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ORIGINAL PAPER

Hepatocyte growth factor inhibits TGF-β1-induced myofibroblast differentiation in tendon fibroblasts: role of AMPK signaling pathway

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Abstract The transforming growth factor-β1 (TGF-β1)-induced myofibroblastic differentiation in tendon fibroblasts was thought to be one of the most important features of scar fibrosis formation, which is associated with occurrence of re-rupture. Previously, we reported that hepatocyte growth factor (HGF) inhibited TGF-β1-induced myofibroblast differentiation and extracellular matrix deposition in the Achilles tendon of rats. Here, we investigated the potential molecular mechanisms underlying the inhibitory effect of HGF on TGF-β1-induced myofibroblast differentiation. We found that treatment with HGF (10, 20, and 40 ng/ml) increased phosphorylation of adenosine monophosphate kinase (AMPK) and acetyl-CoA carboxylase (ACC) in tendon fibroblasts. Pharmacological inhibition of the AMPK signaling pathway using compound C, a specific blocker of AMPK signaling, remarkably attenuated the inhibitory effect of HGF on TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. Moreover, small interfering RNA (siRNA)-mediated knockdown of AMPKα1 subunit decreased the inhibitory effect of HGF on TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. Finally, overexpression of constitutively active AMPKα1, which led to constitutive activation of the AMPK signaling pathway in tendon fibroblasts, mimicked the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation. Our study therefore suggests that HGF inhibits TGF-β1-induced myofibroblastic differentiation via an AMPK signaling pathway-dependent manner in tendon fibroblasts.

Keywords HGF · TGF-β1 · Myofibroblastic differentiation · AMPK

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Introduction

Fibroblasts are active participants in creating connective tissue and are instrumental in wound healing owing to their exceptional ability to undergo various interconversions between related but distinctly different cell types. In the 1970s, the transient appearance and disappearance of "myofibroblasts" in the granulation tissue of healing cutaneous wounds was described [1]. Myofibroblasts are cells that are in between a fibroblast and a smooth muscle cell in terms of differentiation and have been defined by their ability to express contractile proteins, including α-smooth muscle actin (α-SMA), collagen, type I, alpha 1 (Col I-α1), collagen, type III (Col III), fibronectin, etc. [2]. The responsive profile of transforming growth factor β1 (TGF-β1) in healing tissue is thought to play a critical role in the healing of tendons and ligaments. It induces proliferation of connective tissue cells [3, 4]. Importantly, TGF-β1 stimulates matrix contraction, cell growth, and collagen production in fibroblast and then produces tissue-like fibroplasia, a fibronectin matrix-dependent event [5, 6]. TGF-β receptor type II (TGF-βRII) is found to be responsive and essential for TGF-β's function [7].

Tendon or ligament injury, a common problem caused by physical and recreational activities, often results in scar fibrosis formation, characterized by myofibroblast differentiation and overproduction of extracellular matrix (ECM) [8, 9]. After scar fibrosis formation, the biochemical and

mechanical properties of the healed tendon were greatly decreased. For example, after the first incidence of Achilles tendon rupture, the re-rupture rate can be as high as approximately 20 % [10]. TGF-β1 was found to be the major inducer of scar tissue formation in tendon fibroblasts [11] by activating myofibroblastic differentiation. Thus, to blunt or disrupt the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts may be a promising therapeutic target for tendon injury.

Recently, cytokines such as hepatocyte growth factor (HGF) were reported to attenuate TGF-β1-induced myofibroblastic differentiation. HGF is a heterodimer molecule composed of a large 69-kDa subunit and a small 34-kDa subunit [12], and is a humoral mediator of liver regeneration [13]. HGF prevents the initiation and progression of chronic renal fibrosis in a wide variety of animal models [14]. HGF suppresses type III collagen expression in human renal fibroblasts [15] and in rat ligament fibroblasts [16]. Our group previously demonstrated that HGF inhibits TGF-β1-induced myofibroblast differentiation and ECM deposition in the Achilles tendon of rats [17]. However, how HGF inhibits TGF-β1-induced myofibroblast differentiation is largely unknown. In this study, we explored the role of the AMPK signaling pathway in the inhibitory effect of HGF on TGF-β1-induced myofibroblast differentiation.

Materials and methods

Animals

Adult male Sprague–Dawley rats weighing 250–260 g were supplied by the Beijing Vital River Laboratory Animal, Inc. Rats were housed in a facility with controlled temperature (23 ± 2 °C) and lighting (0800–2000 hours), with free access to tap water and food. All procedures were performed in compliance with and approved by the Animal Care and Usage Committee at Harbin Medical University.

Isolation and culture of primary tendon fibroblasts

The tendon fibroblasts were isolated and cultured as described previously [17]. Briefly, the Achilles tendons of Sprague–Dawley rats were harvested to culture tendon fibroblasts. The tendons were isolated and washed in sterile saline containing 1 % penicillin/streptomycin and cut into 1-mm sections. The sections were subjected to collagenase type II (0.1 %) digestion overnight in DMEM (Sigma, St. Louis, MO, USA) containing 5 % FBS, penicillin/streptomycin, and amphotericin. Then, samples were placed in an orbital shaker at 37 °C [18]. The solution was spun for 10 min at 3000×*g*. The supernatant was removed and the pellet was washed two times with the above medium. The isolated cells and remaining tissue were plated in a 75-mm^2 tissue culture flask and placed in an incubator (5 % CO_2 and 37 °C) [19]. All experiments were performed using the cells at passages 4–6.

Experiments and groups

Tendon fibroblasts were grown to approximately 70 % confluence, and switched to serum-starved medium (0.1 % FBS) for 8 h before further treatments. Then, the cells were treated with TGF-β1 (5 ng/ml, Peprotech, Rocky Hill, NJ) or TGF-β1 plus different concentrations of HGF (10, 20, and 40 ng/ml, Peprotech, Rocky Hill, NJ). For the following mechanism study, the AMPK inhibitor compound C (Sigma-Aldrich) was also added into cell culture wells to block the AMPK signaling pathway [20].

siRNA-mediated RNA interference

We knocked down AMPK using siRNAs targeting AMPK-α1 (sc-270142, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in tendon fibroblasts with Lipofectamine-LTX with Plus Reagent (Invitrogen, Carlsbad, CA) [21]. The transfection was performed according to the manufacturer's instructions. Western blotting was used to confirm the efficiency of knockdown.

Transfection of constitutively active AMPK-α1 subunit

We overexpressed plasmid of constitutively active AMPK-α1 (caAMPK-α1, addgene, plasmid no. 27632) in tendon fibroblasts using Lipofectamine-LTX with Plus Reagent (Invitrogen, Carlsbad, CA). Western blotting analysis with specific primary antibody against the N-terminal epitope of both exogenous AMPK and caAMPKα2 was performed to confirm the constitutive activation of AMPK signaling in transfected cells. At 3 days post-transfection, the cells were treated with TGF-β1.

RNA extraction and quantitative real-time PCR analysis

Quantitative real-time PCR analysis was performed as previously described [22, 23]. Total RNA was extracted from cell tissues using Trizol, and 1 μg RNA was reverse transcribed to cDNA. Quantitative PCR was performed using Opticon Monitor 3 real-time PCR system (Bio-Rad) and SYBR Premix Ex Taq Mixture (Takara) with specific primers. The PCR reactions were initiated with denaturation at 95 °C for 10 s, followed by amplification with 40 cycles at 95 °C for 10 s, and annealing at 60 °C for 20 s

(two-step method). Finally, melting curve analysis from 60 to 85 °C was performed [24]. Data were evaluated with Opticon Monitor 3 software [25]. The sequences of the primers were as follows: α-SMA, CTAAACCCTAAAG CCAACA (sense), CAGTGCATAGCCCTCGT (anti-sense); Col I-α1, CACCAGACGCAGAAGTCATAGG (sense), GCAAAGTTTCCTCCAAGACCAG (anti-sense); Col III, CTACACCTGCTCCTGTCATTCC (sense), GCCACCCA TTCCTCCGACT (anti-sense); Fibronetin, ACTCGCTTTG ACTTCACCACC (sense), TCCTTCCTCGCTCAGTTCGT (anti-sense); β-actin, ATCCTGCGTCTGGACCTGG (sense), CCGCTCATTGCCGATAGTG (anti-sense). All data were normalized to β-actin expression ($2^{-\Delta\Delta Ct}$ methods) [26].

Immunoblotting

Immunoblotting analyses were performed as described previously [26, 27]. Cell samples were subjected to 10 % SDS-PAGE, and transferred onto PVDF membranes at 100 V for 1–2 h. After being blocked in blocking buffer with 5 %(w/v) nonfat milk and 0.1 % (v/v) Tween 20 in phosphate-buffered saline for 4 h, the membrane was incubated with specific primary antibody (phospho-AMPK, total-AMPK, phospho-acetyl-CoA carboxylase [ACC] and total-ACC; Cell Signaling Technology, Beverly, MA) and then followed by HRP-labeled secondary antibody [28]. The membranes were then detected using the enhanced chemiluminescence system as described previously [29].

Statistical analysis

Data are expressed as mean ± SD. Differences were evaluated by two-tailed Student's *t* test or ANOVA. Statistical significance was set at $P < 0.05$.

Results

HGF activates AMPK signaling pathway in tendon fibroblasts

As shown in Fig. 1a, treatment with different concentrations of HGF (10, 20, and 40 ng/ml) induced phosphorylation of AMPK in tendon fibroblasts. We also measured the phosphorylation of ACC, a critical downstream factor of AMPK. Accordingly, phosphorylation of ACC was increased significantly by HGF in tendon fibroblasts (Fig. 1b). Because there was no difference in the increase of AMPK phosphorylation between the three concentrations, the moderate concentration of HGF (20 ng/ml) was used in the following experiments.

Administration of AMPK inhibitor compound C abolishes the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts

Previously, we showed that HGF attenuated TGF-β1-induced fibrogenic responses in the tendon in vivo [17]. In this investigation, we studied this phenotype in tendon fibroblasts. Before the incubation of TGF-β1, the tendon fibroblasts were flat. At 72 h post TGF-β1 treatment, some tube-like structures were observed (Fig. 2a). As shown in Fig. 2b–e, administration of TGF-β1 (10 ng/ml) for 48 and 72 h induced significant upregulation in mRNA levels of several markers for myofibroblastic differentiation (α-SMA, Col I α1, Col III, and fibronectin), suggesting that TGF-β1 induced myofibroblastic differentiation in tendon fibroblasts. In agreement with previous in vivo data [17], supplementation with HGF obviously inhibited the changes of mRNA levels of these markers (Fig. 2b–e). Notably,

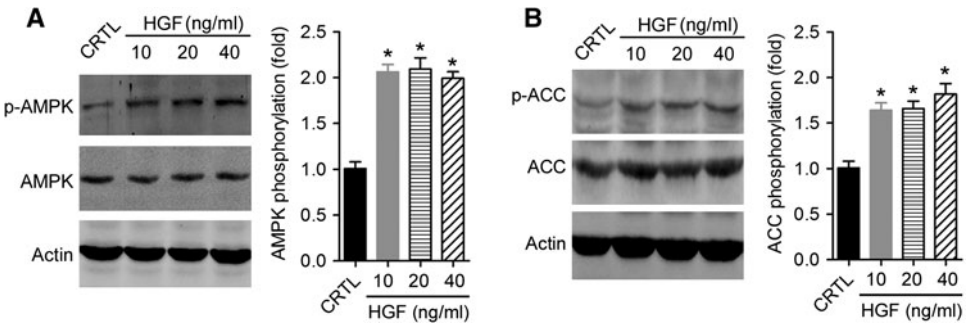

Fig. 1 Treatment with HGF activated AMPK signaling pathway in tendon fibroblasts. **a** Primary tendon fibroblasts were treated with three different concentrations of HGF (10, 20, and 40 ng/ml) and then the intracellular AMPK phosphorylation (p-AMPK to AMPK ratio) was determined by Western blotting analysis. Actin was used as a loading control. $N = 6$. $^*P < 0.05$ versus CTRL (control). **b** The phosphorylation of ACC (p-ACC to ACC ratio), a critical downstream factor of AMPK, in HGF-treated tendon fibroblasts was also determined. $N = 6$. $^*P < 0.05$ versus CTRL

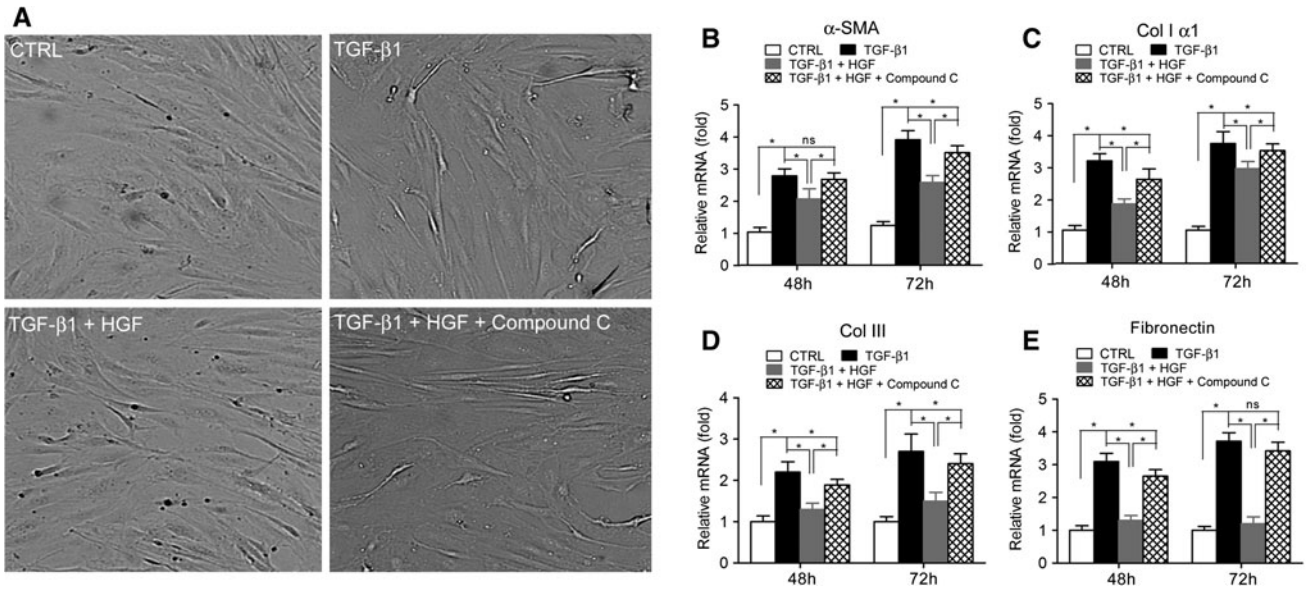

Fig. 2 AMPK inhibitor compound C attenuated the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. **a** Typical cell morphology of tendon fibroblasts under stimulation by TGF-β1 and HGF. **b–e** Tendon fibroblasts were treated with TGF-β1 (10 ng/ml), TGF-β1 (10 ng/ml) + HGF (20 ng/ml), or TGF-β1 (10 ng/ml) + HGF (20 ng/ml) + compound C (20 μM) for 48 and 72 h. Then, the mRNA levels of α-SMA (**b**), Col I α1 (**c**), Col III (**d**), and fibronectin (**e**), four markers of myofibroblastic differentiation, were measure by real-time quantitative PCR analysis; β-actin was used as a housekeeping gene for reference. All data were normalized to β-actin expression ($2^{-\Delta\Delta Ct}$ methods). $N = 8$. $^*P < 0.05$

treatment with compound C, a well-established AMPK signaling pathway inhibitor, greatly attenuated the inhibitory effect of HGF on TGF-β1-induced effects (Fig. 2b–e). These results indicate that HGF may decrease TGF-β1-induced myofibroblastic differentiation via the AMPK signaling pathway partly.

Blocking AMPK signaling by knocking down of AMPKα1 subunit disrupts the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts

Considering that the chemical agent (compound C) may have potential unspecific effects, we used a more specific method to block the AMPK signaling pathway. Knocking down of AMPKα1 using AMPKα1-targeting siRNA significantly decreased the AMPKα1 subunit protein level (>70 %, data not shown). As shown in Fig. 3a, HGF treatment stopped the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts in wild-type (WT) and siRNA-scramble transfected cells but not in siRNA-AMPKα1 transfected cells (Fig. 3a). The AMPKα1 knockdown disrupted the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts (Fig. 3b–e).

Constitutive activation of AMPK signaling mimics the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts

Next, we further studied whether constitutive activation of AMPK signaling can mimic the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. We overexpressed the caAMPKα1 plasmid (Fig. 4a) into tendon fibroblasts, which induced constitutive activation of AMPK signaling (Fig. 4b). Interestingly, compared with WT and vector-control transfected cells, the caAMPKα1-transfected cells showed blunted TGF-β1-induced myofibroblastic differentiation (Fig. 5a). The increases of mRNA levels α-SMA, Col I α1, Col III, and fibronectin (Fig. 5b–e), indicating constitutive activation of AMPK signaling, mimics the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts.

Discussion

In the present study, using an in vitro cell model, we found that HGF inhibited TGF-β1-induced myofibroblastic differentiation via an AMPK signaling pathway-dependent manner in tendon fibroblasts. First, HGF activated the

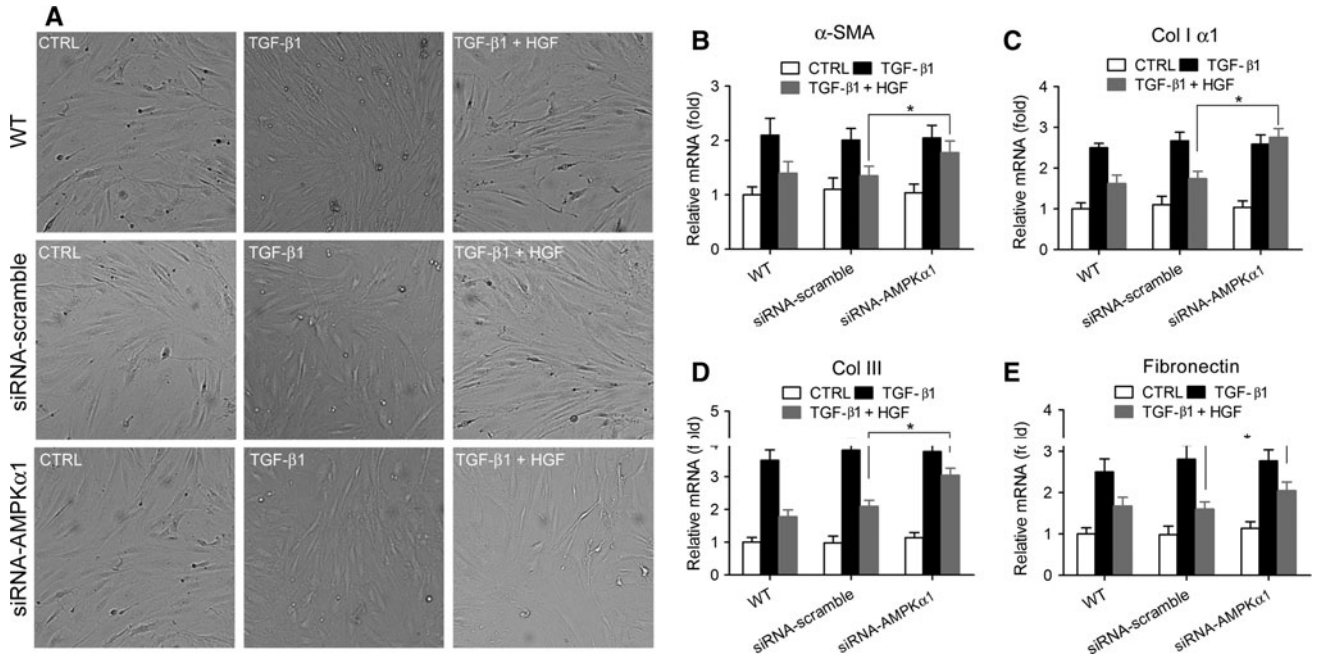

Fig. 3 Knocking down of AMPKα1 disrupted the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. Wild-type tendon fibroblasts, scramble-siRNA-transfected tendon fibroblasts, and AMPKα1-targeting siRNA-transfected tendon fibroblasts were treated with TGF-β1 (10 ng/ml) or TGF-β1 (10 ng/ml) + HGF (20 ng/ml) for 72 h. **a** Typical cell morphology of control, siRNA-scramble transfected, and siRNA-AMPKα1 transfected tendon fibroblasts under stimulation by TGF-β1 and HGF. **b–e** The mRNA levels of α-SMA (**b**), Col I α1 (**b**), Col III (**d**), and fibronectin (**e**), four markers of myofibroblastic differentiation, were measure by real-time quantitative PCR analysis; β-actin was used as a housekeeping gene for reference. All data were normalized to β-actin expression ($2^{-\Delta\Delta Ct}$ methods). $N = 8$. $^*P < 0.05$

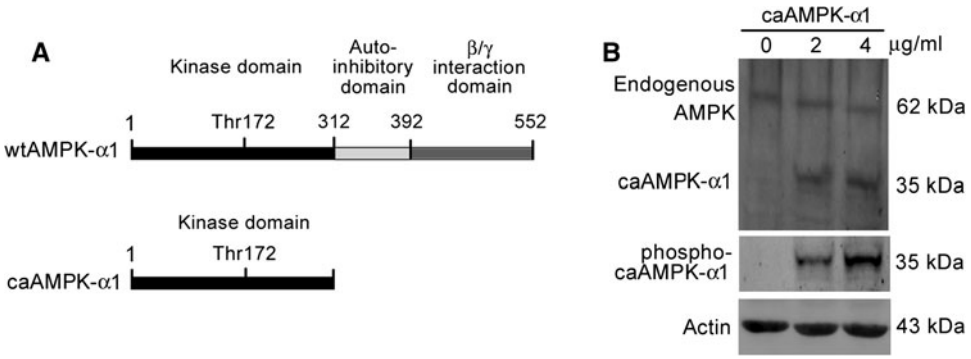

Fig. 4 Overexpression of constitutively active AMPKα1 plasmid (caAMPKα1). **a** Schematic diagram of the wild-type and constitutive active AMPKα1 protein. **b** Tendon fibroblasts cells were transfected with either caAMPKα1 (2 or 4 μg/ml) or control vector. Whole cell lysates were examined for AMPK, caAMPKα1, and phosphorylation of caAMPKα1 by immunoblotting. Anti-AMPK antibody having an N-terminal epitope detected exogenous caAMPKα1 (approximately 35 kDa) and endogenous wild-type AMPK (approximately 62 kDa). Actin was used as an internal loading control

AMPK signaling pathway in tendon fibroblasts. Second, either pharmacological or genetic inactivation of the AMPK signaling pathway greatly revoked the inhibitory effect of HGF on TGF-β1-induced myofibroblastic differentiation. Finally, constitutive activation of AMPK signaling using caAMPKα1 overexpression in tendon fibroblasts mimicked the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation.

HGF has a remarkable ability to block myofibroblast transition. Yang and Liu [30] demonstrated that HGF abrogated the α-SMA expression and E-cadherin depression triggered by TGF-β1 in renal tubular epithelial cells in a dose-dependent manner. Moreover, injections of exogenous HGF blocked myofibroblast activation and drastically prevented renal interstitial fibrosis in the obstructed kidneys [30]. HGF also blocked the IL-1α-induced myofibroblastic

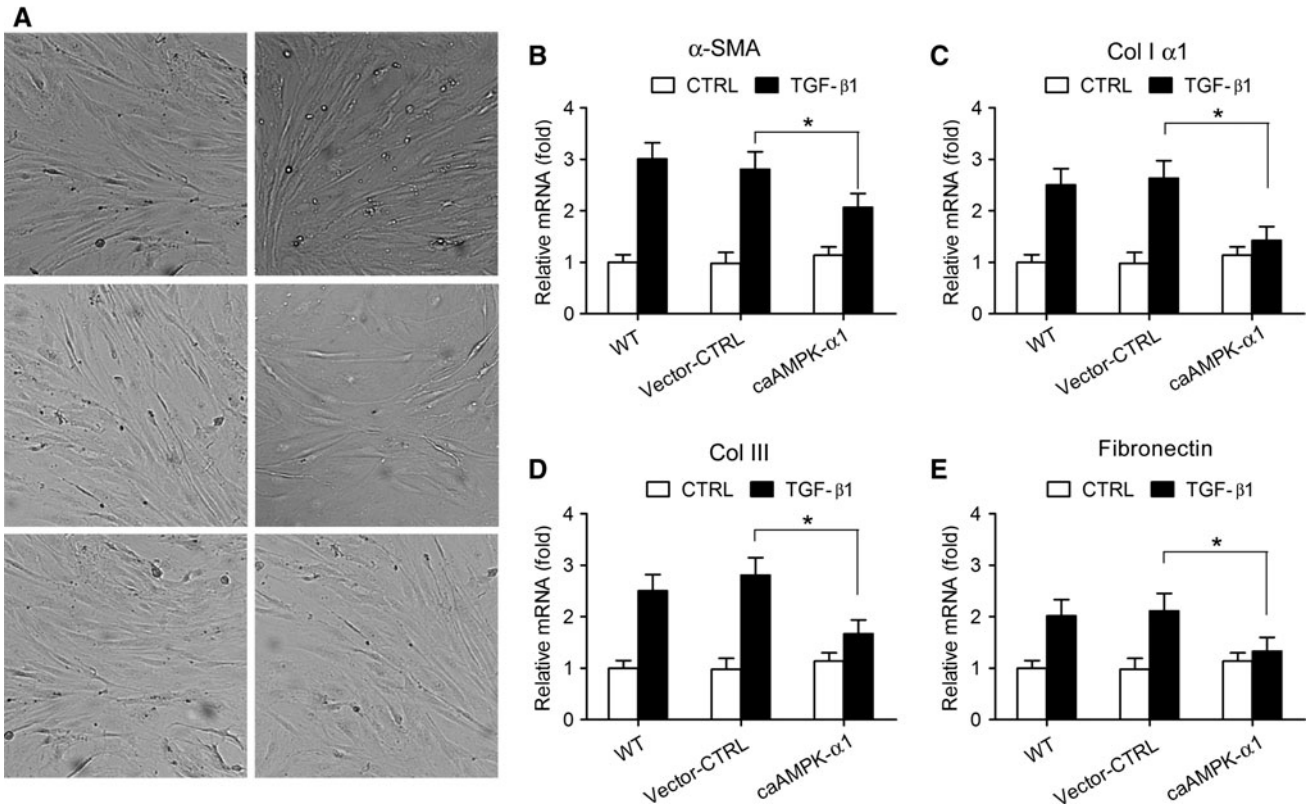

Fig. 5 Overexpression of constitutively active AMPKα1 plasmid (caAMPKα1) mimics the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. **a** Typical cell morphology of control, vector transfected, and ca-AMPKα1 transfected tendon fibroblasts under stimulation by TGF-β1. **b–e** The mRNA levels of α-SMA (**b**), Col I α1 (**c**), Col III (**d**), and fibronectin (**e**) were measure by real-time quantitative PCR analysis; β-actin was used as a housekeeping gene for reference. All data were normalized to β-actin expression ($2^{-\Delta\Delta Ct}$ methods). $N = 8$. $^*P < 0.05$

differentiation of renal tubular epithelial cells and inhibited the secretion of fibronectin from these cells [31]. In addition, HGF contributed to the inhibition of vitamin D on renal interstitial myofibroblast activation [32]. Besides renal cells, HGF was found to inhibit TGF-induced myofibroblastic differentiation in human retinal pigment epithelial cells [33], primary vocal fold fibroblasts [34], hepatic stellate cells [35], and human cardiac fibroblasts [36]. Our group also demonstrated the anti-fibrotic effect of HGF in the Achilles tendon [17]. Therefore, the inhibitory effect of HGF on fibrosis is well documented. However, the underlying mechanisms have not been fully elucidated. Kwiecinski et al. [35] provided an explanation for this inhibitory effect: upregulation of miRNA-29 by HGF takes part in the anti-fibrogenic effects of HGF. Apart from this, there was no other study on the detailed molecular mechanisms of HGF in TGF-β1-induced myofibroblastic differentiation.

AMPK is a heterotrimeric protein that is highly conserved from yeast to humans and functions as a key sensor of fuel and energy status [37]. The stimulation of the AMPK signaling pathway by AMP or nutrition shortage results in the repression of many anabolic processes (such as protein, fatty acid, and cholesterol synthesis) and activation of several catabolic processes (such as glucose/fatty acid uptake and oxidation) [37]. Thus, AMPK plays an important role in the adaptive response of cells to nutrient deprivation in vitro and to exercise- or ischemia-induced energy stresses in vivo. Recently, there were two reports on the relationship between myofibroblast differentiation and the AMPK signaling pathway. Cai et al. [38] found that adiponectin, an adipokine, inhibited lipopolysaccharide-induced adventitial fibroblast migration and transition to myofibroblasts via an AMPK-dependent manner. Conversely, Cieslik et al. [39] showed that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a chemical AMPK activator, induced TGF-β-dependent development of myofibroblasts but did not affect adipogenesis in cardiac fibroblasts from aged mice. In our study, we observed that HGF activated the AMPK signaling pathway. We also demonstrated that treatment with the chemical AMPK inhibitor compound C or knocking down of the AMPKα1 subunit using siRNA-mediated RNA interference abolished the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. Moreover,

overexpression of the constitutive activation of AMPKα1 mimicked the inhibitory effect of HGF on the TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts. Our data supported the notion that activation of the AMPK signaling pathway by HGF inhibited but did not promote TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts.

In summary, we demonstrated a critical role of AMPK in the inhibitory effect of HGF on TGF-β1-induced myofibroblastic differentiation in tendon fibroblasts, which may provide a promising therapeutic strategy for the treatment of fibrosis during tendon/ligament healing.

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Conflict of interest All the authors declared no conflict of interests.

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