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Original Article

miR-183 inhibits connective tissue growth factor (CTGF) production in TGF-β1-treated keloid fibroblasts in vitro

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Abstract: CTGF plays a critical role in the development and progression of keloid (KL) fibrogenesis. However, the potential mechanisms of ECM deposition induced by overexpressing CTGF in keloid have not been completely elaborated. In the present study, we hypothesized that a post-translational mechanism of miRs regulated the expression of CTGF in keloid fibroblasts (KFs). In this study, we collected 22 KL tissues and paired corresponding adjacent normal tissues from clinical patients and measured the expression of CTGF and differential expressed miRs. The results demonstrated that the expression of miR-183 was down-regulated in human KL tissues and KFs. In contrast to that CTGF was overexpressed in human KL tissues and KFs exposure to TGF-β1. We found that miR-183 suppressed CTGF by direct binding to the 3'-UTR of CTGF, with subsequently inhibition of KFs growth and induction of apoptosis in the present of TGF-β1. miR-183 loss-of-function combination with TGF-β1 treatment significantly increased CTGF protein expression and exhibited a significant increase in proliferation and decrease in apoptosis in KFs. However, CTGF knockdown was able to inhibit proliferation and induce apoptosis in KFs when miR-182 was knockdown and exposed to TGF-β1. In conclusion, we defined an antiproliferative role of miR-183 in human KFs, and the underlying mechanism was mediated, at least partially, through the inhibition of CTGF expression.

Keywords: miR-183, keloid fibrogenesis, fibroblasts, TGF-β1, CTGF

Introduction

Keloid is a benign dermal fibroproliferative disease, which is characterized by excessive extracellular matrix (ECM) production after skin injury [1]. Migratory fibroblast beyond the original wound border is one of the key features in the initiation and development of keloid [2]. In the process of fibroblasts migration, a variety of factors, including matrix metalloproteinases [3], transcription factors [4] and growth factors, have been proposed [5] to explain keloid pathogenesis. Though not malignant in nature, treatment of keloid is extremely difficult because keloid is highly recurrent after surgical excision [6]. Therefore, there is an urgent need for a better understanding of keloid pathogenesis in order to develop better prevention and treatment approaches in clinical trials.

Connective tissue growth factor (CTGF) is a cysteine-rich peptide and can be transcriptionally activated by TGF-β in various types of fibrotic disorders [7]. In human lung epithelial cells, TGF-β1-induced epithelial-to-mesenchymal transition (EMT) accelerates collagen I deposition, which may be involved in the regulation of CTGF signaling pathway [8]. In skin fibrosis, the levels of CTGF and TGF-β have been observed to be synchronously upregulated [9]. Studies have revealed that TGF-β1 increases CTGF expression markedly in human foreskin fibroblasts [9, 10]. Pharmacological studies have shown that genistein [11] or Simvastatin [12] protects against dermal fibrosis of keloids by inhibiting CTGF mRNA and protein expression in keloid fibroblasts. CTGF plays a major role in the adverse remodeling through the promotion of fibroblast proliferation and ECM production in connective tissues [13]. Emerging evidence shows that CTGF plays a critical role in keloid pathogenesis by promoting collagen synthesis and deposition [9]. Interestingly, the expression of intrinsic CTGF is up-regulated in

hypertrophic scar fibroblasts, and CTGF is significantly higher in response to TGF-β1 stimulation than untreated hypertrophic scar fibroblasts [14]. However, the post-translational mechanism of microRNAs (miRs) regulated the expression of CTGF in keloid fibroblasts remains unclear.

Increasing evidence implicates miRs gene therapies may inhibit tissue fibrosis in interstitial fibrosis and cardiac hypertrophy [15]. Recently, some miRs have been reported to participate in the initiation and development of keloid by accelerating fibrosis and ECM deposition, including miR-196a, miR-29a, miR-200b and miR-21 [2, 16-18]. In the present study, we demonstrated that miR-183 was under-expressed and CTGF was overexpressed in keloid tissues compared with corresponding adjacent normal tissues. In addition, we reported for the first time that miR-183 inhibited the expression of CTGF in TGF-β1-induced fibrogenesis in keloid fibroblasts in vitro. Overexpressed miR-183 could inhibit keloid fibroblasts growth and induce apoptosis. Therefore, we have reason to believe that miR-183 may be a potential therapeutic target to develop novel strategies for keloid fibrogenesis prevention and treatment.

Materials and methods

Patients' samples

Twenty-two keloid tissues (KL) and paired corresponding adjacent normal tissues (NC) were collected from patients who had undergone surgical excision at the Department of Dermatology and Plastic Surgery, Beijing Chaoyang Hospital Xijing Campus, Capital Medical University (Beijing, China) between January 2014 and September 2015. All collected tissue samples were immediately stored at liquid nitrogen until use. Human samples were obtained with written informed consent from all patients. The study was approved by the Ethics Committee of the Capital Medical University (Beijing, China).

Cell culture

Human keloid fibroblasts (KFs) and normal fibroblasts (NFs) were isolated from six different KL tissues and paired corresponding adjacent normal tissues, respectively, as previously described [19]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies) that contained 10% fetal calf serum (Gibco Life Technologies), 10% L-glutamine, 0.5% penicillin/streptomycin, 10% non-essential amino acids and 10% pyruvate, in a 5% CO_2 atmosphere and incubated at 37°C.

MTT assay

Cell proliferation was monitored by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (R&D SYSTEMS) in according to the guidelines.

TUNEL staining analysis

The induction of apoptosis was also monitored by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The TUNEL assay was performed by TUNEL Apoptosis Kit (R&D SYSTEMS) in according to the guidelines.

Luciferase reporter gene activity assay

The 3'-UTR of CTGF gene containing the putative binding site for miR-183 was obtained by PCR amplification. The fragment was inserted into the firefly luciferase gene of the dual-luciferase miRNA target expression vector luciferase reporter vector (pGL3) (Promega, WI, USA). KFs were co-transfected with 0.1 μg of luciferase reporters containing CTGF 3'-UTR and miR-183 inhibitors. We harvested the cell lysates after 24 hours transfection and measured the luciferase activity with a dual luciferase reporter assay kit according to manufacturer's instruction (Promega, WI, USA).

Transfection of miR-183 mimics and inhibitors

The FAM modified 2'-OMe-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography (GenePharma, Shanghai, China). The 2'-OMe-miR-183 mimics were composed of RNA duplexes with the following sequence: 5'-UAUGGCACCGUGGUAAGAAUUCACU-3'. The sequences of 2'-OMe-miR-183 inhibitor and 2'-Ome-scramble oligonucleotides were as follows: 5'-AGUGAAUUCUUACCACGGUGCCAUA-3'; and 5'-CGUAAGUCGAGCCGGUGAAGGCAGU-3'. Cells were transfected using Lipofectamine2000 (Invitrogen, CA, USA) at a final concentration of 100

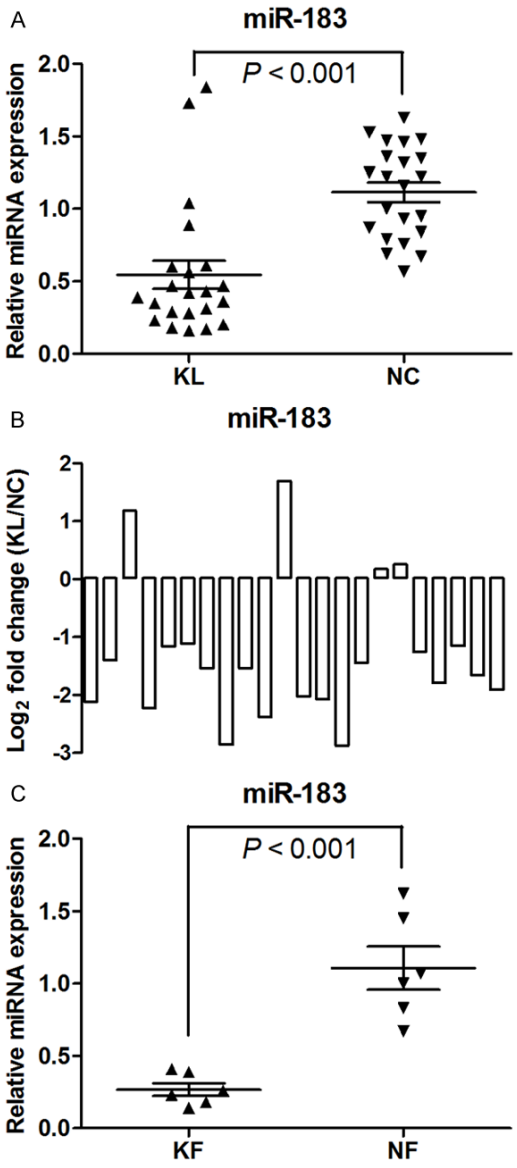

Figure 1. Down-regulated miR-183 in keloid tissues and fibroblasts. miR-183 expression was examined by qRT-PCR and normalized to U6 expression in 22 keloid tissues and paired corresponding adjacent normal tissues (A). Pair-wise comparison of miR-183 expression between keloid tissues and paired corresponding adjacent normal tissues, more than 80% (18/22) of KL tissues showed reduction of miR-183 (B). Relative expression of miR-183 in KF and NF was measured by qRT-PCR (C).

nM. At 24 h post-transfection, the culture medium was changed.

Retrovirus package and transduction

Specific sh-RNAs targeting CTGF were purchased from Invitrogen, and the corresponding sequences were cloned into the pSIREN-RetroQ plasmid (Addgene) for retrovirus production with 293T cells. For transduction, 293T cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 hours, infected cells were selected for with puromycin (2 mg/ml). Then the clones were picked and cultured for further experiment.

Quantitative RT-PCR for miRNA

The quantitative RT-PCR for miRNA was performed using TaqMan MicroRNA Assays (Applied Biosystems). Briefly, 10 ng of total RNA were reverse transcribed using a specific looped RT primer for each miRNA using a corresponding TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). U6 was used as an internal control. The cycle threshold value, which was determined using second derivative, was used to calculate the normalized expression of the indicated miRNAs using the Q-Gene software [20].

Western blotting

Proteins were extracted by NP-40 buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylimide gel electrophoresis and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), and incubated with the following primary antibodies: CTGF (1:1000), FN (1:1000), type I collagen (1:1000) and β-actin (1:500) all from Santa Cruz Biotechnoogy, Inc. (Dallas, TX, USA), at 4°C overnight. After being washed, the membranes were incubated with HRP-conjugated anti-IgG at room temperature for 2 hour. Signal detection was carried out with an ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

All values are expressed as the mean ± SEM of at least three independent experiments. Statistical differences between two groups were determined using Student's t test. The correlation of CTGF level and miR-183 level was analyzed with linear regression analysis. All statistical analyses were performed using

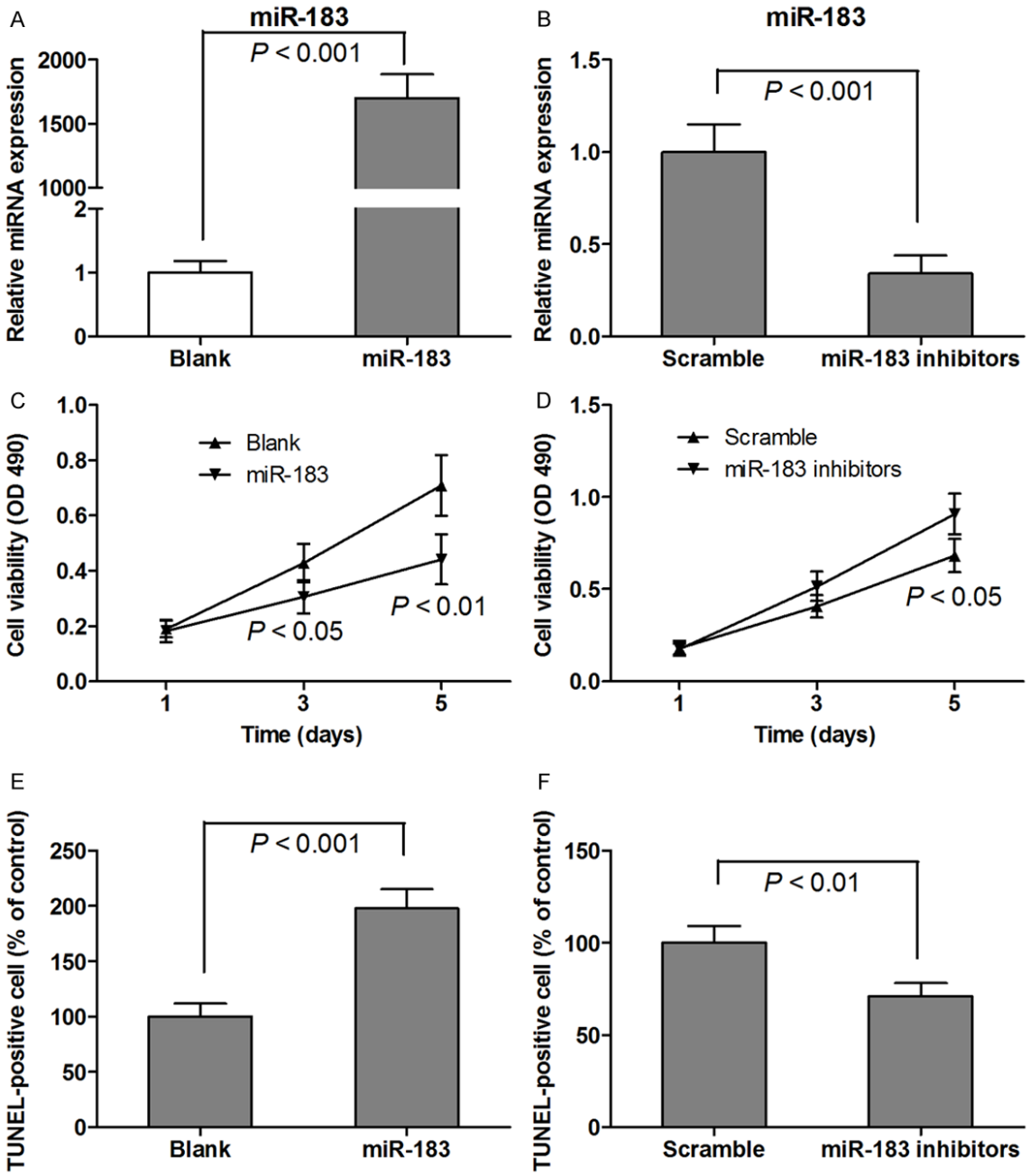

Figure 2. KFs were transfected with miR-183 mimic (A), miR-183 inhibitor (B) or scrambled oligonucleotide. Non-transfected cells were used as blank control group (Blank). miR-183 expression levels in KFs were measured by qRT-PCR analysis after 24 hours transfection. MTT assays were performed to measure cell viability in the present of miR-183 mimic (C) or miR-183 inhibitor (D). KFs were transfected with miR-183 mimic (E) or miR-183 inhibitor (F) for 3 days, and the cells apoptosis were measured by TUNEL analysis.

GraphPad Prism software, version 5.0 (Graph Pad Software, Inc., La Jolla, CA, USA). Groups were compared using one-way analysis of variance, followed by Turkey's multiple comparison tests as a post hoc test to compare the mean values of each group. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Down-regulated miR-183 in keloid tissues and fibroblasts

To explore the miRs expression profiles in KL tissues, we compared miRs expression be-

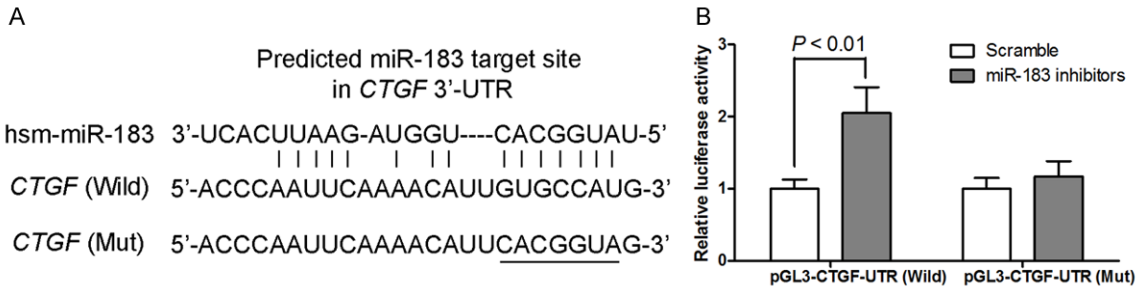

Figure 3. miR-183 directly targets to the 3'-UTR of CTGF. Base pairing between miR-183 and CTGF was predicted by bioinformatics analysis. Schematic representation of the putative miR-183 binding site in the 3'-UTR of CTGF was predicted by miRanda (A). The wild or mutant (Mut) reporter plasmid was cotransfected into KFs with miR-183 inhibitor or scrambled oligonucleotide, and luciferase activity was significantly increased by miR-21 inhibitor (B).

tween KL tissues and adjacent normal tissues (NC) using by qRT-PCR analysis. A total of 36 miRNAs could bind to the 3'-untranslated regions (3'-UTR) of CTGF and were selected by miRanda (http://www.microrna.org). Among these miRs, miR-183 showed the highest fold change (0.17-fold in KL as compared to NC, data no shown). We then focused on miR-183 in the following analysis. As shown in **Figure 1A**, the levels of miR-183 in 22 KL tissues were 2-fold lower than that of the adjacent normal tissues. Pair-wise comparison indicated that over 80% (18/22) of KL tissues showed reduction of miR-183 expression compared with their matching adjacent normal tissues, with only four pairs showing increase in KL tissues (**Figure 1B**). As expected, the expression of miR-183 was also decreased in KF as compared to NF group (**Figure 1C**).

miR-183 regulates proliferation and apoptosis in KF

First, KFs were transfected with the miR-183 mimics or inhibitors to evaluate the expression capacity of miR-183 in KFs. qRT-PCR confirmed the elevated level of miR-183 in KFs transfected with miR-183 mimics and reduced level of miR-183 in KFs transfected with miR-183 inhibitors (**Figure 2A** and **2B**). KFs transfected with miR-183 mimics exhibited a significant decrease in proliferation compared with blank control group (**Figure 2C**). In contrast to that inhibition of miR-183 increased the growth of KFs as compared to control group (**Figure 2D**). To investigate whether DNA damage was involved in apoptosis induced by abnormal miR-183 expression, and TUNEL assay was performed. KFs transfected with miR-183 mimics significantly increased number of TUNEL-positive cells compared with blank control group (**Figure 2E**). However, the decrease of TUNEL-positive cells transfected with miR-183 inhibitors (**Figure 2F**). Taken together, miR-183 can regulate proliferation and apoptosis in KFs.

miR-183 directly targets to the 3'-UTR of CTGF

To further investigate the post-translational mechanism of miR-183, the miRanda prediction algorithms were used in this study, and we found that CTGF contained one putative target of miR-488 in the 3'-UTR of CTGF (**Figure 3A**). Next, the 3'-UTR of CTGF containing the potential miR-183 binding site was cloned for use in a firefly luciferase reporter assay. The wild type sequence of CTGF or its mutant sequence was transfected into KFs along with the miR-183 inhibitor or scramble control. Compared with the scramble control group, transfected with miR-183 inhibitor increased the relative luciferase activity in the present of CTGF wild-type sequence. However, the mutant reporter plasmid reversed the miR-183 inhibitor-induced increase in luciferase activity (**Figure 3B**). These findings suggest that miR-183 suppresses CTGF by direct binding to the 3'-UTR of CTGF.

miR-183 inhibits TGF-β1-induced CTGF expression in KFs

Western blotting analysis showed an elevated expression of CTGF in KL tissues (**Figure 4A**) or KFs (**Figure 4B** and **4C**) as compared to the adjacent normal tissues or NFs, respectively. These results indicate a possible role of the CTGF in keloid pathogenesis. As shown in **Figure 4D**, TGF-β1 treatment significantly inhib-

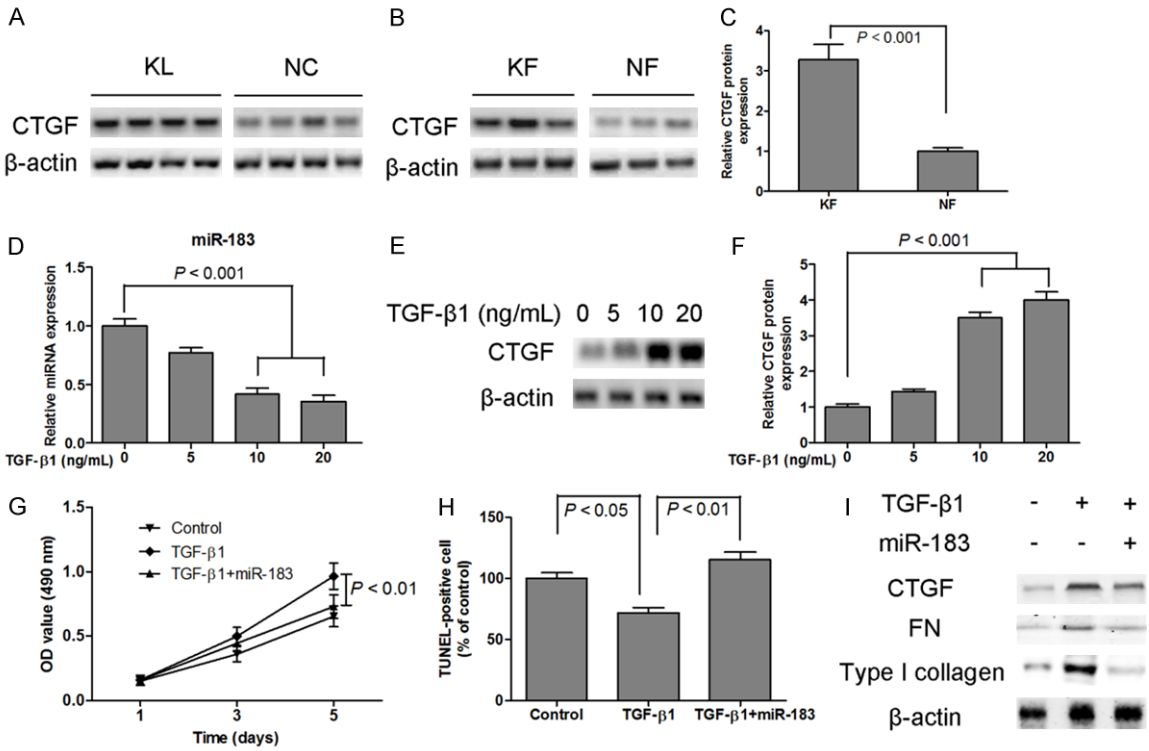

Figure 4. miR-183 inhibits TGF-β1-induced CTGF expression in KFs. The expression of CTGF in KL tissues was measured by western blotting analysis (A). KFs or NFs were isolated from KL tissues or corresponding adjacent normal, respectively, and the expression of CTGF was measured by western blotting after 3 days culture in vitro (B and C). miR-183 expression levels in KFs were measured by qRT-PCR analysis in the present of TGF-β1 for 3 days (D). The expression of CTGF in KFs was measured by western blotting analysis in the present of TGF-β1 for 3 days (E and F). MTT assays were performed to measure cell viability in the present of TGF-β1 and with or without miR-183 mimics (G), and cells apoptosis were measured by TUNEL analysis (H). The protein expression of CTGF, FN and type I collagen was performed by western blotting analysis in KFs in the present of TGF-β1 and with or without miR-183 mimics for 3 days culture in vitro (I).

ited miR-183 expression in KFs. Simultaneously, TGF-β1 induced significant expression of CTGF in KFs in a dose-dependent manner (**Figure 4E** and **4F**). There was no obvious different of TGF-β1 at concentration of 10 or 20 ng/mL to regulate miR-183 and CTGF expression (**Figure 4D-F**). As shown in **Figure 4G** and **4H**, 10 ng/mL of TGF-β1 treatment caused a significant increase in cell viability and decrease in apoptosis of KFs. However, overexpressed miR-183 could inhibit TGF-β1-induced cell growth and reverse TGF-β1-induced the decrease of apoptosis. To examine whether miR-183 inhibited CTGF expression in the present of TGF-β1, KFs were incubated with TGF-β1 (10 ng/mL) and transfected with miR-183 mimics. Western blotting results demonstrated that miR-183 mimics markedly inhibited TGF-β1-induced up-regulation of CTGF in KFs (**Figure 4I**). Moreover, we also found that overexpressed miR-183 resulted in the down-regulation of FN and type I collagen in the present of TGF-β1 at the concentration of 10 ng/mL (**Figure 4I**).

miR-183/CTGF signaling is involved in TGF-β1-induced KFs growth in vitro

Based on these studies, we concluded that miR-183 could inhibit CTGF expression by targeting its 3'-UTR, which was further confirmed by linear regression analysis between the miR-183 levels and CTGF protein expression in KL tissues. The linear regression analysis demonstrated that miR-183 levels were significantly and negatively correlated with CTGF protein expression in human KL tissues (**Figure 5A**). In this study, miR-183 loss-of-function combination with TGF-β1 treatment significantly increased CTGF protein expression in KFs as compared to TGF-β1 single treatment group (**Figure**

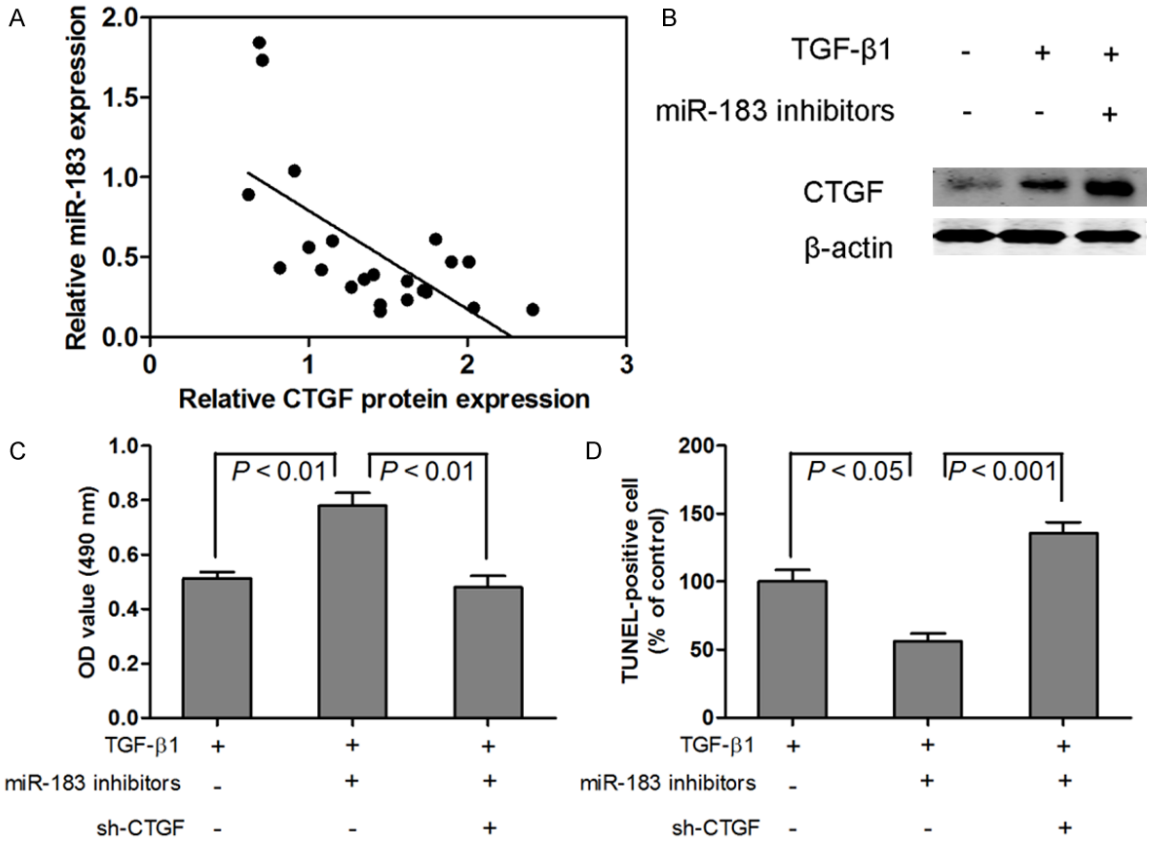

Figure 5. miR-183/CTGF signaling is involved in TGF-β1-induced KFs growth in vitro. Linear regression analysis was performed to analyze the correlation between miR-183 level and CTGF protein expression, n = 22 (A). The protein expression of CTGF was performed by western blotting analysis in KFs in the present of TGF-β1 and with or without miR-183 inhibitors for 3 days culture in vitro (B). MTT assays were performed to measure cell viability in the present of TGF-β1 and with or without miR-183 inhibitors (C), and cells apoptosis were measured by TUNEL analysis for 3 days culture in vitro (D).

5B). miR-183 loss-of-function exhibited a significant increase in proliferation (**Figure 5C**) and decrease in apoptosis (**Figure 5D**) in KFs in the present of TGF-β1 as compared to TGF-β1 single treatment group. However, CTGF knockdown was able to inhibit proliferation and induce apoptosis in KFs when miR-183 was knockdown and exposed to TGF-β1 (**Figure 5C** and **5D**). These results demonstrated that CTGF was critically essential for the function of miR-183 in KFs proliferation and apoptosis in the present of TGF-β1.

Discussion

Here, we define an antiproliferative role of miR-183 in human keloid. We discovered that CTGF is overexpressed in human KL tissues and KFs exposure to TGF-β1, which can be a keloid fibrogenic cell model. In this process, a multitude of cytokines, growth factors, and proteins are released at the wound-healing site, these compounds stimulate profibrotic activity [21]. Interestingly, miR-183 suppresses CTGF by direct binding to the 3'-UTR of CTGF, with subsequently inhibition of KFs growth and induction of apoptosis in the present of TGF-β1. These data provide new insights into the mechanism underlying KL formation and therapeutic strategies for this disorder.

Overexpressed miR-183 is a frequent event in various cancers, including colorectal cancer [22], breast cancer [23] and non-small cell lung cancer [24]. In contrast to that miR-183 is significantly downregulated in gastric cancer, and up-regulation of miR-183 significantly inhibits gastric cancer cell proliferation and invasion [25]. However, the role of miR-183 in keloid fibrogenesis remains to be elucidated. Previous

study indicates that miR-29a is significantly decreased in keloid as compared to healthy fibroblasts, miR-29a loss-of-function up-regulates the mRNA and protein expression of type I and type III collagen in the fibroblasts [16]. In our study, we found that miR-183 inhibited TGF-β1-induced type I collagen protein expression. Collagen family is a dominant component of ECM, increased ECM deposition is the major findings in keloid pathology [2, 26]. Overproduction of collagen and ECM in fibroblasts is easy to induce fibrotic diseases, including keloid and hypertrophic scars [27]. Therefore, inhibition of collagen and ECM production and deposition in fibroblasts is an important goal for pharmaceutical agents used in the treatment of keloid. In the present study, we found that miR-183 might be a new therapeutic approach for fibrotic disorders.

CTGF is a downstream effector of TGFβ signaling and can be secreted in fibroblasts [11]. CTGF is known to elicit fibroblast-specific mitogenesis, chemotaxis and ECM synthesis in vitro [28]. A growing body of evidence suggests that CTGF can mediate TGF-β-induced fibrogenesis, including fibroblast proliferation and the production of extracellular matrix proteins such as collagen and fibronectin [29]. Elevated levels of CTGF have frequently been associated with fibrotic skin disorders such as scleroderma and keloids [30]. Upregulation of secretory CTGF in keratinocyte-fibroblast coculture contributes to keloid pathogenesis [9]. Gene array profiling has also indicated CTGF may be up-regulated in isolated KF in the present of hydrocortisone [31]. In our study, we demonstrated that the protein expression of CTGF was markedly increased in KL tissues and KFs. This observation was consistent with a statistically significant increase in downstream protein markers, FN and type I collagen, in the present of TGF-β1. Overexpressed miR-183 decreased TGF-β1-induced expression of CTGF, FN and type I collagen in cultured KFs. In addition, we showed that CTGF knockdown inhibited KFs growth and induced apoptosis in the present of TGF-β1. Noticeably, CTGF knockdown increased number of TUNEL-positive cells in KFs, indicating that CTGF knockdown induced DNA damage. Collectively, our evidence indicates that CTGF regulates cellular proliferation and apoptosis behaviors in KFs, which can be regulated by miR-183.

To the best of our knowledge, overexpressed miR-183 can inhibit KFs growth and induce apoptosis, and the underlying mechanism is mediated, at least partially, through the suppression of CTGF expression. But above all, these findings offer a treatment for abnormal wound-healing processes that respond to multiple upstream events, such as TGF-β1 stimulation.

Disclosure of conflict of interest

None.

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