Isorhamnetin attenuates the proliferation, invasion, migration and fibrosis of keloid fibroblasts by targeting S1PR1

XIAOSHU PU[1], XIAOLEI CAO[2], HONGYAN LIU[1], WENLIAN HUANG[3], LANFANG ZHANG[1] and TING JIANG[1]

[1]Department of Burn and Plastic Surgery, Nanchong Central Hospital; [2]General Surgery Department, The People's Hospital of Shunqing District; [3]Department of Critical Care Medicine, Nanchong Central Hospital, Nanchong, Sichuan 637000, P.R. China

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Abstract. Isorhamnetin (IH) is a type of flavonoid with multiple biological activities, including cardioprotective, anti-tumor, anti-inflammatory and antioxidant activities. However, the role and potential mechanism of IH in keloids are still not completely understood. The aim of the present study was to explore how IH affects keloid progression. In the present study, cell proliferation was evaluated using the Cell Counting Kit-8 assay and immunofluorescence. Wound healing and Transwell assays were performed to assess cell migration and invasion, respectively. The expression levels of fibrosis-related proteins were measured using western blot analysis and immunofluorescence. In addition, the binding between IH and sphingosine-1-phosphate receptor-1 (S1PR1) was analyzed using the TargetNet database, and molecular docking was performed using Zinc, PubChem, AutoDockTools 1.5.6 and Discovery Studio 4.5 software. The expression levels of proteins in the PI3K/AKT pathway were detected by western blot analysis. The results showed that IH inhibited the proliferation, invasion, migration and fibrosis of keloid fibroblasts. The binding of IH and S1PR1 was verified and molecular docking was performed. Notably, IH significantly suppressed the expression levels of S1PR1, phosphorylated (p)-PI3K and p-AKT. Furthermore, the silencing of S1PR1 suppressed the cell proliferation, migration, invasion and fibrosis of keloid fibroblasts, as well as the expression of the PI3K/AKT pathway proteins. Conversely, S1PR1 upregulation reversed the inhibitory effects of IH on keloid fibroblast proliferation, migration, invasion and fibrosis. In conclusion, the results revealed that IH suppressed the proliferation, migration, invasion and fibrosis of keloid fibroblasts by targeting the S1PR1/PI3K/AKT pathway, suggesting that IH may be a promising drug for the treatment of keloids.

Correspondence to: Dr Xiaoshu Pu, Department of Burn and Plastic Surgery, Nanchong Central Hospital, 97 Renmin Nan Lu, Nanchong, Sichuan 637000, P.R. China
E-mail: puxiaoshu_11122@163.com

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Introduction

A keloid is a hyperplastic skin disease characterized by excessive fibroblast proliferation, excessive deposition of extracellular matrix (ECM) and inflammatory cell infiltration (1). Keloids result in excessive fibrosis of local tissues caused by the abnormal healing of the skin following injury or inflammation (2). They can be accompanied by pain, hypersensitivity and itching, which can significantly affect the quality of life of patients (3). Their formation is mainly caused by changes in the regulation of growth factors, abnormal conversion of collagen, genetics, immune dysfunction and sebum reaction (4). At present, surgical resection is the main treatment for keloids; however, treatment efficiency is low and relapse is common (5). Alternative treatments include steroid injections, radiotherapy, and laser, silicone and stress therapy (6). Although keloids benign dermal tumors, they have high recurrence rates (7). Keloid fibroblasts are considered the key effector cells for keloid formation (8). Studies have found that fibroblast dysfunction, including abnormal proliferation, invasive migration and invasion, and excessive ECM production, is involved in keloid formation (9). Therefore, identifying treatments or drugs that can inhibit fibroblast dysfunction may be an effective way to treat keloids.

Isorhamnetin (IH) is a type of flavonoid widely found in sea buckthorn, ginkgo biloba and sophora japonica (10). Recent studies have shown that IH has biological activities, including cardiovascular and cerebrovascular protective, anti-tumor, anti-inflammatory, antioxidant activities, and is also associated with obesity prevention (11). IH can inhibit the migration and invasion of non-small-cell lung cancer cells by inhibiting the Akt/ERK-mediated epithelial-mesenchymal transition (EMT) process (12). Another study showed that IH alleviates bleomycin-induced endoplasmic reticulum stress and PERK signal activation to play a protective role in bleomycin-induced pulmonary fibrosis in mice (13). In addition, Yang *et al* (14) found that IH suppressed hepatic stellate cell activation and prevented hepatic fibrosis by inhibiting the TGF-β/Smad signaling pathway and alleviating oxidative stress. However, the biological roles of IH in keloid progression remain unclear. Therefore, identifying the roles of IH in keloid fibroblasts and the potential underlying mechanisms is crucial.

Materials and methods

Bioinformatics analysis. The TargetNet database (http://targetnet.scbdd.com/calcnet/calc_text/) was used to predict the binding between IH and sphingosine-1-phosphate receptor-1 (S1PR1) in keloids. In addition, the structure of IH was obtained from the Zinc (http://zinc.docking.org) and PubChem databases (https://pubchem.ncbi.nlm.nih.gov), and protein structure was obtained from the PDB database (http://www.rcsb.org). AutoDockTools 1.5.6 software was used to process proteins and Discovery Studio 4.5 software was used to visualize the molecular docking results to obtain 2D and 3D images.

Cell culture and treatment. Keloid fibroblasts (ATCC®CRL-1762™) were obtained from the American Type Culture Collection and cultivated in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C. The cells were then treated with different doses of IH (20, 50 and 100 μM) for 24, 48 or 72 h. IH (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and was adjusted to final concentrations using complete culture medium. The final DMSO concentration was <0.05% in all experiments (i.e., a non-cytotoxic range).

Cell transfection. To knock down S1PR1, specific siRNA targeting S1PR1 (siRNA-S1PR1-1: 5'-GCUAUUCAUUAG AUAGUAAUU-3'; siRNA-S1PR1-2:5'-GCAUUGUCA AGCUCCUAAAGG-3') and corresponding control siRNA (siRNA-NC: 5'-UUCUCCGAACGUGUCACGU-3'; both synthesized by Gene Pharma) were used. To overexpress S1PR1, the pc-DNA3.1 vector containing the whole length of S1PR1 (Ov-S1PR1) and the empty vector (Ov-NC; both synthesized by Gene Pharma) were used. These recombinants were transfected into keloid fibroblasts using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Following 48 h of transfection, cells were harvested for subsequent experiments.

Cell counting kit-8 (CCK-8) assay. Keloid fibroblasts were treated with different doses of IH (20, 50 and 100 μM) with or without Ov-S1PR1, seeded into 96-well plates at a density of $5x10^4$ cells/ml and cultured in DMEM with 10% FBS for 24, 48 and 72 h. Next, 10 μl WST-8 (Beyotime Institute of Biotechnology) was added to each well followed by incubation at 37˚C for 2 h. The absorbance value was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Immunofluorescence staining. The treated keloid fibroblasts were fixed in 4% polyoxymethylene and permeabilized with 0.5% Triton-X100. Following blocking with 10% BSA in PBS for 1 h at room temperature, they were incubated with primary antibodies against proliferating cell nuclear antigen (PCNA;1:100, ab29, Abcam Inc., Cambridge, UK) and smooth muscle α-actin (α-SMA; 1:2,000, ab7817, Abcam Inc., Cambridge, UK) at 4˚C overnight. Next, Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) secondary antibody (1:400, ab150113, Abcam Inc., Cambridge, UK) was added and incubated for 1 h at room temperature. The cells were subsequently counter-stained with DAPI and examined under a fluorescence microscope (Nikon Eclipse 80i; Nikon Corporation).

Wound healing assay. Transfected cells were seeded into a 6-well plate and cultured to 80-90% confluence. A 20-μl tip was used to make a straight scratch. The cells were then washed three times and cultured with serum-free medium. After a 24-h incubation, the area occupied by migrated cells in the scratch was evaluated. Five randomly selected fields were analyzed in each well. The migration rate was calculated based on the following formula: (wound width at 0 h-wound width at 24 h)/wound width at 0 h x 100%. Five fields per well were randomly selected for analysis.

Transwell assay. An invasion assay was performed to examine tumor invasion using a Transwell chamber (6.5 mm in diameter, 8 μm pore-size, Corning, Inc.). The Transwell chambers (Corning, Inc.) were first coated with 0.1 ml Matrigel (Nippon Becton Dickinson) at 37˚C for 1 h. The cells were then collected and suspended at a final concentration of $2x10^5$ cells/ml in serum-free DMEM. Cell suspensions were then loaded onto the upper compartment, and medium with 10% fetal bovine serum was added in the lower compartment. Following incubation for 24 h, the non-invaded cells on the upper face of the Transwell membrane were wiped off with a cotton swab. The invaded cells on the lower face were fixed with 100% methanol, stained with hematoxylin and eosin, and counted under a microscope.

Western blot analysis. The total proteins were extracted from cells using RIPA buffer (Auragene). The protein concentration was determined using Detergent Compatible Bradford Protein Assay Kit (Beyotime Institute of Biotechnology). Proteins (20-30 μg) were resolved using 10% PAGE (Bio-Rad Laboratories, Inc.), transferred to a nitrocellulose membrane (Pall Life Sciences) at 25 V for 30 min, and blocked for 1 h in 10% non-fat milk in 1x Tris-buffered saline/0.1% (v/v) Tween 20 at room temperature. The membranes were incubated at 4˚C overnight with the following primary antibodies: Collagen I (1:1,000, ab138492, Abcam), α-SMA (1:1,000, ab7817, Abcam), fibronectin (FN; 1:1,000, ab2413, Abcam), S1PR1 (1:1,000, 55133-1-AP, Proteintech), p-PI3K (1:1,000, ab191606, Abcam), PI3K (1:1,000, ab182651, Abcam), p-AKT (1:1,000, ab192623, Abcam), AKT (1:1,000, ab179463, Abcam), and GAPDH (1:2,500, ab9485, Abcam). Next, the membranes were washed with PBST four times and then incubated with the HRP-conjugated goat anti-rabbit or mouse secondary antibodies (cat. nos. sc-2004 or sc-2005; 1:5,000; Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the protein bands were visualized using an ECL detection system (Beyotime Institute of Biotechnology) and quantified using ImageJ software 1.49 (National Institutes of Health).

Statistical analysis. Statistical analysis was conducted using SPSS 22.0 software (IBM Corp). Data are presented as the mean ± standard deviation of three independent experiments. Differences among multiple groups were analyzed using one-way ANOVA with a post-hoc Bonferroni multiple

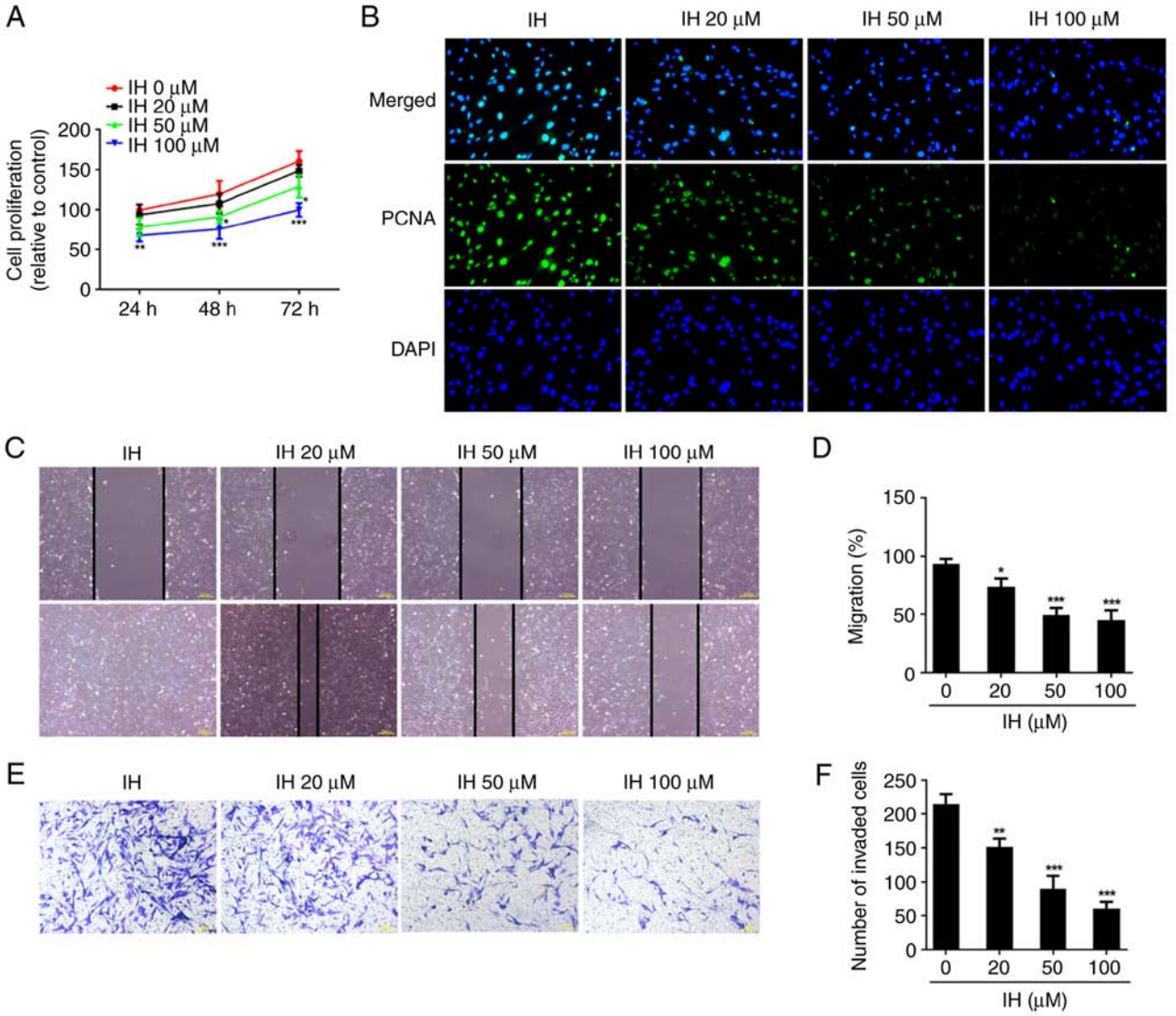

Figure 1. IH inhibits the proliferation, migration, invasion of keloid fibroblasts. (A) CCK-8 assay was used to assess cell proliferation. (B) Immunofluorescence was used to detect the expression of PCNA. Original magnification, x400. (C and D) The migration were evaluated by wound healing assay. Original magnification, x40. (E and F) The invasiveness were evaluated by Transwell assay. Original magnification, x100. Results are the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. IH 0 μM. IH, isorhamnetin; PCNA, proliferating cell nuclear antigen; DAPI, 4',6-diamidino-2-phenylindole.

comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

IH inhibits the proliferation, migration, invasion and fibrosis of keloid fibroblasts. To investigate the roles of IH in keloid progression, the effects of IH on keloid fibroblast proliferation were first detected. As shown in Fig. 1A, cell proliferation was markedly suppressed following treatment with IH, as compared with the control cells in a dose-dependent manner. In addition, the protein levels of PCNA was reduced in IH-treated cells (Fig. 1B). In addition, 20-100 μM IH reduced the cell migration rate, when compared with the control cells (Fig. 1C and D). Transwell assay results indicated that the invasive ability was restrained by the different doses of IH (Fig. 1E and F). Western blot analysis showed that IH treatment specifically decreased the protein levels of collagen I, α-SMA and FN (Fig. 2A). Consistent with these results, the immunofluorescence staining data revealed that the number of positive cells in IH groups were significantly decreased (Fig. 2B).

IH suppresses S1PR1/PI3K/AKT signaling in keloid fibroblasts. Autodock software was used to dock IH with S1PR1 protein at the molecular level, and the results showed that the binding free energy of the active ingredient and S1PR1 was -8.5 kcal/mol (Fig. 3A); the molecular docking diagram is shown in Fig. 3B. Next, western blot analysis was used to detect the expression of the S1PR1/PI3K/AKT pathway. The results revealed that IH reduced the protein levels of S1PR1, p-PI3K and p-AKT in a dose-dependent manner in keloid fibroblasts (Fig. 3C).

S1PR1 silencing suppresses keloid fibroblast proliferation, migration, invasion and fibrosis. To explore the biological roles of S1PR1 in keloid fibroblasts following IH treatment, cells were transfected with siRNA-S1PR1-1/2 to silence S1PR1.

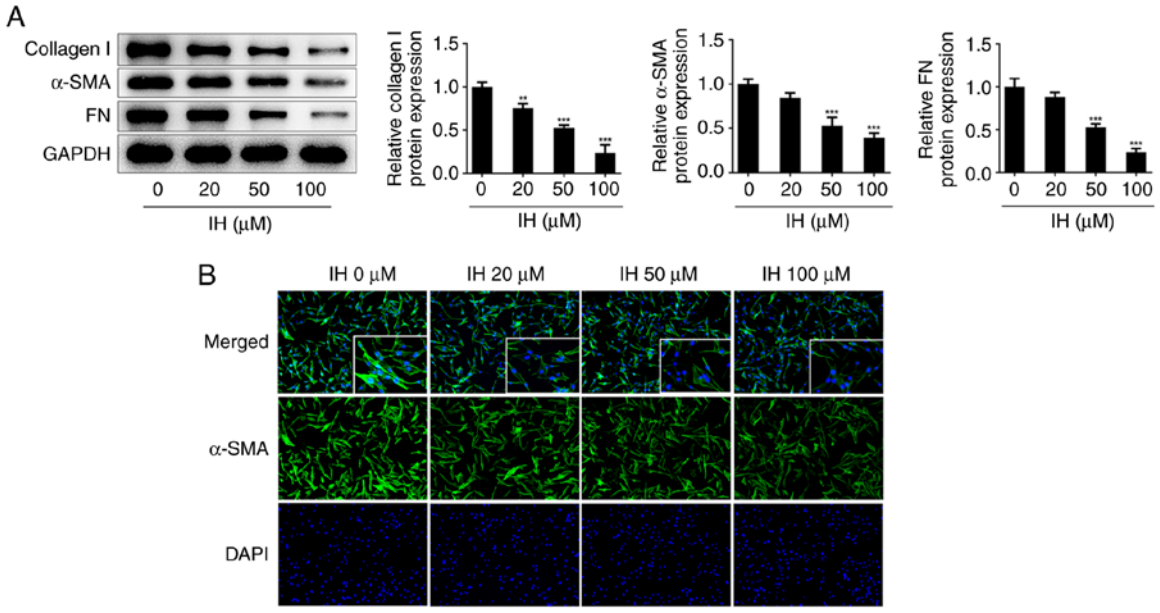

Figure 2. IH suppresses the fibrosis of keloid fibroblasts. (A) Western blot assay was performed to detect the protein level of Collagen I, α-SMA and FN. (B) Immunofluorescence was used to detect the expression of α-SMA. Original magnification, x200. Results are the mean ± SD. **P<0.01, ***P<0.001 vs. IH 0 μM. IH, isorhamnetin; α-SMA, smooth muscle α-actin; FN, fibronectin; DAPI, 4',6-diamidino-2-phenylindole.

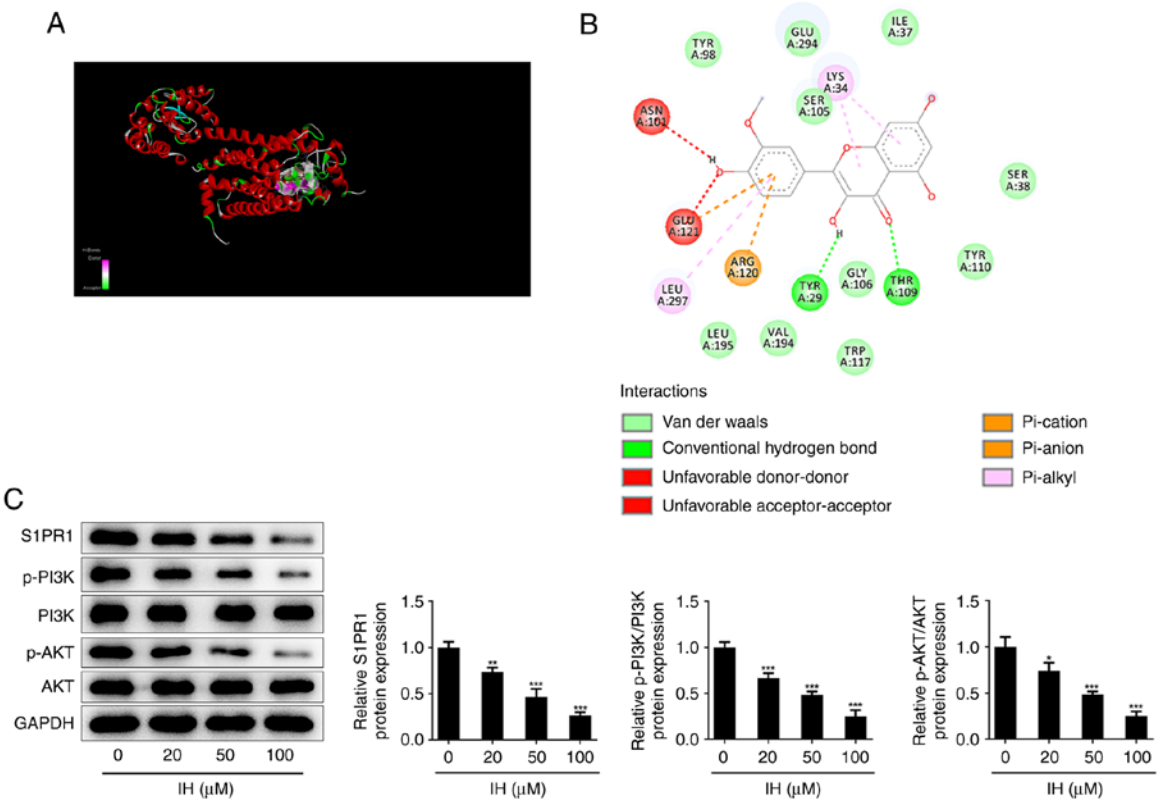

Figure 3. IH suppresses the expression of S1PR1/PI3K/AKT signaling in keloid fibroblasts. Molecular docking (A) 3D and (B) 2D diagram of the binding of IH and S1PR1. (C) Western blot assay was performed to detect the protein level of S1PR1, p-PI3K, PI3K, AKT and p-AKT. Results are the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. IH (0 μM). IH, isorhamnetin; S1PR1, sphingosine-1-phosphate receptor-1; p-PI3K, phosphorylated phosphatidylinositol-3-kinase; PI3K, phosphatidylinositol-3-kinase; p-AKT, phosphorylated protein kinase B; AKT, protein kinase B.

Western blot analysis showed that siRNA-S1PR1-1 exhibited a better transfection efficiency. Thus, siRNA-S1PR1-1 (termed siRNA-S1PR1) was selected for the following assays (Fig. 4A). The CCK-8 assay results showed that S1PR1 silencing suppressed cell proliferation compared with the negative control (Fig. 4B). Similarly, immunofluorescence revealed that the PCNA level was significantly decreased following transfection with siRNA-S1PR1 (Fig. 4C). In addition, wound

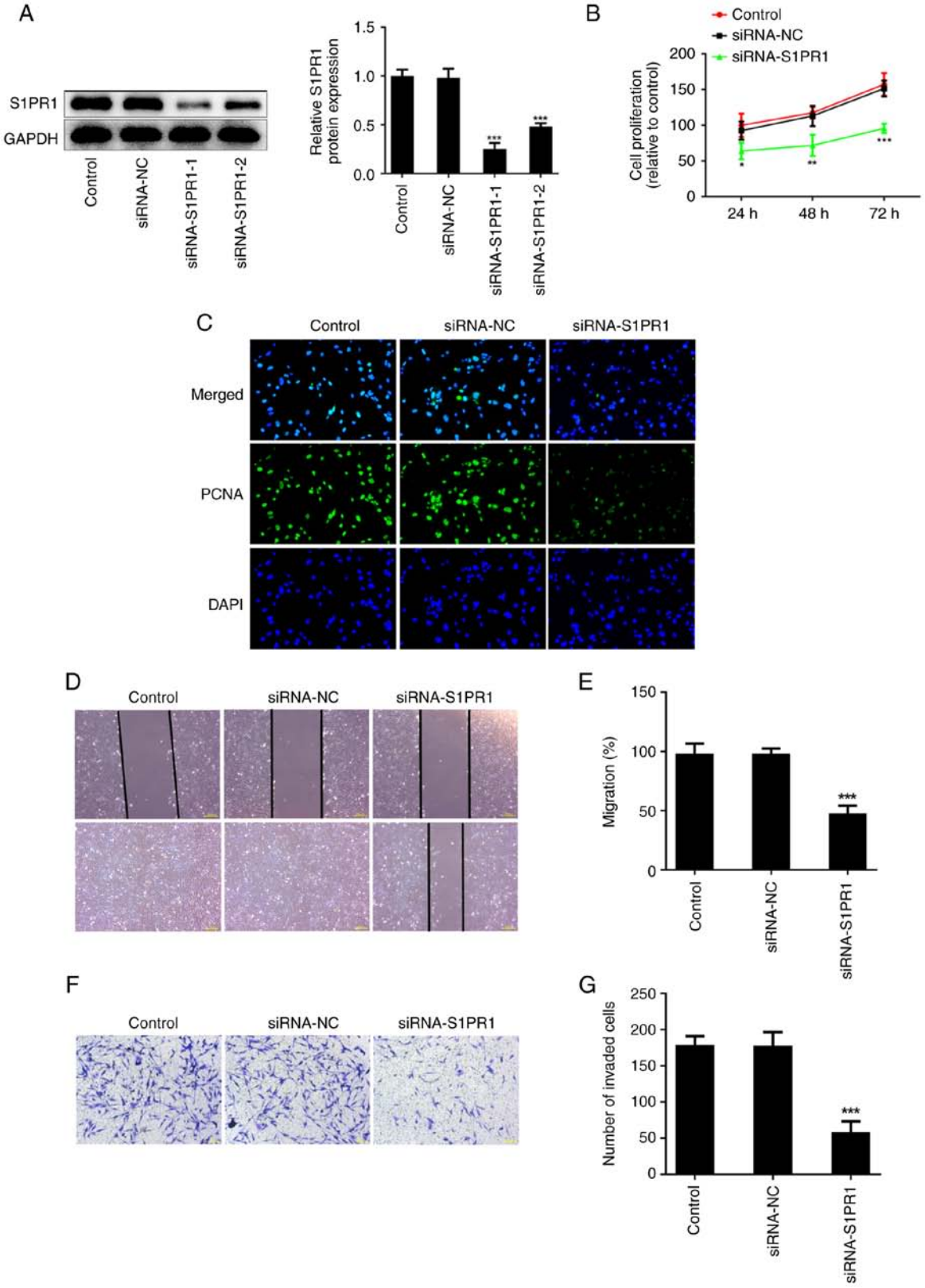

Figure 4. S1PR1 silencing restrains keloid fibroblasts proliferation, migration and invasion. (A) Western blot assay was performed to detect the protein level of S1PR1. (B) CCK-8 assay was used to assess cell proliferation. (C) Immunofluorescence was used to detect the expression of PCNA. Original magnification, x400. (D and E) The migration were evaluated by wound healing assay. Original magnification, x40. (F and G) The invasiveness were evaluated by Transwell assay. Original magnification, x100. Results are the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. siRNA-NC. S1PR1, sphingosine-1-phosphate receptor-1; PCNA, proliferating cell nuclear antigen; DAPI, 4',6-diamidino-2-phenylindole.

healing and Transwell assays showed that keloid fibroblast migration and invasion were decreased following S1PR1 silencing (Fig. 4D-G). Furthermore, a reduction in the levels of collagen I, α-SMA and FN was observed in cells transfected

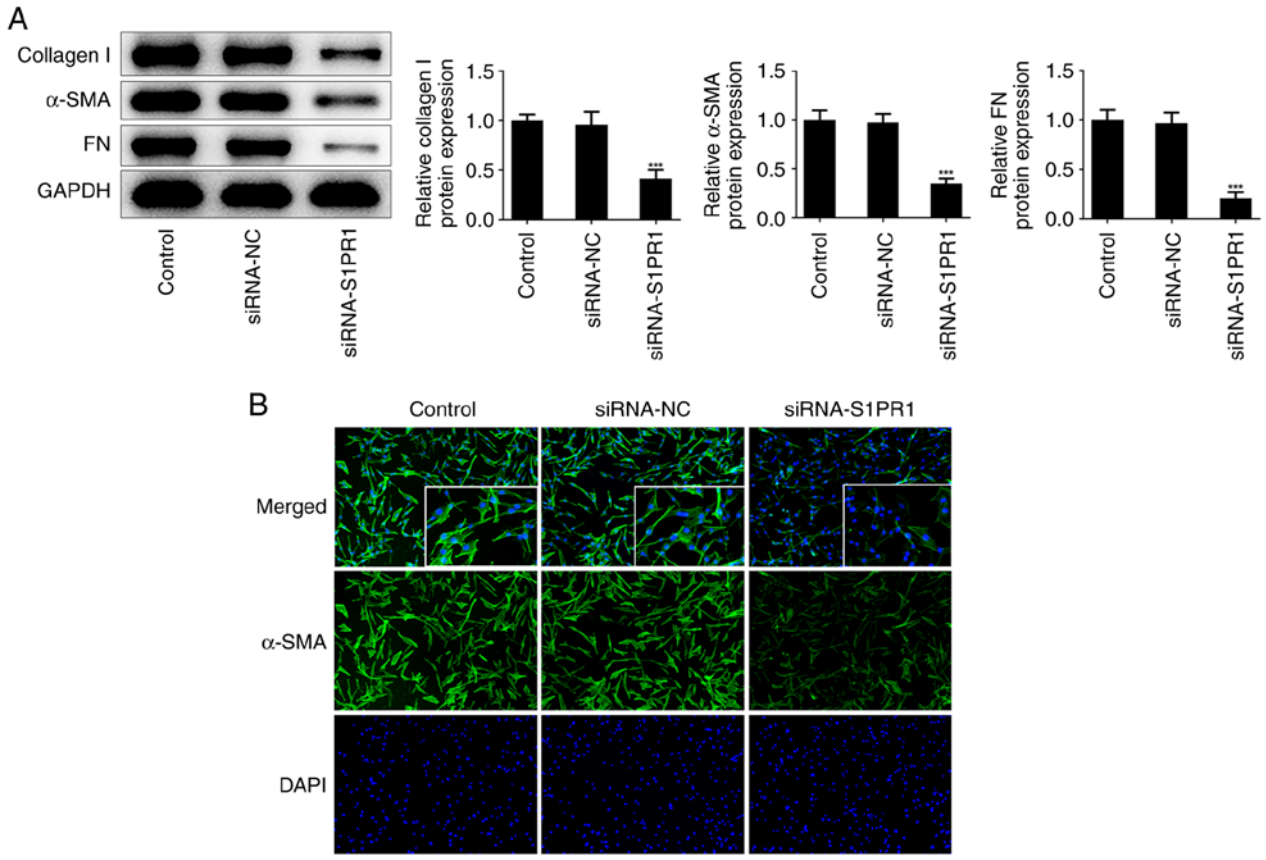

Figure 5. S1PR1 silencing restrains keloid fibroblast fibrosis. (A) Western blot assay was performed to detect the protein level of Collagen I, α-SMA and FN. (B) Immunofluorescence was used to detect the expression of α-SMA. Original magnification, x200. Results are the mean ± SD. ***P<0.001 vs. siRNA-NC. S1PR1, sphingosine-1-phosphate receptor-1; α-SMA, smooth muscle α-actin; FN, fibronectin; DAPI, 4',6-diamidino-2-phenylindole.

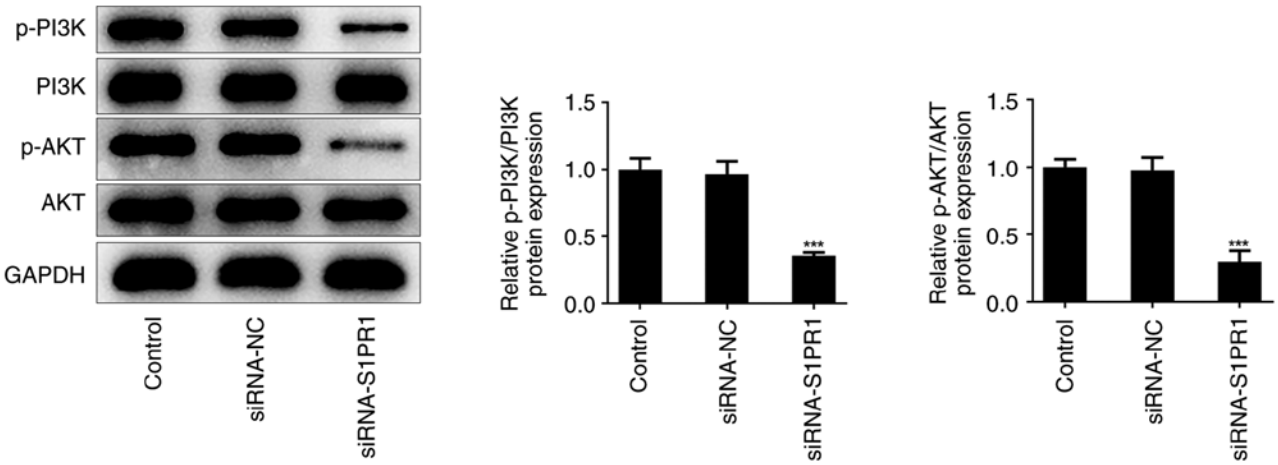

Figure 6. Knockdown of S1PR1 inhibits the PI3K/AKT pathway. Western blot assay was performed to detect the protein level of p-PI3K, PI3K, AKT and p-AKT. Results are the mean ± SD. ***P<0.001 vs. siRNA-NC. S1PR1, sphingosine-1-phosphate receptor-1; p-PI3K, phosphorylated phosphatidylinositol-3-kinase; PI3K, phosphatidylinositol-3-kinase; p-AKT, phosphorylated protein kinase B; AKT, protein kinase B.

with siRNA-S1PR1 (Fig. 5A), which was consistent with the immunofluorescence results (Fig. 5B).

S1PR1 knockdown inhibits the PI3K/AKT pathway. Next, the association between S1PR1 and the PI3K/AKT pathway was explored in IH-treated keloid fibroblasts. Western blot analysis showed that the levels of p-PI3K and p-AKT were markedly reduced, but the total levels of PI3K and AKT did not change following S1PR1 silencing, which indicated that S1PR1 may regulate the expression of the PI3K/AKT pathway in keloid fibroblasts (Fig. 6).

IH suppresses keloid fibroblast proliferation, metastasis and fibrosis by targeting the S1PR1/PI3K/AKT pathway. To verify the role of the S1PR1/PI3K/AKT pathway in IH-treated keloid fibroblasts, S1PR1 was overexpressed, and western

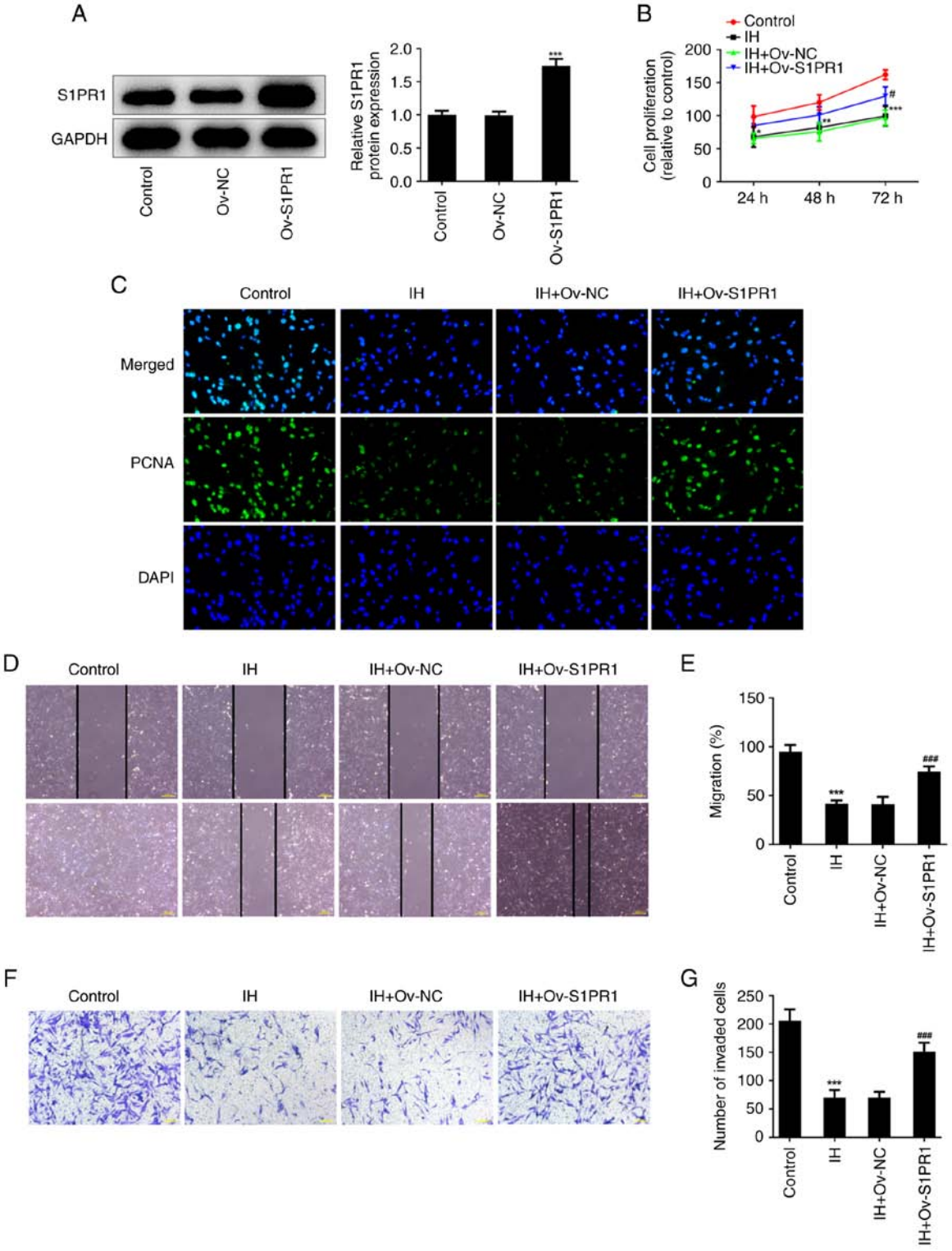

Figure 7. IH suppresses keloid fibroblasts proliferation and metastasis by targeting S1PR1/PI3K/AKT pathway. (A) Western blot assay was performed to detect the protein level of S1PR1. ***P<0.001 vs. Ov-NC. (B) CCK-8 assay was used to assess cell proliferation. (C) Immunofluorescence was used to detect the expression of PCNA. Original magnification, x400. (D and E) The migration were evaluated by wound healing assay. Original magnification, x40. (F and G) The invasiveness were evaluated by Transwell assay. Original magnification, x100. Results are the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. Control; #P<0.05, ###P<0.001 vs. IH + Ov-NC. IH, isorhamnetin; S1PR1, sphingosine-1-phosphate receptor-1; PCNA, proliferating cell nuclear antigen; DAPI, 4',6-diamidino-2-phenylindole.

blot analysis was used to determine transfection efficiency (Fig. 7A). S1PR1 overexpression noticeably enhanced the cell proliferation rate and the level of PCNA in IH-exposed cells (Fig. 7B and C). In addition, the cell migration and invasion rates were increased following S1PR1 overexpression (Fig. 7D-G). In addition, S1PR1 upregulation reversed the effects of IH on the protein levels of collagen I, α-SMA and FN in keloid fibroblasts (Fig. 8A). Immunofluorescence

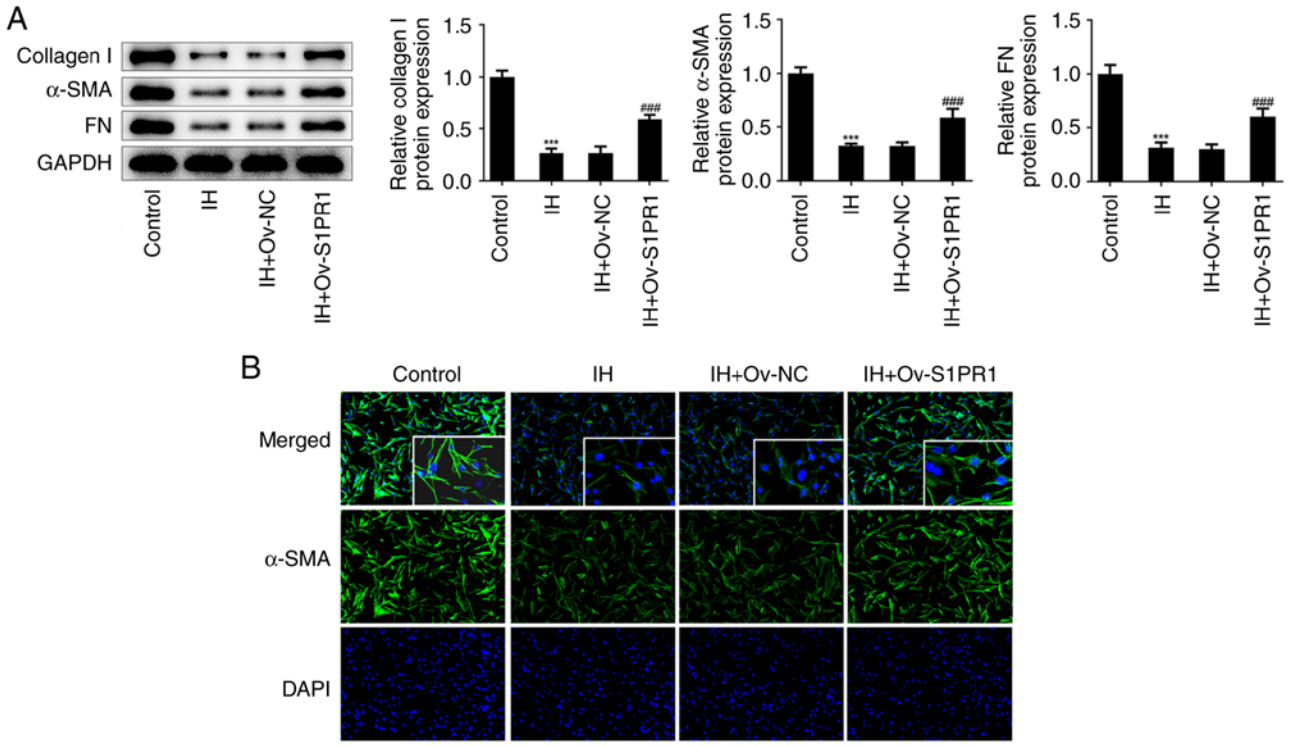

Figure 8. IH abates keloid fibroblast fibrosis by targeting S1PR1/PI3K/AKT pathway. (A) Western blot assay was performed to detect the protein level of Collagen I, α-SMA and FN. (B) Immunofluorescence was used to detect the expression of α-SMA. Original magnification, x200. Results are the mean ± SD. ***P<0.001 vs. Control; ###P<0.001 vs. IH + Ov-NC. IH, isorhamnetin; S1PR1, sphingosine-1-phosphate receptor-1; α-SMA, smooth muscle α-actin; FN, fibronectin; DAPI, 4',6-diamidino-2-phenylindole.

revealed that the relative fluorescence intensity of α-SMA was elevated in cells with S1PR1 overexpression (Fig. 8B).

Discussion

A keloid is a skin lesion secondary to connective tissue hyperplasia following trauma (15). Currently, the development of keloids is considered to be associated with the accumulation of ECM collagen and excessive fibroblast proliferation (16). The fibrosis pathway and fibrosis-related cytokines play a promoting role in keloid development (17). At present, the etiology of keloids is unknown, and despite the existence of several treatment methods, relapse is common, which has a great impact on the work and life of patients. Thus, developing more effective and feasible strategies for keloid treatment is critical. The aim of the present study was to explore the effects of IH in keloids and investigate whether IH can attenuate keloid fibroblast proliferation, invasion, migration and fibrosis by inhibiting S1PR1 signaling.

IH is a flavonoid isolated from sea buckthorn, which has anti-inflammatory, antioxidant and antitumor activities (18-20). A previous study showed that IH improved liver injury markers, reduced collagen deposition and decreased gene expression of fibrogenic markers in mice with nonalcoholic steatohepatitis (21). Liu *et al* (22) reported that IH protected against liver fibrosis by suppressing ECM formation and autophagy through the inhibition of the TGF-β1-mediated Smad3 and p38 MAPK signaling pathways. In addition, Luo *et al* (12) found that IH suppressed migration and invasion through the inhibition of Akt/ERK-mediated EMT in human non-small-cell lung cancer cells. Another study showed that IH suppressed cell proliferation and metastasis, triggered apoptosis and arrested the G2/M phase through the inactivation of the PI3K/AKT signaling (23). In the present study, the effects of IH on proliferation, metastasis and fibrosis in keloid fibroblasts was detected, and it was found that IH can inhibit cell proliferation, migration, invasion and fibrosis in a dose-dependent manner.

S1P can regulate multiple physiological and pathological processes (24). It has been shown that the S1P/S1PR1 signaling could enhance tumorigenesis and stimulate the growth, angiogenesis, expansion, survival and metastasis of cancer cells (25). In addition, S1P influences different aspects of fibrosis modulating the recruitment of inflammatory cells, as well as cell proliferation, migration and transdifferentiation into myofibroblasts, the cell type mainly involved in fibrosis development (26). Jung *et al* (27) reported that S1P-induced signal transduction was associated with increased collagen synthesis in the keloid tissue through the S1PR-mediated signaling pathways, and the mRNA and protein levels of S1PR1 were increased in keloid tissues. Using the TargetNet database, it was found that IH targets S1PR1, and molecular docking analysis verified the docking of IH and S1PR1. Next, S1PR1 was silenced in keloid fibroblasts and the results showed that S1PR1 silencing significantly repressed keloid fibroblast proliferation, metastasis and fibrosis. In addition, S1PR1 overexpression reversed the effects of IH on keloid fibroblasts, which indicated that IH may regulate keloid fibroblasts by targeting S1PR1.

The PI3K/AKT pathway was also found to be associated with keloid progression (28). A previous study showed that

the PI3K/AKT pathway promoted keloid formation (27). Xin *et al* (29) revealed that CD26 may be closely associated with proliferation and invasion in keloids through the IGF-1-induced PI3K/AKT/mTOR pathway. Meanwhile, Liu *et al* (30) found that the S1P/S1PR1 axis activated the AKT/nitric oxide synthase 3 signaling pathway to regulate pressure overload-induced cardiac remodeling. Rostami *et al* (31) revealed that S1PR1 signaling can trigger various signaling pathways involved in carcinogenesis, including the activation of PI3K/AKT. In the present study, IH treatment reduced the phosphorylation of PI3K and AKT in the PI3K/AKT pathway. In addition, S1PR1 silencing decreased the levels of p-PI3K and p-AKT in keloid fibroblasts, suggesting that the S1PR1-mediated PI3K/AKT pathway may be involved in the regulation of IH in keloids. In conclusion, the results of the present study suggested that IH suppressed cell proliferation, migration and invasion, and inhibited fibrosis in keloid fibroblasts. These protective effects may be dependent on the regulation of the S1PR1-mediated PI3K/AKT pathway, which may provide a novel insight into keloid pathogenesis and develop a therapeutic strategy for patients with keloids. However, we preliminarily explored the combination of IH and S1PR1 and the effects of IH on expressions of S1PR1/PI3K/AKT signaling, but how IH affects specifically S1PR1/PI3K/AKT signaling needs to be further explored.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XP and XC designed the study, and drafted and revised the manuscript. HL and WH analyzed the data and searched the literature. XP, XC, HL, WH, LZ and TJ performed the experiments. All authors read and approved the final manuscript. XP and XC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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