Low-dose halofuginone inhibits the synthesis of type I collagen without influencing type II collagen in the extracellular matrix of chondrocytes

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Abstract. Full-thickness and large area defects of articular cartilage are unable to completely repair themselves and require surgical intervention, including microfracture, autologous or allogeneic osteochondral grafts, and autologous chondrocyte implantation. A large proportion of regenerative cartilage exists as fibrocartilage, which is unable to withstand impacts in the same way as native hyaline cartilage, owing to excess synthesis of type I collagen in the matrix. The present study demonstrated that low-dose halofuginone (HF), a plant alkaloid isolated from *Dichroa febrifuga*, may inhibit the synthesis of type I collagen without influencing type II collagen in the extracellular matrix of chondrocytes. In addition, HF was revealed to inhibit the phosphorylation of mothers against decapentaplegic homolog (Smad)2/3 and promoted Smad7 expression, as well as decrease the synthesis of type I collagen synthesis. Results from the present study indicated that HF treatment suppressed the synthesis of type I collagen by inhibiting the transforming growth factor-β signaling pathway in chondrocytes. These results may provide an alternative solution to the problems associated with fibrocartilage, and convert fibrocartilage into hyaline cartilage at the mid-early stages of cartilage regeneration. HF may additionally be used to improve monolayer expansion or 3D cultures of seed cells for the tissue engineering of cartilage.

Introduction

Articular cartilage is a type of hyaline cartilage that provides a unique low-friction and weight-bearing surface in diarthrodial joints. Owing to its aneural and avascular characteristics, articular cartilage may be subject to trauma without causing pain or other symptoms in the early stages, which may subsequently become defects with serious symptoms and a poor regenerative capacity (1). Therefore, patients with cartilage defects frequently require surgical interventions to fix defects, relieve pain and reduce the effects of other symptoms. The current available surgical interventions for serious cartilage defects include reparative methods, such as microfracture and drilling, and reconstructive methods, such as autologous or allogeneic osteochondral grafts, autologous chondrocyte implantation, cell-seeded scaffolds and acellular scaffolds (2). Although these methods may be successful in certain aspects, they exhibit a common limitation: The generation of fibrocartilage (2,3). Fibrocartilage may be observed in the filling of microfractures or implantations, around autografts or allografts, and within scaffolds; however, almost all regenerative cartilage may be affected. In contrast with fibrocartilage, articular hyaline cartilage contains primarily type II collagen with proteoglycan, rather than type I collagen, and it is therefore able to resist more compressive loads and be more durable (4). Owing to its vulnerability, fibrocartilage is considered to be the 'Achilles' heel' of regenerative cartilage.

Halofuginone (HF; 7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone; Fig. 1) is an analogue of febrifugine, a type of alkaloid isolated from a plant used in traditional Chinese medicine, *Dichroa febrifuga* (5,6). In previous studies, various pharmacological effects of HF have been observed in a number of diseases, including malaria, cancer, and fibrosis-associated and autoimmune diseases (5,7-9). The present study focused on the antifibrotic properties of HF. Fibrosis may affect numerous organs and involves multiple signaling pathways. Among these, the transforming growth factor (TGF)-β pathway has been the most well studied in *in vitro* and *in vivo* experiments. According to a previous study, HF treatment may reduce the expression of collagen type I, α1 chain (COL1A1) gene and prevent type I collagen synthesis by inhibiting the phosphorylation of mothers against decapentaplegic homolog (Smad)2/3 in the TGF-β pathway without influencing other types of collagen, including

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type II collagen (10). Additionally, HF has been approved by the Food and Drug Administration of the USA for the treatment of scleroderma, an autoimmune fibrotic disorder (5).

Similar to fibrosis, the generation of fibrocartilage also occurs through a tissue repair process, although it is of relatively poor function and quality. Therefore, the present study hypothesized that the antifibrotic capacity of HF may decrease type I collagen synthesis in fibrocartilage and improve the quality and function of regenerative cartilage, which, to the best of our knowledge, has not been previously reported. The present study aimed to elucidate the cause of, and a potential solution to, the problem of fibrocartilage by investigating the effect of TGF-β1 and HF on rat chondrocytes.

Materials and methods

Materials. The present study was approved by the ethics committee of the General Hospital of Shenyang Military Region (Shenyang, China). Sprague-Dawley rats (total, 20; female, 10; male, 10; age, 2 weeks; weight, 25-30 g) were provided by the Animal Center of Nanjing Medical University (Nanjing, China). The animals were housed in a polycarbonate cage in a temperature and humidity-controlled (23±1˚C; 53±2%) room and maintained on a 12/12 h light/dark cycle with free access to food and water. Dulbecco's modified Eagle's medium (DMEM-low glucose), fetal bovine serum (FBS), PBS and trypsin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Collagenase II and HF were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The Cell Counting Kit-8 (CCK-8) kit was acquired from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Recombinant human TGF-β1 was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). Primers for GAPDH (cat. no. RQP049537), COL1A1 (cat. no. RQP054226), collagen type II, α1 chain (COL2A1; cat. no. RQP049248), TGF-β1 (cat. no. RQP050181), Smad2 (cat. no. RQP049947), Smad3 (cat. no. RQP049401), and Smad7 (cat. no. RQP050884) were provided by GeneCopoeia, Inc. (Rockville, MD, USA). The RNA Extraction kit, PrimeScript RT Master Mix and SYBR Premix Ex Taq were acquired from Takara Bio, Inc. (Otsu, Japan). Anti-collagen I (cat. no. 6308), anti-collagen II (cat. no. 34712) antibodies, Alexa Fluor® 488-conjugated anti-mouse immunoglobulin (Ig)G (cat. no. 150113) and Alexa Fluor® 488-conjugated anti-rabbit IgG (cat. no. 150077) were purchased from Abcam (Cambridge, UK); anti-phosphorylated (p)-Smad2/3 (cat. no. 8828), anti-Smad2/3 (cat. no. 8685), horseradish peroxidase (HRP)-linked anti-rabbit IgG (cat. no. 7074) and HRP-linked anti-mouse IgG (cat. no. 7076) were supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA); anti-Smad7 antibody (cat. no. 365846) was supplied by Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); and HRP-conjugated anti-GAPDH antibody was provided by KangChen Bio-tech, Inc. (Shanghai, China). The Bicinchoninic Acid (BCA) protein assay and the Enhanced Chemiluminescence (ECL) kits were purchased from Thermo Fisher Scientific, Inc.

Cell culture. Articular hyaline cartilage was isolated from the knees of two-week-old rats under sterile conditions and sliced into pieces (1 mm^3) for subsequent digestion. The cartilage was first incubated with 0.25% trypsin for 30 min at 37˚C, followed by 0.2% collagenase II for 4 h at 37˚C. The digested samples were purified using a cell strainer and cultured in DMEM-low glucose with 10% FBS in a Heraeus BB 5060 incubator (Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. Chondrocytes were passaged by trypsinization when cells reached >90% confluence. When passaged to the second generation, chondrocytes were seeded onto slides in 6-well or 96-well plates for the following experiments.

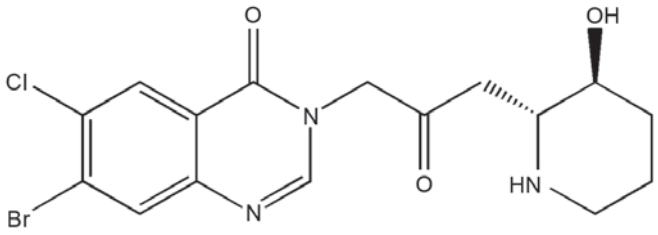

Figure 1. Chemical structure of halofuginone.

Experimental design. The experiments consisted of three parts. In the first part, second-generation cells were seeded in 6-well plates ($5x10^6$ cells/well) with gradually increasing concentrations of TGF-β1 (0, 0.1, 1 and 10 ng/ml). In the second set of experiments, cells were seeded in 96-well plates ($5x10^3$ cells/well) with gradually increasing concentrations of HF (0, 1, 3, 10, 30, 100, 300, 1,000, 3,000 and 10,000 ng/ml) for 24 h, and subsequently the concentrations (15, 30 and 60 ng/ml) which exhibited no side effects on proliferation for 24 h was cultured with the cells for 24, 48 and 72 h; these cells were analyzed by CCK-8 assays as described below. Subsequent to the above screenings, 10 ng/ml TGF-β1, 30 ng/ml HF as a low-dose (safe dose) and 100 ng/ml HF as high-dose (overdose) were used for the further experiments. Cells were seeded in 6-well plates, divided into 6 groups and treated with HF and/or TGF-β1 as follows: i) Control, which did not receive HF or TGF-β1 treatment; ii) low-dose HF; iii) high-dose HF; iv) TGF-β1; v) TGF-β1 with low-dose HF; and vi) TGF-β1 with high-dose HF. Each experiment was incubated at 37˚C and repeated at least three times.

CCK-8 assay. The cytotoxicity of HF was determined by CCK-8 assay, according to the manufacturer's protocol. Cells ($5x10^3$ cells/well) were seeded in a 96-well plate and treated with HF as aforementioned. CCK-8 solution (10 μl) was added and the plates were incubated for 3 h at 37˚C; following incubation, the absorbance was determined using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 450 nm. Cell viability was calculated as the ratio of the absorbance at 450 nm of the treatment groups vs. the control group, using SkanIt software version 2.4.2 (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using an RNA Extraction kit by adding 350 μl Buffer RL per well of the 6-well plates, and the concentration and purity was measured using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Inc.). RNA with a 260/280 ratio of 1.8-2.0 was

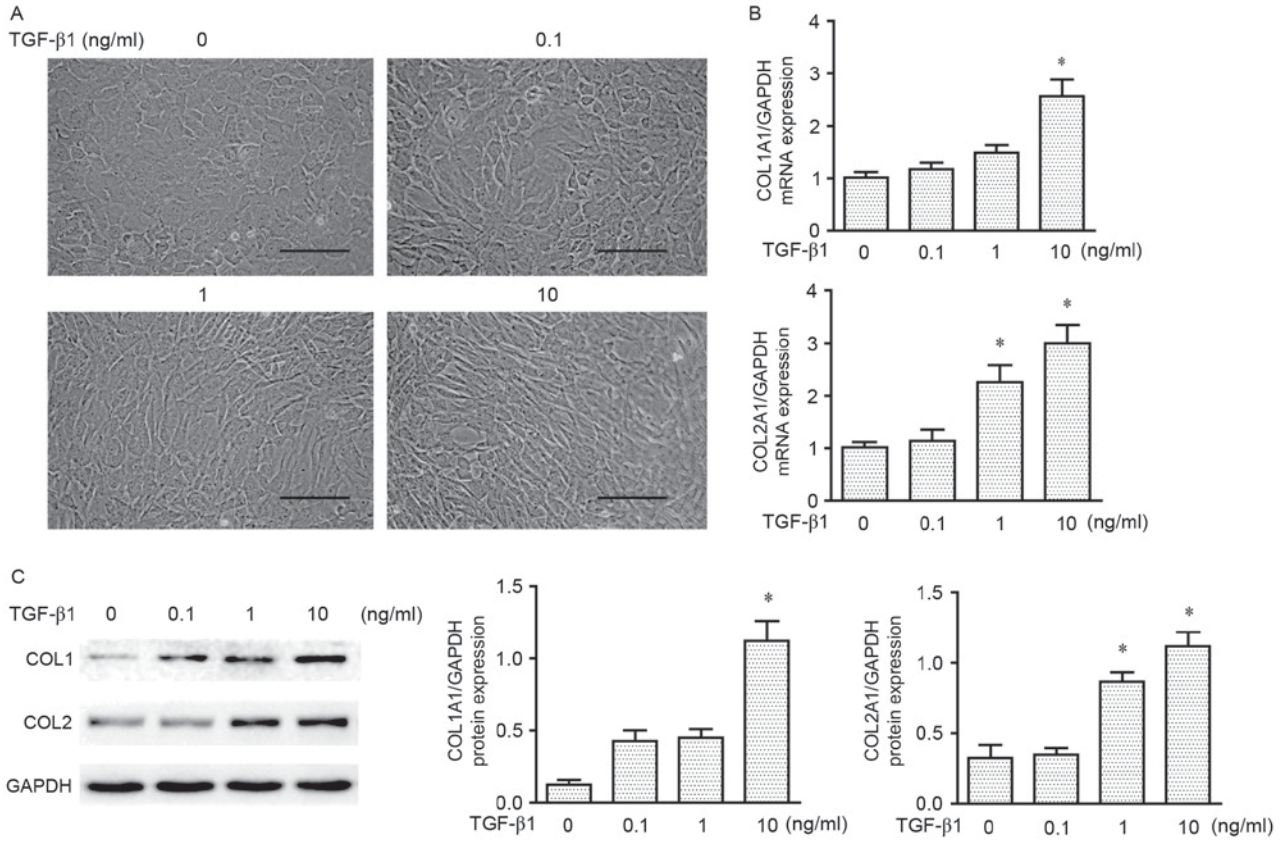

Figure 2. TGF-β1-induced expression of type I and type II collagen in chondrocytes. (A) Gross morphology of chondrocytes treated with various concentrations of TGF-β1 (0, 0.1, 1 and 10 ng/ml) for 3 days. Scale bar, 100 μm. (B) mRNA expression levels of COL1A1 and COL2A1 (normalized to GAPDH) in chondrocytes treated with different concentrations of TGF-β1 (0, 0.1, 1 and 10 ng/ml) for 3 days. (C) Representative western blot analysis and densitometric analysis of COL1 and COL2 in chondrocytes treated with gradual concentrations of TGF-β1 (0, 0.1, 1 and 10 ng/ml) for 3 days. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05 vs. 0 ng/ml. COL1, type I collagen; COL2, type II collagen; COL1A1, collagen type I, α1 chain; COL2A1, collagen type II, α1 chain; TGF-β1, transforming growth factor-β1.

reverse transcribed into cDNA using the PrimeScript RT Master Mix. qPCR analysis was performed in a 10-μl mixture using a LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). Briefly, 1 μl cDNA was mixed with 1 μl specific primers, 4 μl water and 5 μl SYBR Premix Ex Taq and amplified under the following cycling conditions: 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec, 60˚C for 20 sec, and extension at 72˚C for 10 min. The gene expression of COL1A1, COL2A1, TGFB1, Smad2, Smad3 and Smad7 was normalized to that of GAPDH using the $2^{-\Delta\Delta Cq}$ method, as previously described (11). Each experiment was repeated at least three times.

Western blot analysis. Total protein was extracted by adding 50 μl radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) to each well with 1% phenylmethylsulfonyl fluoride, and the BCA assay was used to determine the protein concentration. Protein samples (30 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Following blocking with 5% skimmed milk in TBS + Tween-20 for 1 h at room temperature, membranes were incubated with primary antibodies (anti-collagen I antibody, 1:1,000; anti-collagen II antibody, 1:5,000; anti-Smad2/3 antibody, 1:1,000; anti-p-Smad2/3 antibody, 1:1,000; anti-GAPDH antibody, 1:4,000) at 4˚C overnight and with secondary antibodies (HRP-linked anti-mouse IgG, 1:5,000; HRP-linked anti-rabbit IgG, 1:5,000) at room temperature for 1 h. Blots were visualized using an ECL kit and a gel imaging system (ChemiDoc XRS+ system; Bio-Rad Laboratories, Inc., Hercules, CA, USA); protein bands were normalized to GAPDH and densitometric analysis was performed using Image Lab 5.0 Software (Bio-Rad Laboratories, Inc.). Each experiment was repeated at least three times.

Immunofluorescence assay. The cells on the slides in the 6-well plates were cultured for 3 days and fixed with 4% paraformaldehyde for 30 min at room temperature. Following washing with PBS and cell membrane breaking with Triton X-100 (Wuhan Goodbio Technology Co., Ltd., Wuhan, China), the cells were incubated with primary antibodies (anti-collagen I antibody, 1:1,000; anti-collagen II antibody, 1:100) at 4˚C overnight and subsequently incubated with secondary antibodies (Alexa Fluor® 488-conjugated anti-mouse IgG, 1:500; Alexa Fluor® 488-conjugated anti-rabbit IgG, 1:500) at room temperature for 50 min. The cells were counterstained with DAPI for 10 min in the dark at the room temperature. Images were captured using a fluorescent microscope (Nikon Corporation, Inc., Tokyo, Japan) with image capture software (CapturePro 2.8; Jenoptik AG, Jena, Germany).

Statistical analysis. Statistical analyses were performed using SPSS software, version 20 (IBM Corp., Armonk, NY, USA),

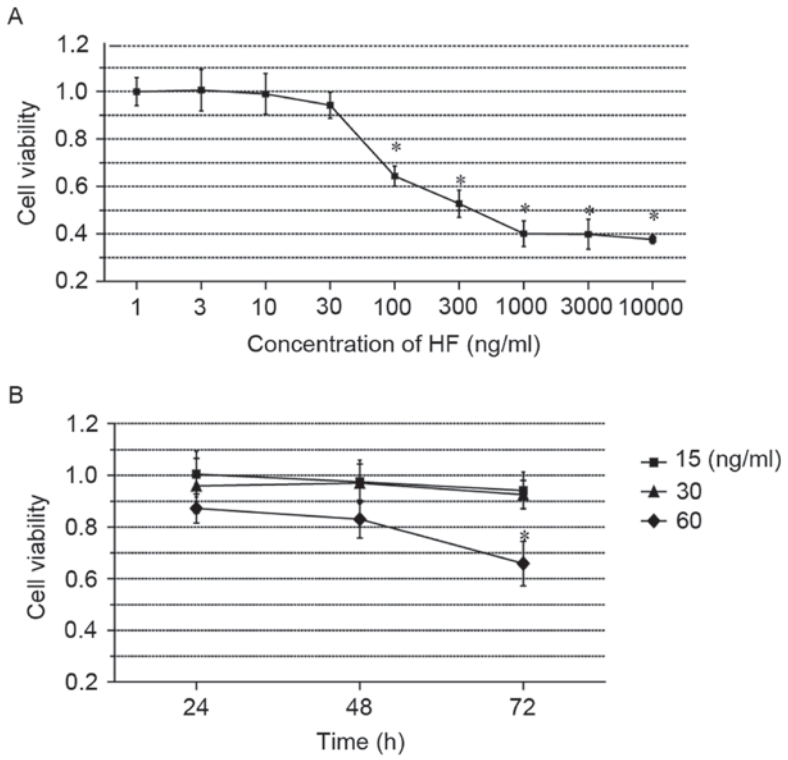

Figure 3. HF is nontoxic at concentrations ≤30 ng/ml. (A) Dose-dependent cytotoxicity analysis of HF. Chondrocytes were treated with 10 gradually increasing concentrations of HF (0, 1, 3, 10, 30, 100, 300, 1,000, 3,000 and 10,000 ng/ml) for 24 h. (B) Time-dependent cytotoxicity analysis of HF. Chondrocytes were treated with 4 concentrations of HF (0, 15, 30 and 60 ng/ml) for 24, 48, 72 h. Data are presented as the mean ± standard error of the mean; n=6; *P<0.05 vs. control. HF, halofuginone.

and results are presented as the mean ± standard error of the mean. One-way and multi-way analysis of variance with Student-Newman-Keuls post hoc tests were used to determine the statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β1 induces type I collagen expression in chondrocytes. TGF-β1 is a known stimulant of fibrosis in various tissues, and the present study aimed to investigate whether TGF-β1 is able to induce the production of type I collagen in chondrocytes *in vitro*. Second passage chondrocytes were treated a range of concentrations of TGF-β1 (0, 0.1, 1 and 10 ng/ml) for 3 days. Gross examination of cell morphology of the four treatment groups revealed that the extracellular matrix of the chondrocytes was altered from subovate to spindle, which indicated the formation of fibrocytes as the concentration of TGF-β1 increased; a marked difference was observed at 10 ng/ml compared with the other treatment groups (Fig. 2A). RT-qPCR and western blot analyses demonstrated that type I and type II collagen mRNAs and proteins were produced in the second-generation chondrocytes during monolayer expansion, and expression increased following treatment with TGF-β1 (Fig. 2B and C, respectively). However, a difference was observed between the two types of collagen expressions: A significant increase was noted for type I collagen at 10 ng/ml, whereas type II collagen also exhibited a significant increased at 1 ng/ml. Therefore, it was decided to use 10 ng/ml TGF-β1 to increase type I collagen in the extracellular matrix of chondrocytes for the experiment discussed below.

Cytotoxicity of HF in chondrocytes. To assess the cytotoxicity of HF treatments, the CCK-8 assay was used to determine a concentration that resulted in minimal toxicity to chondrocytes. Cells were treated with 10 gradually increasing concentrations of HF (0, 1, 3, 10, 30, 100, 300, 1,000, 3,000 and 10,000 ng/ml) for 24 h. The results demonstrated that cell viability dropped markedly from >90 to 40% between 30 and 1,000 ng/ml (Fig. 3A). It was additionally observed that the cytotoxicity was significant at concentrations ≥100 ng/ml HF, compared with the control group. Based on these results, 4 concentrations of HF (0, 15, 30 and 60 ng/ml) were selected to determine whether the cell viability was affected by the duration of treatment. Cells were treated with HF for 24, 48 and 72 h, and the cell viabilities were analyzed every 24 h. The results demonstrated that viability was significantly reduced in cells treated with 60 ng/ml for 72 h, whereas no significant effects were noted in cells incubated with either 15 or 30 ng/ml (Fig. 3B). Therefore, 30 ng/ml HF was determined to be a safe concentration; 30 ng/ml as a low-dose (safe dose) and 100 ng/ml as high-dose (overdose) were used for further cell function tests.

Low-dose HF only suppresses type I collagen expression in chondrocytes. To determine whether HF was able to decrease type I collagen synthesis without decreasing type II collagen synthesis, cells were treated with different concentrations of HF (0, 30 and 100 ng/ml) with or without TGF-β1 (10 ng/ml)

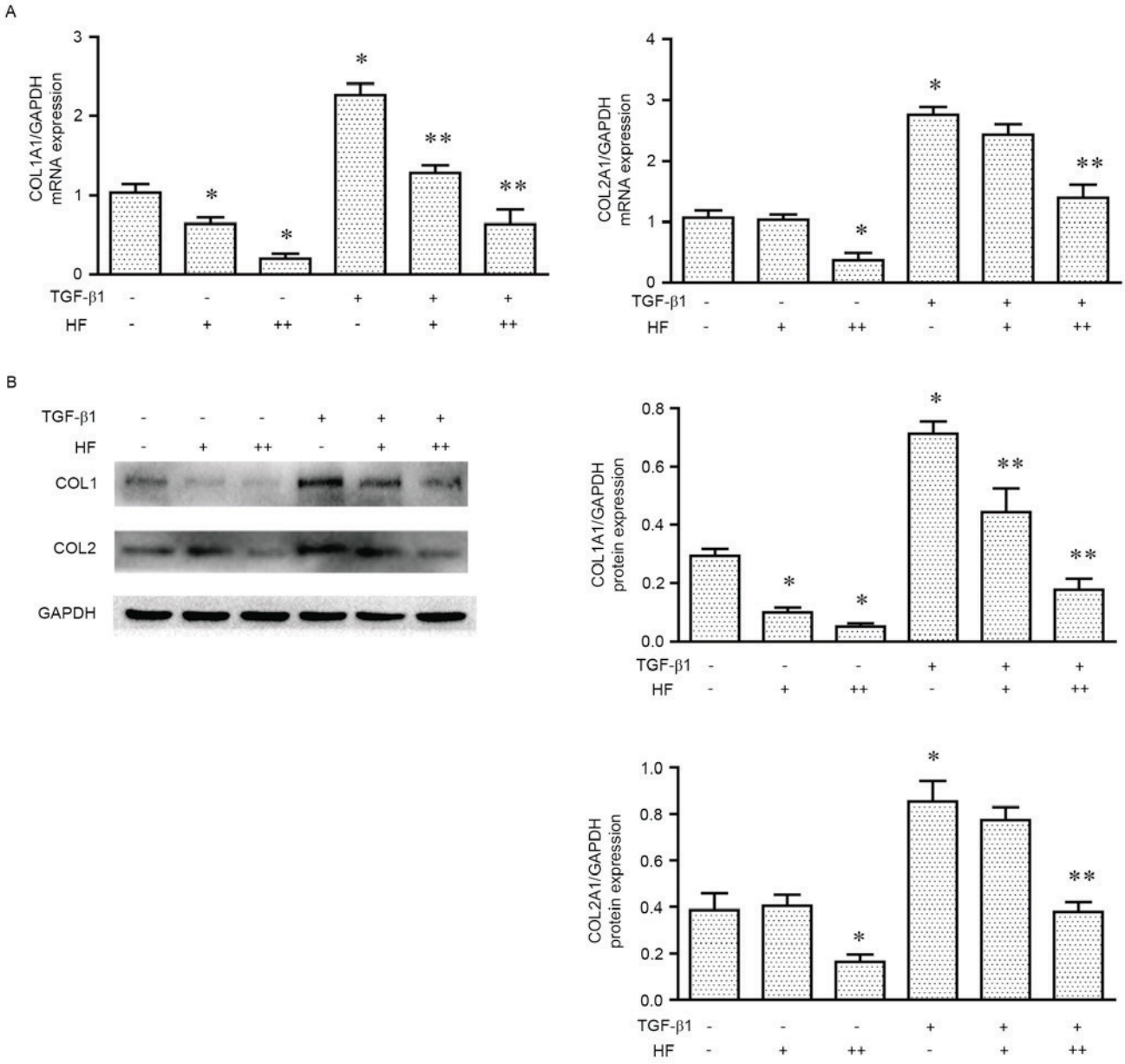

Figure 4. Low-dose HF suppresses type I collagen in chondrocytes without decreasing type II collagen. (A) mRNA levels of COL1A1 and COL2A1 (normalized to GAPDH) of chondrocytes treated with HF with or without TGF-β1 co-treatment. (B) Representative western blot analysis and densitometric analysis of type I and type II collagen protein expression in chondrocytes treated with HF and/or TGF-β1. For HF treatments: (-) indicates 0 ng/ml HF; (+) indicates 30 ng/ml HF; and (++) indicates 100 ng/ml HF. For TGF-β1 treatments: (-) indicates 0 ng/ml TGF-β1; and (+) indicates 10 ng/ml TGF-β1. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05 vs. untreated control. **P<0.05 vs. TGF-β1 (+)/HF (-). COL1, type I collagen; COL2, type II collagen; COL1A1, collagen type I, α1 chain; COL2A1, collagen type II α1 chain; HF, halofuginone; TGF-β1, transforming growth factor-β1.

co-treatment. In the RT-qPCR analysis (Fig. 4A), type I collagen was markedly inhibited with 30 ng/ml HF, whereas type I and type II collagen were inhibited at a concentration of 100 ng/ml HF with or without TGF-β1. Western blot analysis (Fig. 4B) demonstrated a similar concentration-dependent effect on the chondrocytes with or without TGF-β1. In the immunofluorescence assay (Fig. 5A and B), the cells were observed to exhibit a markedly decreased expression of type I collagen at concentrations of 30 and 100 ng/ml HF, while type II collagen only decreased at 100 ng/ml HF.

HF acts via Smad2/3 and Smad7 in the TGF-β pathway in chondrocytes. As TGF-β1 was revealed to increase the expression of type I and type II collagen, whereas their expression levels were decreased with HF treatment, the expression levels of TGF-β pathway proteins were analyzed in chondrocytes, as were observed in cells of other fibrotic tissues and organs (12). The present study examined the mRNA expression levels of TGFB1, Smad2, Smad3 and Smad7 in the six treatment groups by RT-qPCR (Fig. 6A). No significant differences were identified for TGFB1, Smad2 or Smad3 mRNA expression; however, Smad7 mRNA expression levels were significantly higher in cells treated with high doses of HF, either with or without TGF-β1 co-treatment. Western blot analysis of p-Smad2/3 and Smad7 protein expression demonstrated that HF treatment was able to inhibit the phosphorylation of Smad2/3 and increase the expression of Smad7, in cells treated with or without TGF-β1 (Fig. 6B).

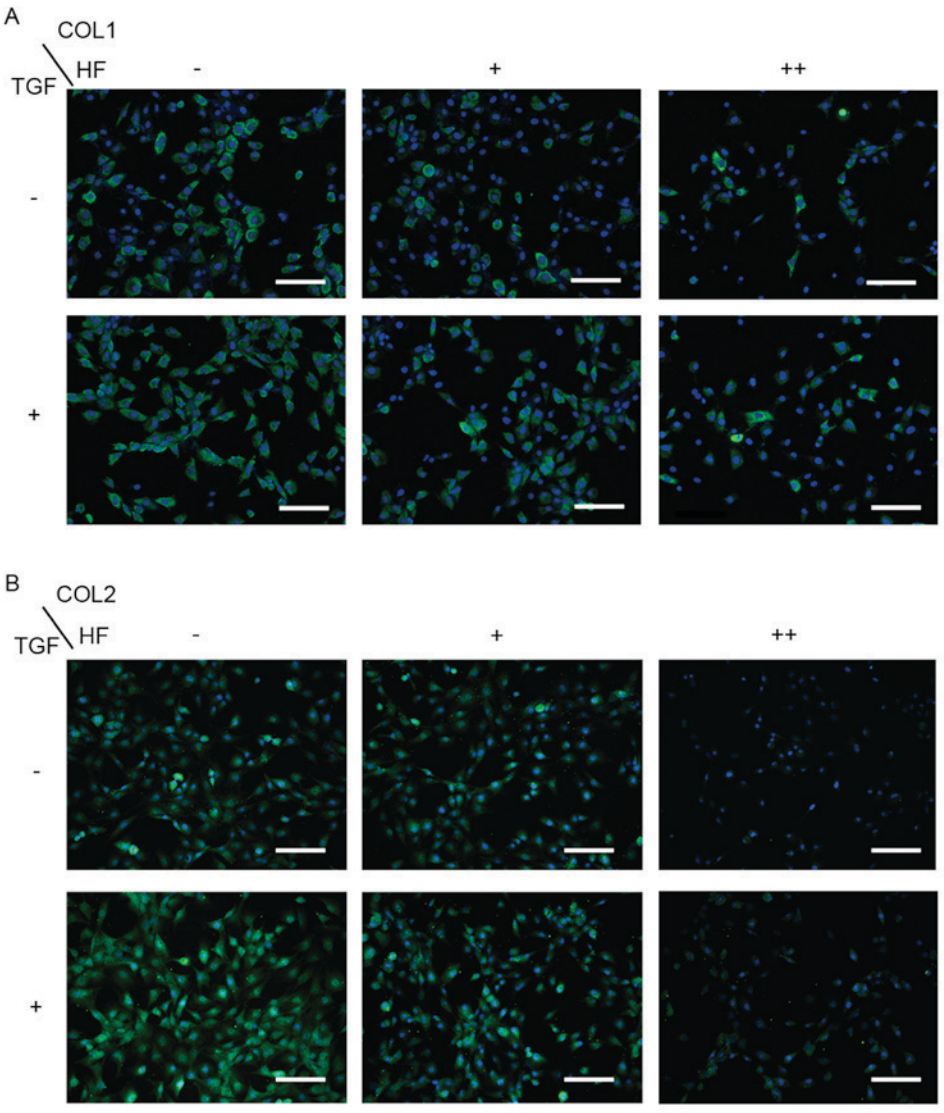

Figure 5. Low-dose HF suppresses type I collagen in chondrocytes without decreasing type II collagen. (A) Representative images of immunostainings for type I collagen. (B) Representative images of immunostainings for type II collagen. For HF treatments: (-) indicates 0 ng/ml HF; (+) indicates 30 ng/ml HF; and (++) indicates 100 ng/ml HF. For TGF-β1 treatments: (-) indicates 0 ng/ml TGF-β1; and (+) indicates 10 ng/ml TGF-β1. Scale bar, 100 μm. COL1, type I collagen; COL2, type II collagen; HF, halofuginone; TGF-β1, transforming growth factor-β1.

Discussion

Full-thickness and large area defects of articular cartilage are unable to repair themselves and require surgical intervention to regenerate (3). The durability of regenerative cartilage depends on the histological structure. Hyaline cartilage with type II collagen is resistant to loading compression, whereas fibrocartilage with type I collagen is more resistant to tension than hyaline cartilage, although it is vulnerable to compression in the joints (Table I) (13). A large proportion of regenerative cartilage exists in the form of fibrocartilage, which cannot withstand impact to the same degree as natural hyaline cartilage (2,14). Therefore, the part of regenerative cartilage that is fibrocartilage is frequently the earliest site of failure.

TGF-β is a central factor in fibrosis, which promotes the expression of proteins of the extracellular matrix in various fibrotic conditions through the TGF-β pathway. During the process of cartilage regeneration, TGF-β is produced and recruited; however, this recruitment may contradictorily both enhance cartilage repair and stimulate tissue fibrosis (15,16). High levels of TGF-β have been demonstrated to induce osteoarthritis (17). In a previous study, TGF-β1 was able to dedifferentiate chondrocytes and cause them to lose their phenotypic characteristics, which led to the production of more type I collagen compared with type II collagen (18); this process additionally occurs in monolayer expansion and 3D regenerative cartilage *in vivo* (19). Therefore, the present study used second-generation monolayer-cultured chondrocytes, with or without high levels of TGF-β1 treatment, to mimic the phenotypic alterations of fibrocartilage. In the second-generation monolayer expansion chondrocytes, type I collagen was increased in the group treated with TGF-β1 compared with the untreated cells.

HF was previously identified to be an antifibrotic agent in the 1990s (20). HF may prevent the increase in collagen synthesis and promote the resolution of established fibrosis (5). In addition, HF exhibited no apparent effects on the collagen in non-fibrotic tissue. In injured rat carotid arteries, HF was

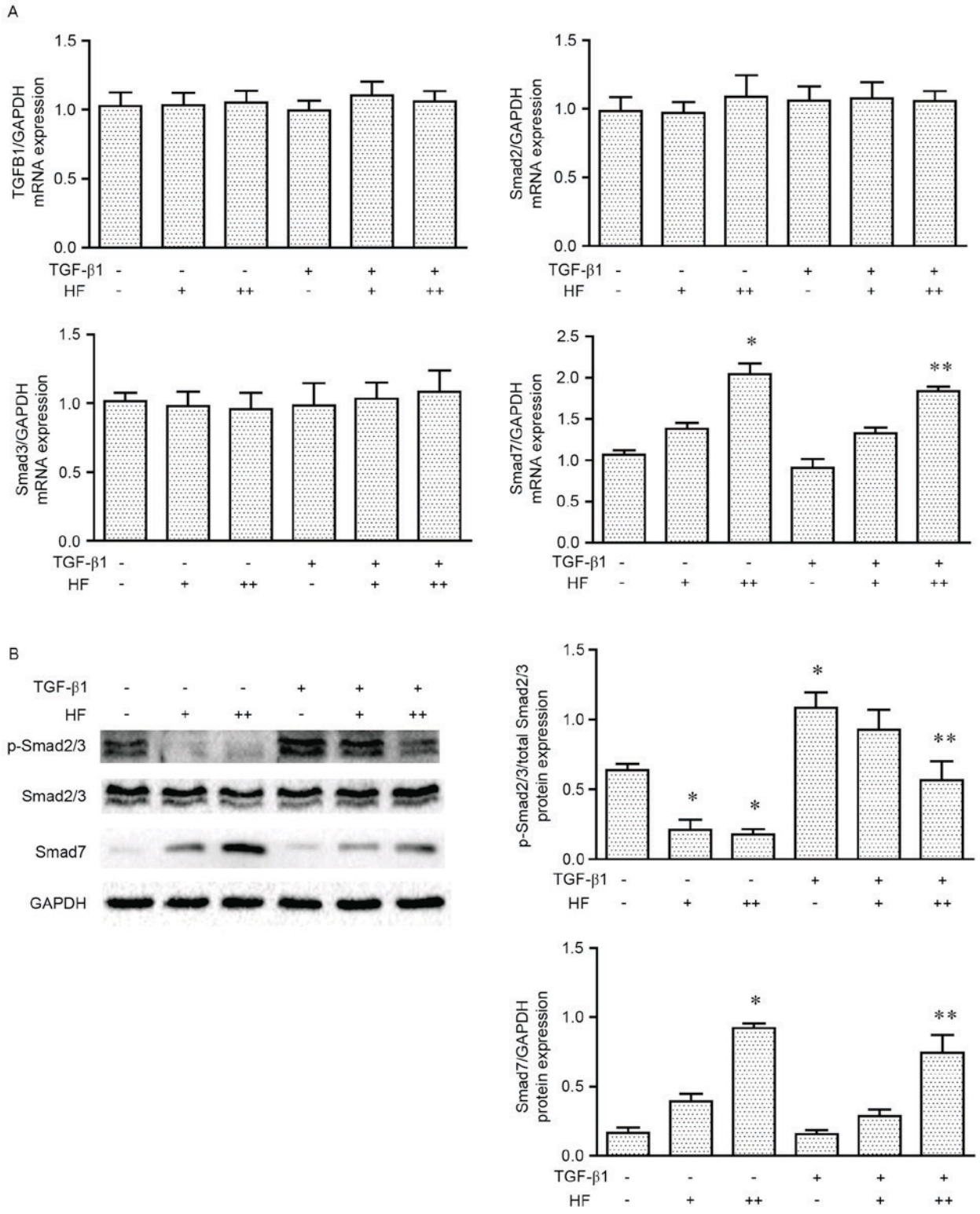

Figure 6. HF acts via Smad2/3 and Smad7 in the TGF-β1 signaling pathway. (A) The mRNA levels of TGFB1, Smad2, Smad3 and Smad7 (compared with GAPDH) of chondrocytes treated with HF and TGF-β1. (B) Representative western blot analysis and quantification of p-Smad2/3, Smad2/3 and Smad7 expression in chondrocytes treated with HF and TGF-β1. HF (-), 0 ng/ml HF; HF (+), 30 ng/ml HF; HF (++), 100 ng/ml HF; TGF-β1 (-), 0 ng/ml TGF-β1; TGF-β1 (+), 10 ng/ml TGF-β1. Data are presented as the mean ± standard error of the mean. n=3. *P<0.05 vs. control. **P<0.05 vs. TGF-β1 (+) HF (-). HF, halofuginone; Smad, mothers against decapentaplegic homolog; TGF-β1, transforming growth factor-β1; TGFB1, TGF-β1; p, phosphorylated.

demonstrated to decrease the synthesis of type I collagen and not type III collagen (21). In liver cirrhosis, chronic graft-versus-host disease and scleroderma models, HF has been observed to inhibit type I collagen gene expression and synthesis without affecting the synthesis of type II or type III collagen (10). In an osteoarthritis model, HF was able to attenuate articular cartilage degeneration and subchondral bone deterioration by decreasing type X collagen and increasing type II collagen synthesis (22). Owing to its selective inhibition of collagen, HF was used to treat fibrocartilage in the

Table I. Composition and loading capacity of fibrocartilage and hyaline cartilage.

	Composition	
Cartilage type	Type I collagen	Type II collagen
Fibrocartilage	+++	+
Hyaline cartilage	-	+++
	Loading capacity	
Cartilage type	Compression	Tension
Fibrocartilage	+	++
Hyaline cartilage	+++	+

-, none; +, low-grade; ++, moderate-grade; +++, high-grade.

present study. A previous study indicated that, during the inhibition of type I collagen, HF inhibited the phosphorylation of Smad2/3, an important element of the TGF-β signaling pathway, and increased Smad7 expression, which acts as an inhibitor of the TGF-β pathway (23). The results of the present study indicated that a low-dose of HF was able to inhibit the TGF-β pathway to decrease the synthesis of type I collagen. Notably, the TGF-β pathway has additionally been reported to be important for the synthesis of type II collagen (24), which indicated that the selective inhibition of HF is complex. There are two possible explanations for this observed effect. The first explanation is that HF may act in a dose-dependent manner. In the present study, only type I collagen was decreased at the low-dose (30 ng/ml) HF treatment, whereas both type I and type II collagen expressions were decreased at the high dose (100 ng/ml). This may indicate that the regulation of normally-expressed genes (type II collagen) may differ from that of abnormally-expressed genes (type I collagen) induced by the fibrogenic stimulus, which is usually an aggressive and rapid process (5). Compared with the native type II collagen, type I collagen may be more sensitive to HF. The second possible explanation is that TGF-β may exert its biological effects by non-Smad pathways. For example, type II collagen synthesis may be stimulated by TGF-β through the TGFR2-Rac-α serine/threonine protein kinase-serine/threonine protein kinase mTOR signaling (25).

In the present study, it was observed that HF was able to inhibit the synthesis of type I collagen, without influencing type II collagen synthesis, at low concentrations. In clinical settings, HF may be used to reduce the proportion of type I collagen in fibrocartilage at the mid-early stage of regeneration and turn fibrocartilage into hyaline-like cartilage, which is more similar to the native hyaline cartilage. Treatment with HF may provide an alternative solution to the problem of fibrocartilage in cartilage regeneration. HF may also be used in the improvement of seed cells in monolayer expansion for the tissue engineering of cartilage.

To the best of our knowledge, the present study presents the first use of HF to treat the issues associated with type I collage in chondrocytes, although certain limitations remain. Owing to the complicated procedures of surgical cartilage regenerative interventions, the present study did not test the effect of HF in animal experiments. In addition, chondrocytes in monolayer expansion may not completely mimic the phenotypic alterations of fibrocartilage *in vivo*. Therefore, the antifibrotic effects of HF in the application of cartilage regeneration require further investigation.

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