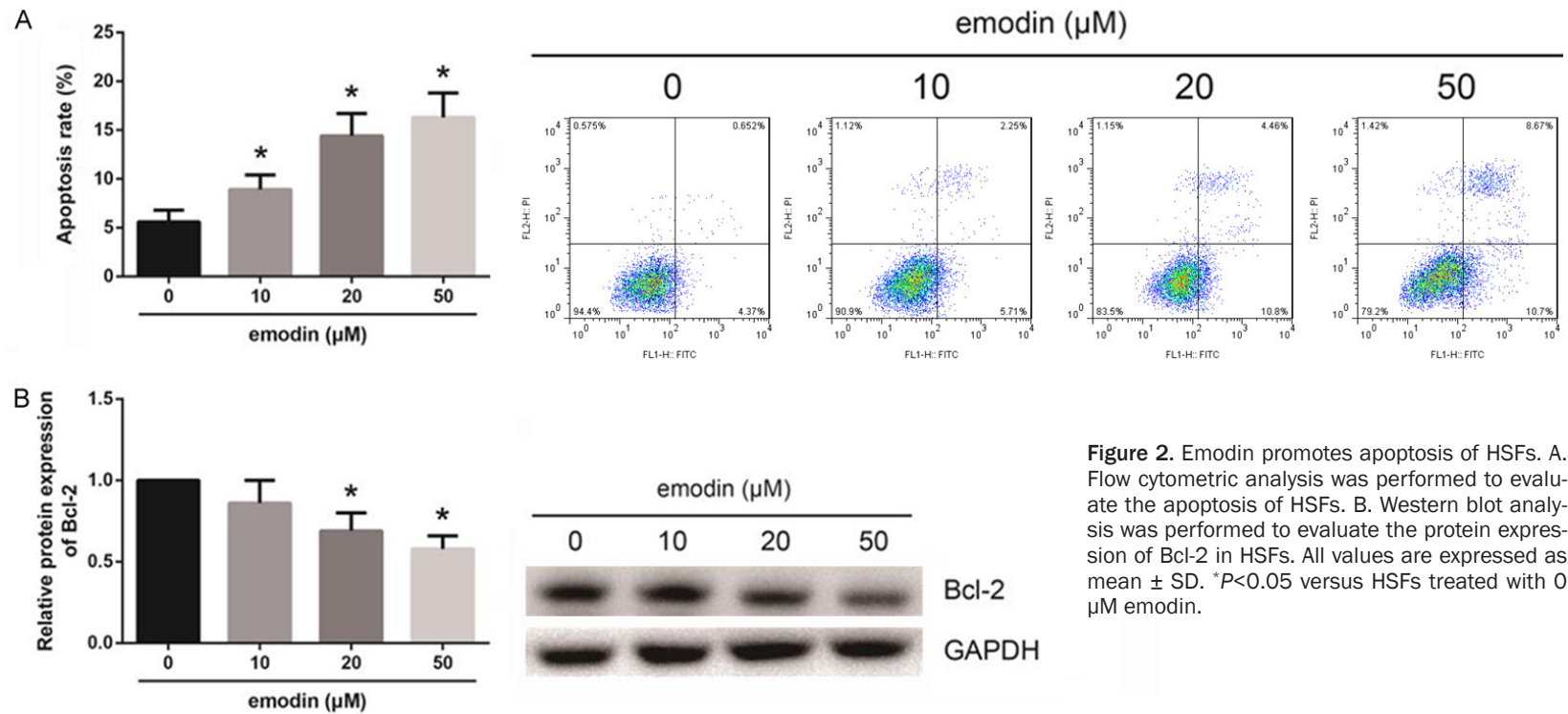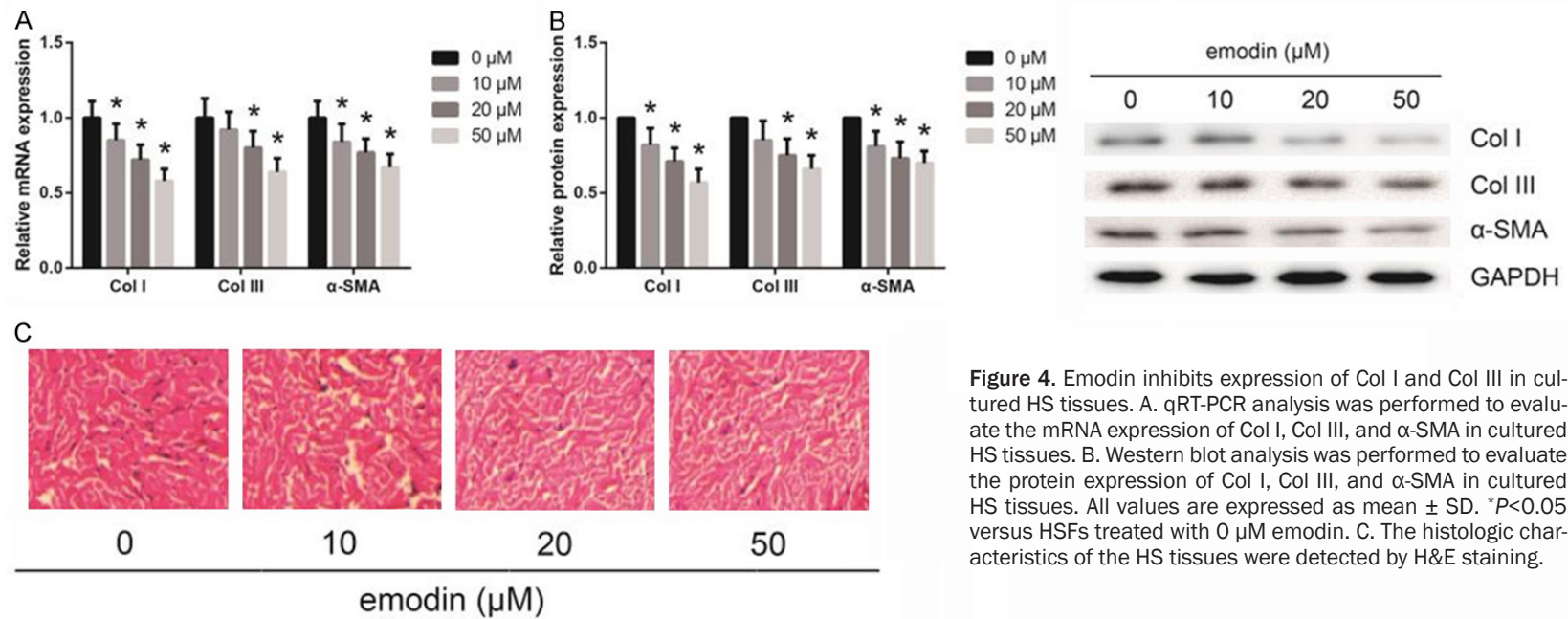

Figure 2. Emodin promotes apoptosis of HSFs. A. Flow cytometric analysis was performed to evaluate the apoptosis of HSFs. B. Western blot analysis was performed to evaluate the protein expression of Bcl-2 in HSFs. All values are expressed as mean ± SD. *P<0.05 versus HSFs treated with 0 µM emodin.

Figure 3. Emodin decreased expression of Col I and Col III in HSFs. A. qRT-PCR analysis was performed to evaluate the mRNA expression of Col I, Col III, and α-SMA in HSFs. B. Western blot analysis was performed to evaluate the protein expression of Col I, Col III and α-SMA in HSFs. All values are expressed as mean ± SD. *$P<0.05$ versus HSFs treated with 0 μM emodin.

Figure 4. Emodin inhibits expression of Col I and Col III in cultured HS tissues. A. qRT-PCR analysis was performed to evaluate the mRNA expression of Col I, Col III, and α-SMA in cultured HS tissues. B. Western blot analysis was performed to evaluate the protein expression of Col I, Col III, and α-SMA in cultured HS tissues. All values are expressed as mean ± SD. *$P<0.05$ versus HSFs treated with 0 μM emodin. C. The histologic characteristics of the HS tissues were detected by H&E staining.
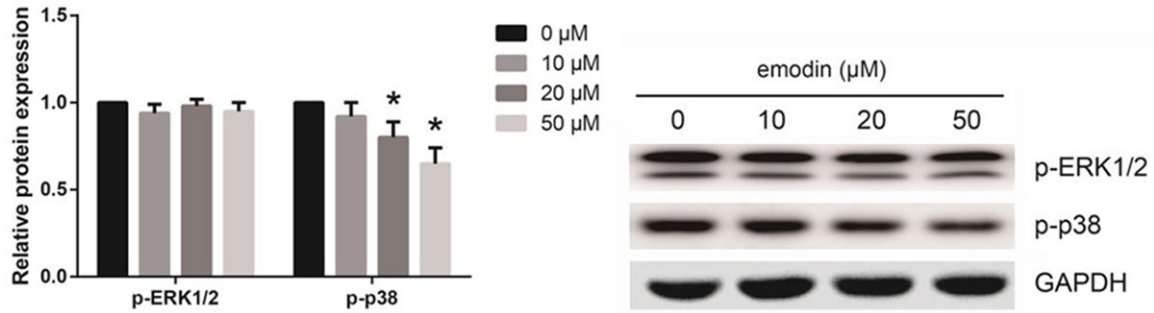
Figure 5. Emodin exerts anti-fibrosis effect through regulation of p38/MAPK signaling. Western blot analysis was performed to evaluate the protein expression of p-ERK1/2 and p-38 in HSFs. All values are expressed as mean ± SD. $^*P < 0.05$ versus HSFs treated with 0 μM emodin.

dose-dependent reduction in the mRNA and protein levels of Col I, Col III, and α-SMA in human HS explants (**Figure 4A**, **4B**), which was in accordance with the results in in vitro HSFs. Furthermore, H&E staining showed that emodin resulted in thinner and orderly arranged collagen fibers (**Figure 4C**). Collectively, these data suggest that emodin also exerts its anti-fibrotic properties in an *ex vivo* HS model.

Emodin exerts anti-fibrosis effects through regulation of p38/MAPK signaling

The p38/MAPK and ERK1/2 members of the MAPK signaling subfamily, are closely associated with fibrogenesis [19]. In the present study, as demonstrated in **Figure 5**, emodin treatment did not significantly affect ERK1/2 phosphorylation, but led to a remarkable, dose-dependent reduction of phosphorylated p38 in HSFs, indicating that emodin exhibits anti-fibrosis activities partly through inactivation of the p38/MAPK signaling pathway.

Discussion

In our present investigation, the potential therapeutic properties of emodin in inhibiting HS formation was investigated. Emodin repressed HSF proliferation, induced HSF apoptosis, and inhibited Col I and Col III expression through inactivating the p38/MAPK signaling pathway.

At present, many of the natural agents that have been utilized as treatment options for HS, including baicalein [20], galangin [21], gallic acid [22] and kaempferol [23]. Some literature has reported that the therapeutic efficacy of emodin, a bioactive compound isolated from plants, is mediated through induction of apoptosis, anti-proliferation, and anti-adhesion [24]

properties, which makes it a very promising drug for HS treatment.

Regulation of dermal fibroblast proliferation and apoptosis is of great importance for controlling HS formation [25]. Recent studies demonstrated a potential anti-fibrotic function of emodin on other diseases, such as corneal fibroblasts [26], renal fibroblasts [27], and pulmonary fibroblasts [28]. In the present study, in vitro validations were performed to further determine whether emodin affects HSFs proliferation and apoptosis. Treatment of emodin displayed a significantly lower HSFs growth rate as evidenced by cell viability assay. Furthermore, FACS was used to assess the effects of emodin on apoptosis and found that treatment of emodin noticeably increased apoptosis of HSFs.

A mounting body of evidence indicates that excessive accumulation of collagen plays a critical role in HS formation, thus the inhibition of collagen synthesis is preferred in HS treatment [3, 6, 29]. Normal fibroblasts synthesize both Col I and Col III, but this synthesis becomes imbalanced with changes in the surrounding environment. Our present study reveals that emodin reduced the levels of Col I, Col III, and α-SMA, indicating that emodin could repress collagen synthesis and trans-differentiation of fibroblasts into myofibroblasts which was featured by less α-SMA expression. Pathological examination also showed that collagen fibers were significantly reduced upon emodin treatments, which was in accordance with the *in vitro* findings.

The molecular mechanisms involved in emodin-mediated repression of collagen accumulation are not well understood. Our present data show that emodin caused a significant reduction of

phosphorylated p38 expression in HSFs, which supports previous studies that emodin had a rapid anti-fibrosis effect in animal models and in vitro experiments by regulation of p38/MAPK signaling pathway [13, 30]. The p38/MAPK pathway is also known to be implicated in HS formation. Li et al. reported that adipose tissue-derived stem cells could exert an anti-fibrotic function through the inhibition of the p38/MAPK signaling pathway [31].

Collectively, our findings disclose that emodin might exert significant anti-proliferative and apoptosis-promoting effects on HSFs *in vitro*, and inhibit collagen synthesis in cultured HS tissues through the p38/MAPK signaling pathway. All of these results indicated that emodin serves as a promising agent for HS treatment and further studies will be needed to verify our current study using an animal model to assess the effect of emodin in suppression of HS formation *in vivo*.

Disclosure of conflict of interest

None.

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