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Original Research

Knockdown of eIF3a ameliorates cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway

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Abstract: Cardiac fibroblasts are key effector cells in the pathogenesis of cardiac fibrosis. The eukaryotic translation initiation factor (eIF) 3a is the largest subunit of the eIF3 complex and has been involved in renal fibrosis. However, the precise role of eIF3a in myofibroblast differentiation and cardiac fibrosis remains unknown. Accordingly, in our present study, we tested the expression of eIF3a in transforming growth factor β1 (TGF-β1)-induced rat CFs and found that eIF3a was upregulated in TGF-β1-induced rat CFs. Then the role and mechanism of eIF3a in cardiac fibrosis were explored. Our results found that the eIF3a expression was significantly up-regulated in TGF-β1-induced CFs. Knockdown of eIF3a significantly inhibited TGF-β1-induced CF proliferation, as well as suppressed the expression levels of α-smooth muscle actin (α-SMA) and SM22α. Mechanistically, knockdown of eIF3a attenuated TGF-β1-induced Smad3 activation in CFs. In summary, our present study firstly demonstrated that silencing eIF3a might alleviate TGF-β1-induced cardiac fibrogenesis in CFs by inhibiting Smad3 activation, and suggest that eIF3a may be positioned as a new and promising target for the prevention and treatment of cardiac fibrosis.

Key words: Eukaryotic translation initiation factor (eIF) 3a, cardiac fibroblasts (CFs), TGF-β1/Smad3 signaling pathway.

Introduction

Cardiac fibrosis plays a central role in the development of cardiac dysfunction in a variety of cardiovascular diseases, including myocardial infarction, cardiac hypertrophy, heart failure and severe arrhythmia (1). A critical event in the progression of cardiac fibrosis is the differentiation of cardiac fibroblasts (CFs) into myofibroblasts, characterized by accumulation of cardiac fibroblasts and excessive deposition of extracellular matrix (ECM) (2). Cardiac myofibroblast differentiation is induced by several pro-fibrotic factors, such as angiotensin II (Ang II) (3), endothelin-1 (4), platelet-derived growth factor (PDGF) (5), and transforming growth factor β (TGF-β) (6). Hence, intervention of TGF-β-induced myofibroblast differentiation might be an effective way to improve and cure cardiac fibrosis.

The eukaryotic translation initiation factor (eIF) 3a, a 170 kDa protein, is component of eIF3 (7). eIF3a can regulate cell cycle progression and proliferation, presumably by controlling the translation of mRNAs (8). Recently, mounting evidence suggests that eIF3a acts as a tumor suppressor in various solid tumors, including urinary bladder, breast and non-small cell lung tumors (9-11). Importantly, Zhang *et al*. reported that eIF3a was up-regulated in renal fibrotic tissues and TGF-β1-treated HK-2 cells and knockdown of eIF3a significantly inhibited TGF-β1-induced expression levels of α-smooth muscle actin (α-SMA) and collagen I in HK-2 cells (12). However, the precise role of eIF3a in myofibroblast differentiation and cardiac fibrosis remains unknown. Accordingly, in our present study, we tested the expression of eIF3a in TGF-β1-induced rat CFs and found that eIF3a was upregulated in TGF-β1-induced rat CFs. Then the role and mechanism of eIF3a in cardiac fibrosis were explored. We found that knockdown of eIF3a ameliorates cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway *in vitro*.

Materials and Methods

Cell culture

Primary cultures of CFs were prepared from 1 to 2-day old neonatal Sprague–Dawley rats as previously described (13). Subconfluent cells from passages 2–3 were used for the experiments, and they were serum deprived for 24 h. A high purity of cells was determined by positive staining for vimentin but negative staining for desmin and von Willebrand factor, markers of vascular smooth muscle cell (VSMC) and endothelial cells, respectively. All studies followed the guideline the Animal Care and Use Committee of Tianjin Medical University Metabolic Diseases Hospital (China).

Small interfering RNA (siRNA) transfections

For transfection, CFs were grown to 70% to 80% confluence. After serum deprivation, the medium was replaced with Opti-MEM (Invitrogen, Carlsbad, CA), and the cells were transfected with siRNA that targeted eIF3a or with non-targeting scrambled siRNA (GenePharma, Shanghai, China) using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The transfection efficiency was evaluated by eIF3a mRNA and protein expression using real-time PCR and Western blot analysis, respectively.

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CFs proliferation assay

Cell Counting Kit-8 (Dojindo, Shanghai, China) was used in CF proliferation assay. Briefly, CFs were seeded at a density of 5×10^4 cells/well into 24-well plates in a final volume of 100 mL and transfected with negative control siRNA or siRNA-eIF3a for 24 h. After the transfection, the cells were treated with TGF-β1 (10 ng/ml) for another 24 h. Then, cells were subjected to assay by adding 10 mL of Cell Counting Kit-8 solution to each well, and the plate was further incubated for 4 h at 37°C. The absorbance at 450 nm was measured with a microplate reader.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from CFs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then, 5 μg of total RNA was transcribed to first-strand cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real-time PCR amplification was performed using SYBR Green master mix (Toyobo, Japan) and real-time PCR (ABI). The following primers were used: eIF3a, 5'-TCAAGTCGCCGGACGATA-3' (sense), 5'-CCTGTCATCAGCACGTCTCCA-3' (antisense); α-SMA, 5'-CTATTCCTTCGTGACTACT-3' (sense), 5'-ATGCTGTTATAGGTGGTGGTT-3' (antisense); SM22α, 5'-TGGTGAACAGCCTGTACCCT-3' (sense), 5'-CACGGTAGTGCCCATCATTC-3' (antisense); and GAPDH 5'-GGCAAATTCAACGGCACAGTC-3' (sense), 5'-GCTGACAAT CTTGAGTGAGTT-3' (antisense). The protocol comprised 40 cycles of 94°C for 5 s, 59°C for 30 s, and 72°C for 1 min each. Relative amounts of mRNA were normalized by GAPDH and calculated using the delta-delta method from threshold cycle numbers.

Western blot

Total protein was extracted from CFs and protein concentrations were determined by BCA protein assay kit (Pierce). Thirty micrograms of protein extract was separated by SDS-PAGE gel and transferred onto nitrocellulose membranes (Millipore, Boston, MA, USA). After blocking with 10% fat-free milk in TBS (20 mmol/l Tris, 0.15 mol/L NaCl (pH 7.0), 0.1% Tween 20), the membranes were incubated at 4°C overnight with the following primary antibodies: anti-eIF3a (1:1000; Abcam, Cambridge, UK), anti-α-SMA (1:800; Abcam, Cambridge, UK), anti-SM22α (1:1000; Abcam, Cambridge, UK), anti-Smad3 (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-Smad3 (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). All membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) after washing in Tris Buffered Saline (TBS) with 5% Tween 20 three times. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Invitrogen, Carlsbad, CA, USA). BandScan 5.0 software was used for the quantification of all the proteins after western blot analysis.

Statistical analysis

Data depicted in graphs represent the means ± SEM. Differences between groups were determined using ANOVA tests with post hoc Bonferroni tests. $P<0.05$ is considered significant.

Results

Expression of eIF3a was upregulated in TGF-β1-induced CFs

We examined the effects of TGF-β1 on mRNA levels of eIF3a in CFs. As indicated in Figure 1A, TGF-β1 treatment significantly increased the mRNA levels of eIF3a. Moreover, Western blot analysis revealed that TGF-β1 also increased the protein expression of eIF3a in a time-dependent manner (Figure 1B), as compared with the untreated group.

Knockdown of eIF3a inhibits CF proliferation

To explore the potential role of eIF3a in cardiac fibrosis, we first knocked down eIF3a in CFs using siRNA. Real-time PCR and Western blot were performed to evaluate the transfection efficiency. As shown in Figure 2A and 2B, siRNA-eIF3a significantly decreased the mRNA and protein expressions of eIF3a by 74% and 86%, respectively, suggesting that the transfection was successful.

TGF-β1 has been shown to promote the proliferation of cultured CFs (14). We investigated the effect of eIF3a

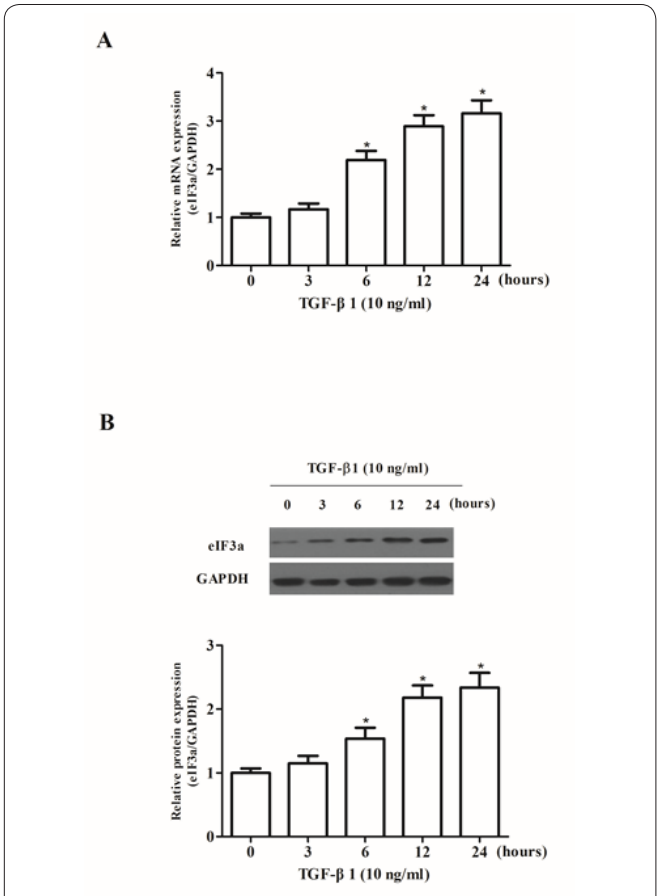

Figure 1. Expression of eIF3a was upregulated in TGF-β1-induced CFs. CFs were stimulated with 10 ng/ml TGF-β1 for different times. (A) qRT-PCR analysis of the mRNA expression of eIF3a. (B) Western blot analysis of eIF3a protein expression, with GAPDH as an internal control. Data represent means ± SEM (n=3). *$P<0.05$ vs. control group.

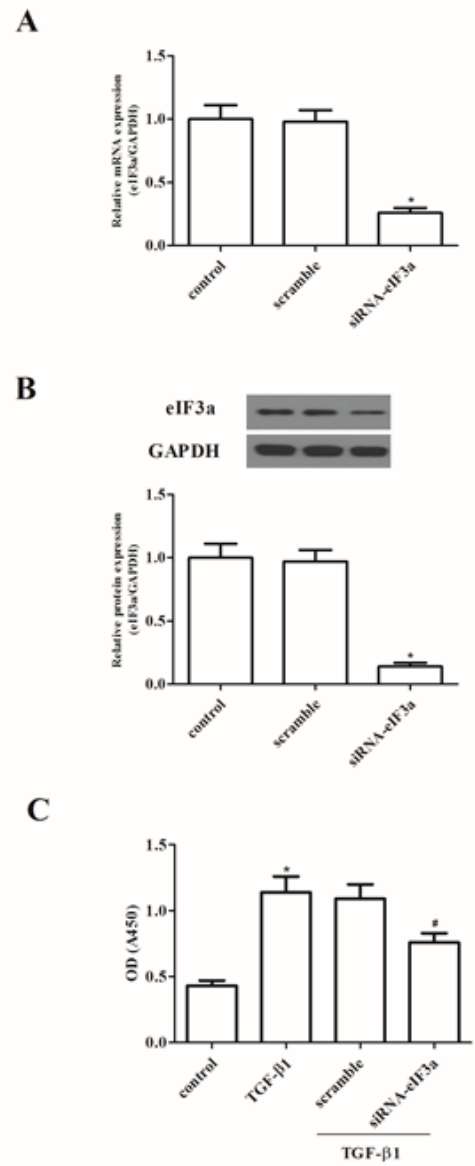

Figure 2. Knockdown of eIF3a inhibits CF proliferation. CFs were transfected with siRNA-eIF3a or scrambled siRNA for 24 h. (A) RT-qPCR analysis was performed to detect the mRNA level of eIF3a. (B) Western blot analysis was performed to detect the protein level of eIF3a. (C) CF proliferation was determined by CCK-8 assay. Data represent means ± SEM (n=3). *P<0.05 vs. control group, #P<0.05 vs. TGF-β1 group.

on CF proliferation under TGF-β1 condition by MTT. As shown in Figure 2C, TGF-β1 significantly increased the proliferation of CFs in a time dependent manner compared with the control group. However, knockdown of eIF3a obviously suppressed TGF-β1-induced CFs proliferation.

Knockdown of eIF3a inhibits CF differentiation into myofibroblasts

To determine whether eIF3a directly modulates myofibroblast differentiation, we examined the effect of eIF3a on TGF-β1-induced differentiation of CFs into myofibroblasts *in vitro*. As shown in Figure 3, compared with the control group, TGF-β1 significantly induced the expression of α-SMA mRNA and protein. In addition, knockdown of eIF3a inhibited the expression of α-SMA mRNA and protein induced by TGF-β1. Similarly, knockdown of eIF3a also suppressed the TGF-β1-induced the expression of SM22α.

Knockdown of eIF3a suppresses Smad3 phosphorylation in TGF-β1-induced CFs

Smad3 is a critical transcription factor that mediates cellular fibrotic response to TGF-β1 (15). Thus, we evaluated the effect of eIF3a on the phosphorylation of Smad3. As shown in Figure 4, TGF-β1 significantly elevated the phosphorylation of Smad3, which was prevented when CFs were pretreated with siRNA-eIF3a.

Discussion

In this study, we found that the eIF3a expression was significantly up-regulated in TGF-β1-induced CFs. Knockdown of eIF3a significantly inhibited TGF-β1-induced CF proliferation, as well as suppressed the expression levels of α-SMA and SM22α. Mechanistically, knockdown of eIF3a attenuated TGF-β1-induced Smad3 activation in CFs.

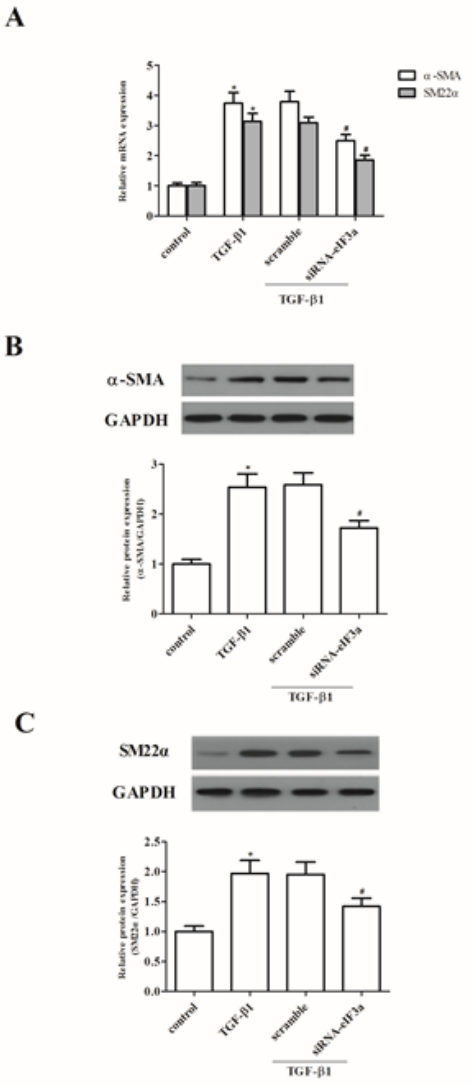

Figure 3. Knockdown of eIF3a inhibits CF differentiation into myofibroblasts. CFs were transfected with siRNA-eIF3a or scrambled siRNA and then stimulated with 10 ng/ml TGF-β1 for 24 h. (A) The mRNA expression of α-SMA and SM22α was determined by qRT-PCR analysis. (B, C) Western blot analysis was performed to detect the protein expression of α-SMA and SM22α. Data represent means ± SEM (n=3). *P<0.05 vs. control group, #P<0.05 vs. TGF-β1 group.

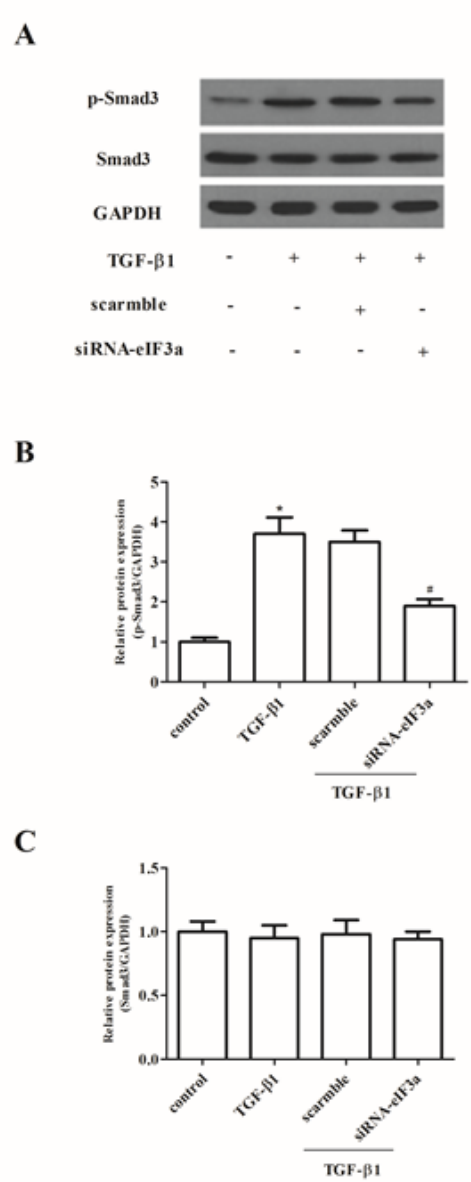

Figure 4. Knockdown of eIF3a suppresses Smad3 phosphorylation in TGF-β1-induced CFs. CFs were transfected with siRNA-eIF3a or scrambled siRNA and then stimulated with 10 ng/ml TGF-β1 for 1 h. (A) Western blot analysis was performed to detect the protein expression of p-Smad3 and Smad3. (B) Protein expression of p-Smad3 was analyzed using BandScan 5.0 software and normalized to GAPDH. (C) Protein expression of Smad3 was analyzed using BandScan 5.0 software and normalized to GAPDH. Data represent means ± SEM (n=3). *P<0.05 vs. control group, #P<0.05 vs. TGF-β1 group.

eIF3a is a multifunctional protein and plays an important role in regulation of fibrosis. Li *et al*. reported that the expression of eIF3a was obviously increased in lungs of pulmonary fibrosis rats, and exogenous TGF-β1 induced eIF3a expression and cell proliferation of in cultured pulmonary fibroblasts (16). Zhang *et al*. showed that eIF3a was up-regulated in renal fibrotic tissues and TGF-β1-treated human proximal tubular epithelial cells (12). These findings are consistent with our results. In our study, we found that eIF3a expression was significantly up-regulated in TGF-β1-induced CFs, and knockdown of eIF3a significantly inhibited TGF-β1-induced CF proliferation. These results suggest that increased eIF3a level might contribute to the pathological cardiac fibrosis.

Cardiac myofibroblasts produce excessive amounts of collagen and ECM proteins and have a contractile phenotype that is characterized by the presence of α-SMA stress fibers. Moreover, previous studies reported that TGF-β1, secreted by cardiac fibroblasts, can stimulate myofibroblast differentiation and excessive ECM production, which induce cardiac fibrosis progression (6, 17), and *in vivo* gene transfer of TGF-β1 can induce myocardial fibrosis (18). Our findings demonstrated that knockdown of eIF3a reversed the up-regulation of α-SMA and SM22α under TGF-β1 stimulation. This finding is in agreement with a previous report on rat pulmonary fibroblasts (16). These data suggest that siRNA-eIF3a exerts anti-fibrotic effect through inhibiting myofibroblast differentiation, and the subsequent ECM production.

The TGF-β/Smad signaling pathway plays an important role in the pathogenesis of cardiac fibrosis (19-21). Once activated by TGF-β1, Smad3 forms a complex with co-Smad, translocates to the nucleus, then recruits co-activators such as p300 and cyclic AMP (cAMP)-response element-binding protein (CREB)-binding protein (CBP), results in the synthesis and release of numbers of cytokine and chemotactic factors, which can induce the expression of α-SMA and other fibrosis-related genes, subsequently contribute to myofibroblast differentiation and cardiac fibrosis (22). Increasing evidences have reported that Smad3 activation is involved in the development of pathologic cardiac fibrosis (23-25). For example, Smad3−/− fibroblasts exhibited attenuated TGF-β1–induced upregulation of extracellular matrix protein synthesis and impact primarily fibrotic remodeling in cardiac injury (26). In this study, we found that knockdown of eIF3a suppressed Smad3 phosphorylation in TGF-β1-induced CFs. These results suggest that silencing eIF3a might alleviate TGF-β1-induced cardiac fibrogenesis in CFs by inhibiting Smad3 activation.

In summary, our present study firstly demonstrated that silencing eIF3a might alleviate TGF-β1-induced cardiac fibrogenesis in CFs by inhibiting Smad3 activation. Our works provide a novel evidence for understanding the possible roles of eIF3a in the development of cardiac fibrosis, and suggest that eIF3a may be positioned as a new and promising target for the prevention and treatment of cardiac fibrosis.

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