



Collagen triple helix repeat containing-1 exerts antifibrotic effects on human skin fibroblast and bleomycin-induced dermal fibrosis models

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Background: Systemic sclerosis (SSc) is an acquired disorder characterized by excessive deposition of extracellular matrix in the skin and internal organs. So far, the molecular mechanisms underpinning the pathogenesis of SSc have remained unknown. Collagen triple helix repeat containing-1 (CTHRC1) has been indicated to be a cell type-specific inhibitor of transforming growth factor- β (TGF- β), which could have the potential for extensive clinical application owing to its ability to reduce collagen deposition. Our previous studies showed that CTHRC1 inhibited TGF- β 1-induced collagen type I synthesis in keloid fibroblasts. In our present research, we attempted to probe the role of CTHRC1 in dermal fibrosis in bleomycin (BLM)-treated mice.

Methods: CTHRC1 and TGF- β 1 expression was detected in dermal tissues from patients with SSc and BLM-treated mice by immunohistochemistry. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was used to assess TGF- β 1-induced proliferation of human dermal fibroblasts. Collagen expression and fibroblast synthesis were evaluated by quantitative real-time polymerase chain reaction and the 3H-proline incorporation. Masson's trichrome staining and western blotting were carried out to analyze the deposits and protein levels of type I collagen, respectively.

Results: Compared with those in normal tissues, the levels of CTHRC1 and TGF- β 1 were elevated in dermal tissues from patients with SSc and in skin tissues from BLM-treated mice, respectively. Furthermore, recombinant CTHRC1 was found to inhibit TGF- β 1-stimulated collagen deposition by fibroblasts. Finally, the in vivo experiments showed that CTHRC1 alleviated BLM-induced dermal fibrotic changes.

Conclusions: CTHRC1 can inhibit human dermal fibroblast collagen deposition and can also exert protective effects against BLM-induced dermal fibrosis in mice. This research provides an indication that CTHRC1 may be a promising treatment choice for dermal fibrosis in SSc patients.

Keywords: CTHRC1; TGF- β 1; bleomycin; dermal fibrosis

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Introduction

Systemic sclerosis (SSc) is an autoimmune disease resulting from genetic predisposition or environmental stimuli. It is characterized by severe microvasculature changes, dramatic inflammatory and immunological alterations, and fibrosis of the skin and internal organs. However, the mechanism of interaction between these 3 major abnormalities has yet to be described (1,2).

Collagen triple helix repeat containing-1 (CTHRC1) is expressed mostly in adventitial fibroblasts and neointimal smooth muscle cells in balloon-injured vessels. It can promote cell migration by decreasing the deposition of collagen matrix and plays a key role in tissue repair following injury (3). A number of studies have revealed that CTHRC1 upregulation is predictive of a poor prognosis in patients with various tumors. The overexpression of *Cthrc1* enhances the migration and invasion of tumor cells, indicating that it is a feasible independent prognostic factor for patients with cancer. LeClair *et al.* observed that adventitial collagen deposition and neointimal lesion formation were reduced in transgenic mice overexpressing CTHRC1 following carotid artery ligation (4). All of the above studies suggest that CTHRC1 could have a major clinical application in reducing collagen deposition.

Transforming growth factor- β (TGF- β) is understood to be the leading profibrotic factor in sclerotic disorders (5). CTHRC1 has been identified as a cell type-specific inhibitor of TGF- β , which, in sequence, impacts collagen deposition, neointimal formation, and smooth muscle cell differentiation (4,6,7). Our previous work investigated the inhibitory effects of CTHRC1 on TGF- β 1-stimulated collagen deposition in keloid fibroblasts (8). However, until now, the expression and regulatory mechanisms of CTHRC1 in SSc have not been explored.

In this study, we firstly measured the expression of CTHRC1 and TGF- β 1 in scleroderma fibroblasts, and then examined the expression and synthesis of collagen type I in normal human dermal fibroblasts stimulated with TGF- β 1. Finally, we constructed a bleomycin (BLM)-induced dermal fibrosis mouse model to investigate the protective effect of CTHRC1 against dermal fibrosis in mice.

We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-21-1884>).

Methods

Cell culture

A normal human dermal fibroblast cell line was bought from Kunming Cell Bank of the Chinese Academy of Sciences. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and grown at 37 °C in a 5% CO₂ atmosphere. The growth medium was changed every 3 to 4 days.

Cell proliferation assay

Cells were seeded on 96-well plates at a density of 5×10^3 /well. Using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, the proliferative activity of fibroblasts treated with TGF- β 1 or recombinant CTHRC1 (rCTHRC1) was assessed. The absorbance was detected at a reference wavelength of 570 nm. Recombinant CTHRC1 was kindly supplied by Prof. Chen (Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China).

Real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Life Technologies, USA). The RNA concentration and quality were tested using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). Complementary DNA (cDNA) was synthesized through reverse transcription of 1 μ g of RNA using the RevertAid First Strand Synthesis kit (Fermentas, Glen Burnie, MD, USA). Then, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was carried out using SYBR[®] Premix Ex Taq (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions. Each sample was analyzed in triplicate, with β -actin serving as an internal control. Sequence of primers used are list in *Table 1*.

Western blot

Proteins were extracted from human tissues and cells using RIPA lysis buffer, and a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was used to measure the protein concentration. Following separation by sodium

Table 1 Sequence of primers used for qRT-PCR

Gene	Sequence
<i>β-actin</i>	5'-CTCCATCCTGGCCTCGCTGT-3'
	5'-GCTGTACCTCCGTTCC-3'
<i>TGF-β1</i>	5'-CAACAATCTGGCGATACCT-3'
	5'-GCTAAGGCGAAAGCCCTCAAT-3'
<i>CTHRC1</i>	5'-CAAGGAAGCCCTGAAATGA-3'
	5'-TCCACTAATCCAGCACCAA-3'

RT-PCR, real-time polymerase chain reaction.

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), equal amounts of protein were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with collagen type I monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) after processing in the blocking buffer. Protein expression was normalized to β -actin expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An imaging densitometer was employed for quantitative analysis of the blotting images.

Collagen synthesis assay

Collagen synthesis was examined using a ^3H -proline incorporation assay as previously described (9), with some optimizations. Cells were counted with a liquid scintillation counter, which indicated the amount of newly synthesized collagen. Collagen synthesis was normalized to the number of cells.

Model establishment and treatment

Six-week-old specific pathogen-free female BALB/c mice (weight: 18 to 22 g) were purchased from the animal research center at Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China. All animal experimental steps were carried out in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the approval of Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China (No.: Sichuan Provincial People's Hospital-D-2013-99). The BLM-induced dermal fibrosis mouse model was established following previously described methods (10). Briefly, BLM (Nippon Kayaku Co., Ltd., Tokyo, Japan) was dissolved in

1 mg·mL⁻¹ phosphate-buffered saline (PBS). In the BLM mice, after sterilization by filtration, 100 μL BLM solution was subcutaneously injected into the shaved back of mice using a 27-gauge needle, the injections were repeated daily for a total of 21 days. The control mice were injected with an equal volume of PBS. After the successful establishment of the model, the BLM mice were randomly divided into the PBS group (8 mice), the rCTHRC1 group (8 mice), the TGF- β 1 group (8 mice), and the rCTHRC1 + TGF- β 1 group (8 mice). The mice were intraperitoneally injected with the designated drugs every 2 days for a 20-day period. The general conditions of the mice including their activity and weight were observed daily.

Histological examination

Dermal tissues from the experimental animals were sliced into 5- μm sections. For structured observation or detection of collagen deposits, the sections were stained with hematoxylin and eosin or Masson's trichrome stain respectively, as instructed by the manufacturers.

Immunohistochemistry

Tissue samples were volunteer contributed by patients with SSc who had received treatment in the Department of Dermatology, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China (No.: Sichuan Provincial People's Hospital-D-2013-350) and informed consent was taken from all the patients. Immunohistochemical staining was carried out to detect the expression of CTHRC1, TGF- β 1, and type I collagen in the dermal tissues using primary antibodies of CTHRC1, TGF- β 1, and type I collagen (all from Abcam, Cambridge, MA), respectively. Immune-specificity controls were included in all experiments.

Statistical analysis

SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses. Data were presented as mean \pm standard error of mean (SEM). A value of $P < 0.05$ was considered statistically significant. Differences were tested for

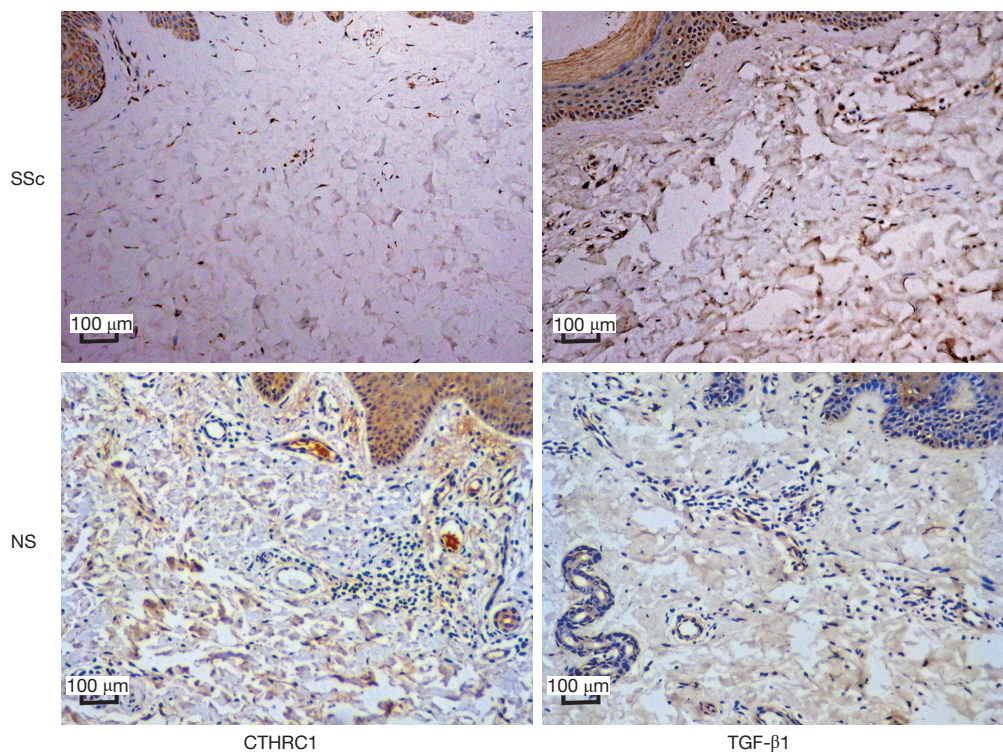


Figure 1 CTHRC1 and TGF- β 1 expression levels are elevated in fibroblasts in normal and SSc tissue by immunohistochemical staining. Paraffin sections of dermal tissue were prepared and labelled with antibodies specific for TGF- β 1 and CTHRC1, and then observed under a microscope ($\times 200$). NS, normal skin; CTHRC1, Collagen triple helix repeat containing-1; TGF- β , transforming growth factor- β ; SSc, systemic sclerosis.

significance using the independent *t* test or Mann-Whitney U test. All experiments were carried out in triplicate and repeated at least 3 times.

Results

CTHRC1 is upregulated in SSc dermal tissues

To clarify the biological function of CTHRC1 in SSc fibroblasts, we firstly investigated the expression of CTHRC1 in SSc dermal tissue samples compared to normal dermal tissues. Immunohistochemical staining revealed CTHRC1 to be dramatically upregulated in SSc dermal fibroblasts. Furthermore, elevated expression of TGF- β 1 was detected in SSc dermal fibroblasts compared to the normal controls (*Figure 1*), which was consistent with the observations of previous studies.

CTHRC1 attenuates TGF- β 1-induced proliferation, collagen expression, and synthesis of fibroblasts

Although the activation mechanism of SSc fibroblasts is unknown, some traits of SSc fibroblasts match those of healthy fibroblasts stimulated with TGF- β 1 (11,12). First, we assessed human dermal fibroblast activity after 24 hours of stimulation with 10 ng·mL⁻¹ TGF- β 1. The results revealed that TGF- β 1 treatment led to a dramatic increase in fibroblast proliferation. Next, we treated TGF- β 1-stimulated human dermal fibroblasts with rCTHRC1 and further observed the effects of CTHRC1 on fibroblasts. We found that CTHRC1 significantly inhibited the proliferation of fibroblasts (*Figure 2A*).

To further probe the inhibitory effects of CTHRC1, we measured the expression of collagen type I. The expression of collagen type I messenger RNA in fibroblasts

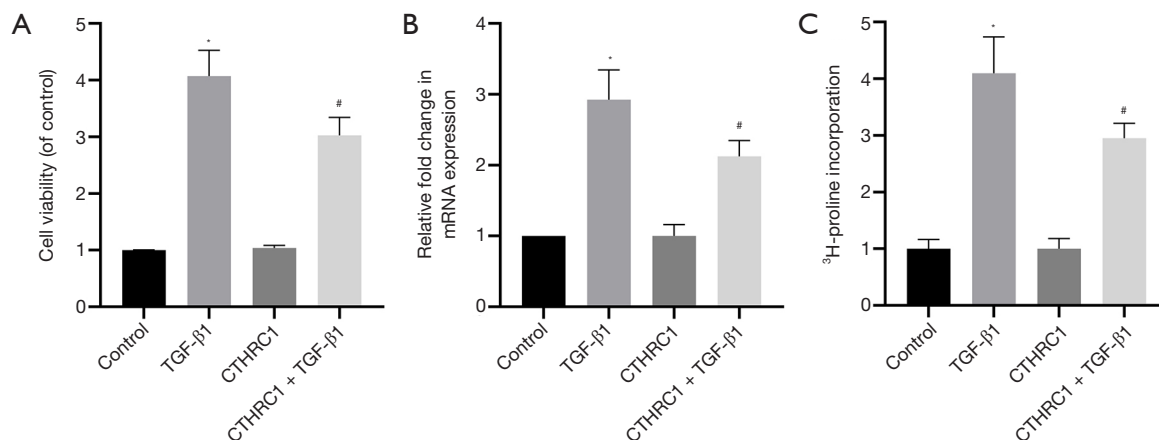


Figure 2 The effects of CTHRC1 on collagen expression in normal human skin fibroblasts. Cultured fibroblasts were either left untreated or treated with 10 ng/mL TGF-β1, 0.1 mg/mL rCTHRC1, or a combination of TGF-β1 and rCTHRC1 for 24 hours. Cell proliferation (A), collagen type I messenger RNA expression (B), and synthesis (C) were assessed by MTT assay, RT-PCR, and ³H-proline incorporation. The Mann-Whitney U test was applied for statistical analysis; n=5, Values represent the mean ± standard error of the mean (SEM). *, P<0.01; #, P<0.05. CTHRC1, Collagen triple helix repeat containing-1; TGF-β, transforming growth factor-β; RT-PCR, real-time polymerase chain reaction.

was significantly increased by TGF-β1, and this effect was reversed by rCTHRC1 at 0.1 mg·mL⁻¹ (Figure 2B). In line with the reduction of collagen expression, collagen synthesis in TGF-β1-stimulated human dermal fibroblasts was inhibited by rCTHRC1, as evidenced by the measurement of ³H-proline incorporation (Figure 2C). Furthermore, rCTHRC1 had no effect on cell viability.

The role of CTHRC1 in BLM-induced skin fibrosis

To unearth the function of CTHRC1 in dermal fibrosis *in vivo*, a BLM-induced dermal fibrosis mouse model was successfully established. First, Masson's trichrome staining was carried out to evaluate collagen deposition, and the type I collagen expression was assessed by immunohistochemical staining. The results showed that BLM significantly increased collagen deposition in dermal tissues and significantly upregulated type I collagen expression compared with the controls. The expression of CTHRC1 was also examined by immunohistochemistry, which showed obvious upregulation of CTHRC1 in fibrotic tissues from BLM-treated mice. Significant upregulation of TGF-β1 was also observed in fibrotic dermal tissues (Figure 3).

CTHRC1 reduces collagen deposition in mice with BLM-induced dermal fibrosis

Following the research findings described above, we next explored the antifibrotic effects of CTHRC1 in a mouse dermal fibrosis model induced by BLM. We observed that BLM significantly increased collagen deposition and increased both the protein level of type I collagen and the hydroxyproline content in the skin tissues, while CTHRC1 treatment markedly reduced these changes. As shown in Figure 4, rCTHRC1 dramatically reduced the protein level of type I collagen and the hydroxyproline content. Thus, there is consistency between our *in vitro* and *in vivo* data from normal human skin fibroblast experiments and BLM-induced dermal fibrosis mouse experiments, respectively, suggesting that CTHRC1 may hold clinical promise for the treatment of fibrotic disorders such as SSC.

Discussion

CTHRC1 is recognized as a positive regulator involved in various physiological and pathological processes, including antifibrotic activity and tissue repair remodeling (3,8,13-15). Moreover, it could have extensive clinical applications, due

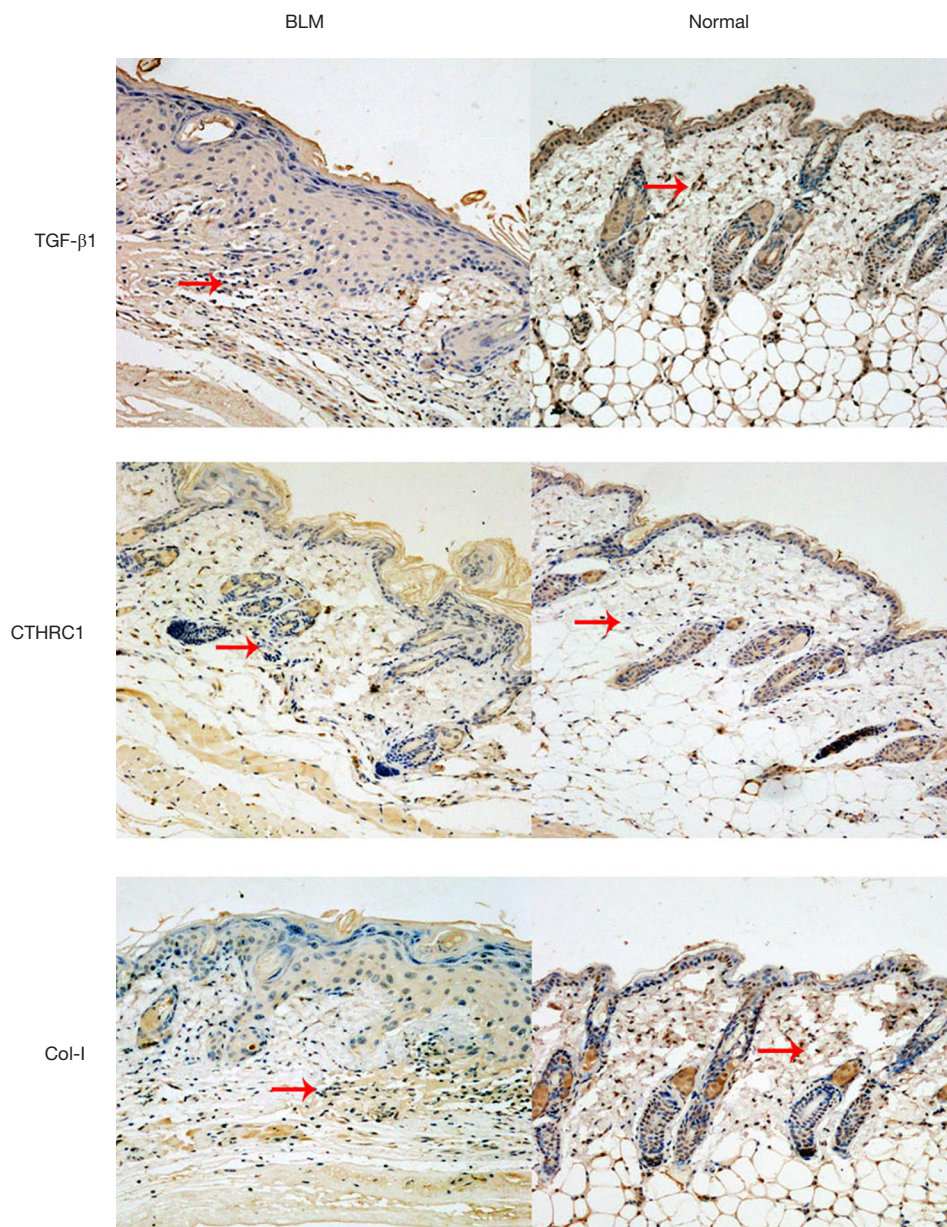


Figure 3 The expression levels of TGF- β 1, CTHRC1, and type I collagen in BLM-induced dermal fibrosis tissues were examined by immunohistochemical staining. Paraffin sections of normal and SSc skin tissues were prepared and labelled with antibodies specific for TGF- β 1, CTHRC1, and type I collagen observed with microscopy ($\times 200$). In each panel, the localization of TGF- β 1, CTHRC1, and type I collagen is indicated by an arrow. CTHRC1, Collagen triple helix repeat containing-1; TGF- β , transforming growth factor- β ; BLM, Bleomycin; SSc, systemic sclerosis.

to its ability to inhibit collagen deposition. An earlier study showed that CTHRC1 upregulation could prevent the progression of cholestatic liver fibrosis and could even cure hepatic fibrosis which had commenced (16).

To a great degree, vascular remodeling after injury

is controlled by TGF- β , and the feasible mechanism of collagen regulation by CTHRC1 is considered to be mutually connected to CTHRC1 and the TGF- β signaling pathway. CTHRC1 is stimulated by TGF- β 1 binding to the promoter of hepatic stellate cells via phospho-

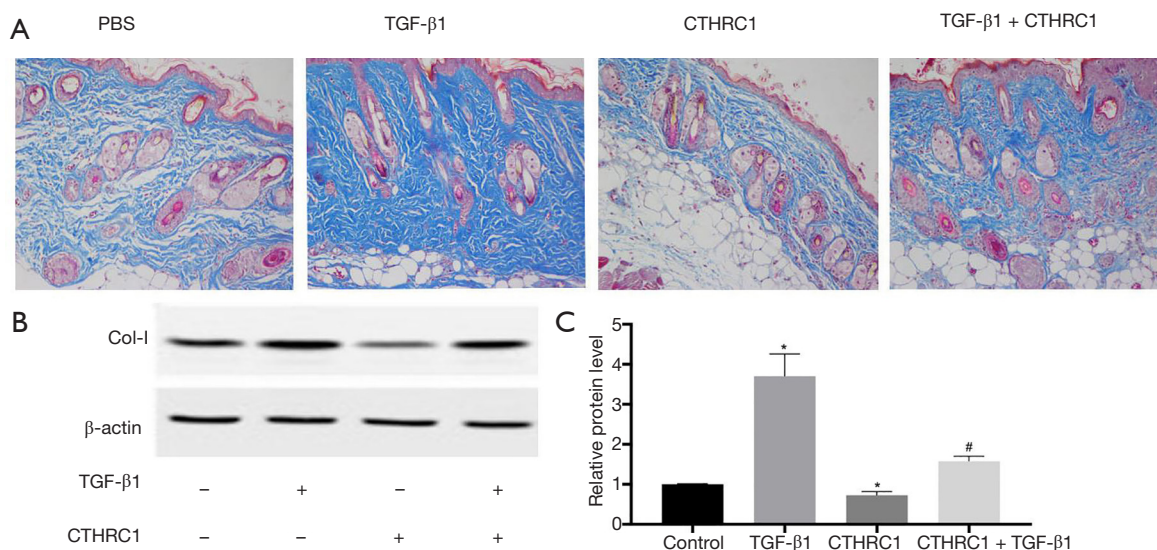


Figure 4 Recombinant CTHRC1 alleviates BLM-induced dermal fibrotic changes. (A) Collagen deposition in the dermis was observed by Masson's trichrome staining ($\times 200$); (B) representative gel image shows collagen type I expression in dermal tissues from BLM-treated mice; (C) summary data from 3 independent experiments. The Mann-Whitney U test was applied for statistical analysis; $n=5$, values represent mean \pm standard error of the mean (SEM). *, $P<0.01$; #, $P<0.05$. CTHRC1, Collagen triple helix repeat containing-1; BLM, Bleomycin.

Smad3, and the signal descends to accelerate the phospho-Smad3 degradation. Thus, CTHRC1 and phospho-Smad3 are deemed to be negatively correlated in the TGF- β pathway (17).

Our previous work demonstrated that overproduction of CTHRC1 inhibited the TGF- β 1-induced effects on keloid fibroblasts (8). In the present study, we firstly showed that CTHRC1 could inhibit human dermal fibroblast collagen deposition induced by TGF- β 1. Further investigation showed that CTHRC1 alleviated BLM-induced dermal fibrotic changes in model mice.

The potent profibrogenic cytokine TGF- β is widely known to play a crucial role in hepatic fibrosis processes. In hepatic fibrosis animal models, encouraging results have been gained through blocking the TGF- β pathway (18,19); however, human data have proved disappointing due to TGF- β playing a dominant role in many aspects of cell biological functions (18). Several studies have suggested that a therapeutic dose of a neutralizing monoclonal antibody against TGF- β 1 alleviated and even reversed fibrosis in several animal models (20-22). The efficacy of a systematically administrated anti-TGF- β 1 drug was non-existent in the treatment of patients with SSc accompanied with severe side effects (23). In short, CTHRC1 is a promising therapeutic target for dermal fibrosis.

To our knowledge, this is the first study to demonstrate

that CTHRC1 exerts antifibrotic effects on dermal fibrosis. We have provided evidence that CTHRC1 may be a potential drug for dermal fibrosis therapy, although further studies are needed.

Conclusions

In the present study, we have evidenced the antifibrotic effects of CTHRC1 in TGF- β 1-stimulated human skin fibroblasts and BLM-induced dermal fibrosis in mice. As far as we know, it is the first report to present CTHRC1 as a potential therapeutic option for dermal fibrosis.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/atm-21-1884>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-21-1884>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China (No.: Sichuan Provincial People's Hospital-D-2013-350) and informed consent was taken from all the patients. All animal experimental steps were carried out in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the approval of Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China (No.: Sichuan Provincial People's Hospital-D-2013-99).

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