NLRC5 silencing ameliorates cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway

HONGTAO ZHOU[1], XUEFANG YU[2] and GUIMING ZHOU[3]

[1]Department of Ultrasound Room, Tianjin Medical University Metabolic Diseases Hospital, Tianjin 300070; Departments of [2]Cardiology and [3]Ultrasound Room, Tianjin Medical University General Hospital, Tianjin 300054, P.R. China

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Abstract. The proliferation of cardiac fibroblasts (CFs) and excessive deposition of extracellular matrix are the predominant pathological characteristics of cardiac fibrosis. As the largest member of the nucleotide-binding domain and leucine-rich repeat (NLR) family, NLRC5 has been shown to be pivotal in the development of hepatic fibrosis. However, whether NLRC5 is involved in the pathogenesis of cardiac fibrosis remains to be elucidated. The present study aimed to investigate the role of NLRC5 and its mechanisms in regulating cardiac fibrosis. CFs were stimulated with transforming growth factor (TGF)-β1 for various times and the mRNA and protein expression of NLRC5 was assessed using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. In addition, CFs were transfected with small interfering (si)RNA targeting NLRC5 or scramble siRNA for 24 h and then stimulated with TGF-β1 for 24 h. Subsequently, cell proliferation was measured using an MTT assay, whereas cell migration was evaluated using a Transwell migration assay. The protein expression levels of α-smooth muscle actin, collagen I, connective tissue growth factor, phosphorylated-Smad3 and Smad3 were measured using western blot analysis. The results demonstrated that NLRC5 was upregulated in TGF-β1-induced CFs. The knockdown of NLRC5 significantly inhibited cell proliferation and migration, and suppressed myofibroblast differentiation and the expression of pro-fibrotic molecules in TGF-β1-treated CFs. Furthermore, the knockdown of NLRC5 attenuated TGF-β1-induced phosphorylation of small mothers against decapentaplegic (Smad)3 in the CFs. The results of the present study indicated that NLRC5 acted as a key regulator of pathological cardiac fibrosis, and NLRC5 silencing ameliorated cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway. These results suggested that NLRC5 may be a novel target for attenuating cardiac fibrosis.

Correspondence to: Dr Guiming Zhou, Department of Ultrasound Room, Tianjin Medical University General Hospital, 154 Anshan Road, Tianjin 300054, P.R. China
E-mail: zguimphd@sina.com

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Introduction

Cardiac fibrosis is an important pathological feature of cardiac remodeling in heart diseases (1) and remains a major contributor to morbidity and mortality rates in a variety of cardiovascular diseases, including myocardial infarction, cardiac hypertrophy, heart failure and severe arrhythmia (2). Although significant therapeutic progress has been made in previous decades (3-5), the molecular mechanisms underlying the development of cardiac fibrosis remain to be elucidated. Cardiac fibroblasts (CFs), the most prevalent cell type in the heart, are key in the regulation of normal myocardial function. The proliferation of CFs and excessive deposition of extracellular matrix (ECM) are the primary pathological characteristics of cardiac fibrosis. It is also known that transforming growth factor-β (TGF-β) is pivotal in mediating CF function and cardiac fibrosis (6). CFs differentiate into cardiac myofibroblasts (CMFs) via TGF-β1, and these differentiated cells are actively involved in cardiac fibrosis.

Nucleotide-binding domain and leucine-rich repeat (NLR) proteins are important in innate immune responses as pattern-recognition receptors. NLRC5, the largest member of the NLR protein family, contains three structural domains, including the N-terminal atypical caspase activation and recruitment domain, the centrally located NACHT (named after neuronal apoptosis inhibitory protein, class II major histocompatibility complex transactivator, HET-E and transition protein 1 proteins) and 27 leucine-rich repeats at the C-terminal. Increasing evidence indicates that NLRC5 is important in regulating immune responses (7-9). For example, Staehli *et al* reported that NLRC5 is expressed at high levels and required for the regulating the expression of major histocompatibility complex I in lymphocytes (10). Another previous study showed that the knockdown of NLRC5 significantly suppressed TGF-β1-induced proliferation, but increased apoptosis, and inhibited the expression levels of collagen 1 and α-smooth muscle actin (α-SMA) in hepatic stellate cells (11). However, whether NLRC5 is involved in the pathogenesis of cardiac fibrosis remains to be elucidated. The aim of the present study was to examine the role of NLRC5 and its mechanisms in regulating cardiac fibrosis.

Materials and methods

Cell culture. A total of 6 female Sprague-Dawley rats (age, 6 weeks; weight, 180-200 g) were obtained from the Animal Breeding Center of Tianjin Medical University Metabolic Diseases Hospital (Tianjin, China). They were housed in barrier facilities under a 12-h light/dark cycle at a temperature of 22±2˚C and had free access to laboratory chow and tap water. Rats were used to harvest CFs. Briefly, rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). Heart ventricles were removed under sterile conditions, placed in cold sterile calcium-free PBS, minced into ~2-mm cubes, and treated with 1 mg/ml type II collagenase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin sulfate and 100 U/ml streptomycin (Sigma-Aldrich; Merck KGaA) at pH 7.4, in an incubator with a humidified atmosphere of 5% CO_2 at 37˚C. All experimental procedures were approved by the guidelines of the Animal Care and Use Committee of Tianjin Medical University Metabolic Diseases Hospital (Tianjin, China).

Small interfering RNA (siRNA) transfection. CFs at a density of $1x10^4$ cells/well were cultured to 80% confluence and transfected with small interfering (si)RNA (2.5 μg) targeting NLRC5 (forward, 5'-GGGACTGAG AGCTTTGTAT-3' and reverse, 5'-CGCACCCTAGACTG AAA-3') or with a non-targeting scrambled siRNA (forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACG UGACACGUUCGGAGAATT-3') at room temperature for 24 h, using Lipofectamine™ RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The siRNAs targeting rat NLRC5 and scrambled siRNA were from GenePharma (Shanghai, China).

Cell proliferation assay. An MTT assay was used to measure cell proliferation. Briefly, the CFs were seeded at a density of $1x10^4$ cells/well into 24-well plates and transfected with siRNA-NLRC5 or scramble siRNA for 24 h. The cells were then treated with TGF-β1 (10 ng/ml; Sigma-Aldrich; Merck KGaA) for another 24 h. Subsequently, 20 μl MTT (5 mg/ml) solution was added to each well and incubation continued at 37˚C for 4 h, followed by removal of the culture medium and the addition of 100 ml of dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). The absorbance at 450 nm was measured using an ELISA microplate reader (Invitrogen; Thermo Fisher Scientific, Inc.).

Transwell migration assay. Cell migration was analyzed using a Transwell chamber (Corning Costar, Cambridge, MA, USA) assay. Briefly, the CFs ($1x10^4$ cells/ml) transfected with siRNA-NLRC5 or scramble siRNA were resuspended in 0.1 ml serum-free DMEM and placed in the upper chambers. The lower chambers were filled with 500 μl DMEM containing 10% FBS as a chemoattractant. After 24 h incubation at 37˚C, the cells on the surface of upper chamber were removed by scraping with a cotton swab. The migrated cells on the lower surface of the filter were washed with TBS containing 0.1% Tween-20, fixed with 100% methanol at 37˚C for 15 min, stained with 0.1% hematoxylin and eosin at 37˚C for 20 min, and counted under an optical microscope (Olympus Corporation, Tokyo, Japan) in 5 randomly selected fields of view.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the CFs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was performed on cDNA using the SYBR green detection system (Bio SYBR-Green Master mix; Takara Bio, Inc., Otsu, Japan). The reaction mixture contained cDNA templates (1 μl), primers (2 μl of each forward and reverse primer) and SYBR-Green qPCR Master mix (5 μl). The specific primers were as follows: NLRC5 forward, 5'-CAGATGGTGGAAACTTTT AGCC-3' and reverse, 5'-AACTTCCTTAGCACCTGGATCA-3'; α-SMA forward, 5'-CTATTCCTTCGTGACTACT-3' and reverse, 5'-ATG CTGTTATAGGTGGTGGTT-3'; collagen I forward, 5'-TGG TGAACAGCCTGTACCCT-3' and reverse, 5'-CACGGT AGTGCCCATCATTC-3'; connective tissue growth factor (CTGF) forward, 5'-CAGGCTGGAGAAGCAGAGTCGT-3' and reverse, 5'-CTGGTGCAGCCAGAAAGCTCAA-3'; and β-actin forward, 5'-GAGGCACTCTTCCAGCCTTC-3' and reverse, 5'-GGATGTCCACGTCACACTTC-3'. The protocol comprised 35 cycles at 94˚C for 5 sec, at 59˚C for 30 sec, and at 72˚C for 1 min. The ratio of the relative expression of target genes to β-actin was calculated using the $2^{-\Delta\Delta Cq}$ method from the quantification cycle numbers (12).

Western blot analysis. The CFs were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) containing a phosphatase inhibitor and a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) on ice for 10 min. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein (40 μg/lane) were loaded and separated by 12% SDS-PAGE, and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Non-specific binding was blocked by incubation with 5% non-fat milk in PBS containing 0.1% Tween-20 at room temperature for 1 h. The membranes were then incubated with primary antibodies overnight at 4˚C, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2,500; cat. no. sc-516087; Santa Cruz Biotechnology Inc., Dallas, TX, USA) at room temperature for 1 h. The proteins were visualized using an enhanced chemiluminescence detection system (Invitrogen; Thermo Fisher Scientific, Inc.). The following antibodies were used: Goat anti-NLRC5 (1:3,000; cat. no. sc-248094; Santa Cruz Biotechnology Inc.), rabbit anti-α-SMA (1:3,000; cat. no. PA5-19465; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-collagen I (1:3,000; cat. no. sc-28657; Santa Cruz Biotechnology Inc.), rabbit anti-CTGF (1:2,500; cat. no. sc-25440; Santa Cruz Biotechnology Inc.), rabbit anti-Smad3 (1:2,500; cat. no. PA5-34774; Invitrogen; Thermo

Fisher Scientific, Inc.), rabbit anti-phosphorylated (p-)Smad3 (1:3,000; cat. no. 44-246G; Invitrogen; Thermo Fisher Scientific, Inc.) and rabbit anti-GAPDH (1:3,000; cat. no. sc-25778; Santa Cruz Biotechnology Inc.) antibodies. Densitometry was performed using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation of triplicate independent samples. Comparisons between two groups and among multiple groups were conducted using Student t-test and one-way analysis of variance followed by Tukey's post hoc test, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NLRC5 is upregulated in TGF-β1-induced CFs. The present study investigated the expression of NLRC5 in TGF-β1-induced CFs. As shown in Fig. 1A, compared with the untreated group, the mRNA expression of NLRC5 was significantly increased by TGF-β1 in CFs and increased in a time-dependent manner. The western blot analysis demonstrated that the protein expression of NLRC5 was also increased when incubated with TGF-β1 (Fig. 1B).

Silencing NLRC5 inhibits cell proliferation and migration induced by TGF-β1 in CFs. To characterize the biological effect of NLRC5 on cell proliferation and migration in CFs, NLRC5 was first knocked down in the CFs using siRNA. The decreased expression levels of NLRC5 were confirmed using RT-qPCR and western blot analyses. The results demonstrated that the downregulation of NLRC5 significantly decreased the mRNA and protein expression levels of NLRC5, respectively (Fig. 2A and B).

The present study then examined the effect of NLRC5 on cell proliferation and migration in CFs induced by TGF-β1. The results of the MTT assays showed that TGF-β1 significantly increased the proliferation of CFs, compared with the control group. However, silencing NLRC5 markedly inhibited TGF-β1-induced CF proliferation (Fig. 2C). Similarly, it was found that silencing NLRC5 markedly inhibited TGF-β1-induced CF migration (Fig. 2D).

Silencing NLRC5 inhibits the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in CFs. As the expression of α-SMA is a hallmark of myofibroblast differentiation, the effects of NLRC5 on TGF-β1-induced mRNA and protein levels of α-SMA were measured using RT-qPCR and western blot analyses, respectively. As shown in Fig. 3, compared with the control group, TGF-β1 treatment markedly induced the expression of α-SMA at the mRNA and protein levels. However, silencing NLRC5 inhibited the TGF-β1-induced expression of α-SMA at the mRNA and protein levels. Similarly, silencing NLRC5 suppressed the TGF-β1-induced expression levels of collagen I and CTGF.

Silencing NLRC5 attenuates TGF-β1-induced phosphorylation of Smad3 in CFs. It has been reported that activation of

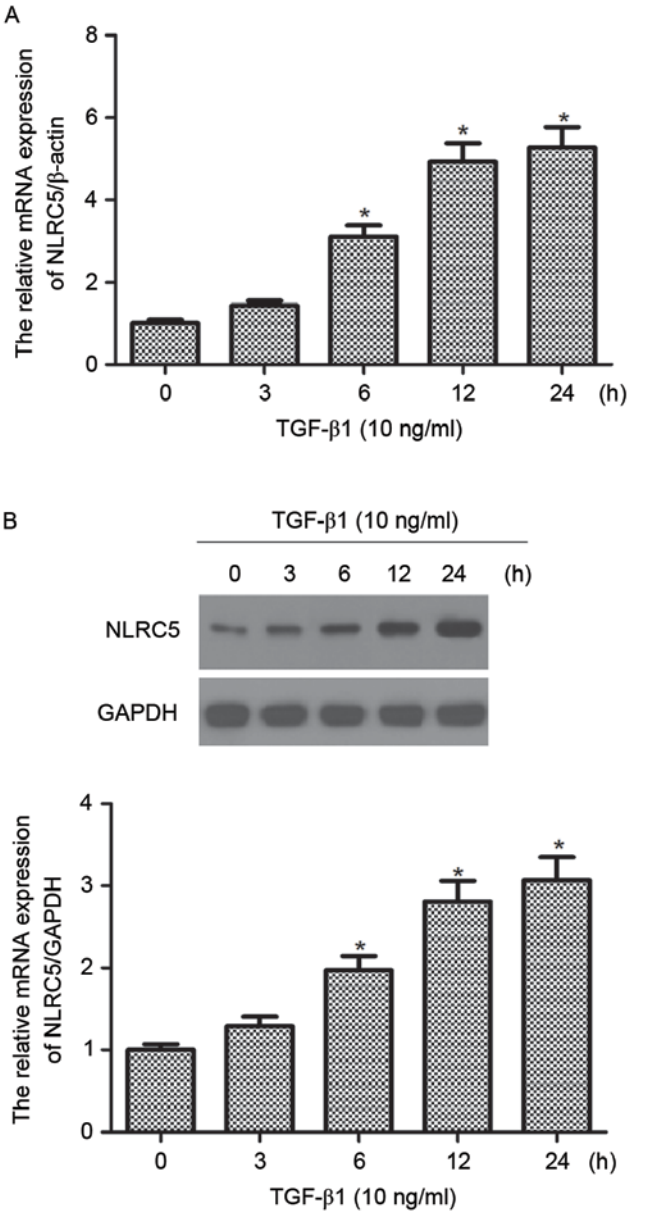

Figure 1. NLRC5 is upregulated in TGF-β1-induced CFs. CFs were seeded at a density of $1x10^4$ cells/well into 24-well plates and stimulated with 10 ng/ml TGF-β1 for 3, 6, 12 and 24 h. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA level of NLRC5. (B) Western blot analysis was performed to detect the protein level of NLRC5. GAPDH was used as the loading control. The results are expressed as the mean ± standard deviation of three independent experiments. *$P<0.05$, vs. control group. CFs, cardiac fibroblasts; TGF-β1, transforming growth factor-β1.

TGF-β1/Smad3 signaling is important in the development and progression of cardiac fibrosis (13). Therefore, the present study examined the effect of siRNA-NLRC5 on TGF-β1/Smad3 signaling in CFs. The results showed that TGF-β1 treatment increased the phosphorylation of Smad3 in the cultured rat CFs. However, silencing NLRC5 significantly inhibited the phosphorylation of Smad3 induced by TGF-β1 (Fig. 4).

Discussion

In the present study, it was demonstrated that NLRC5 was upregulated in TGF-β1-induced CFs. The knockdown of

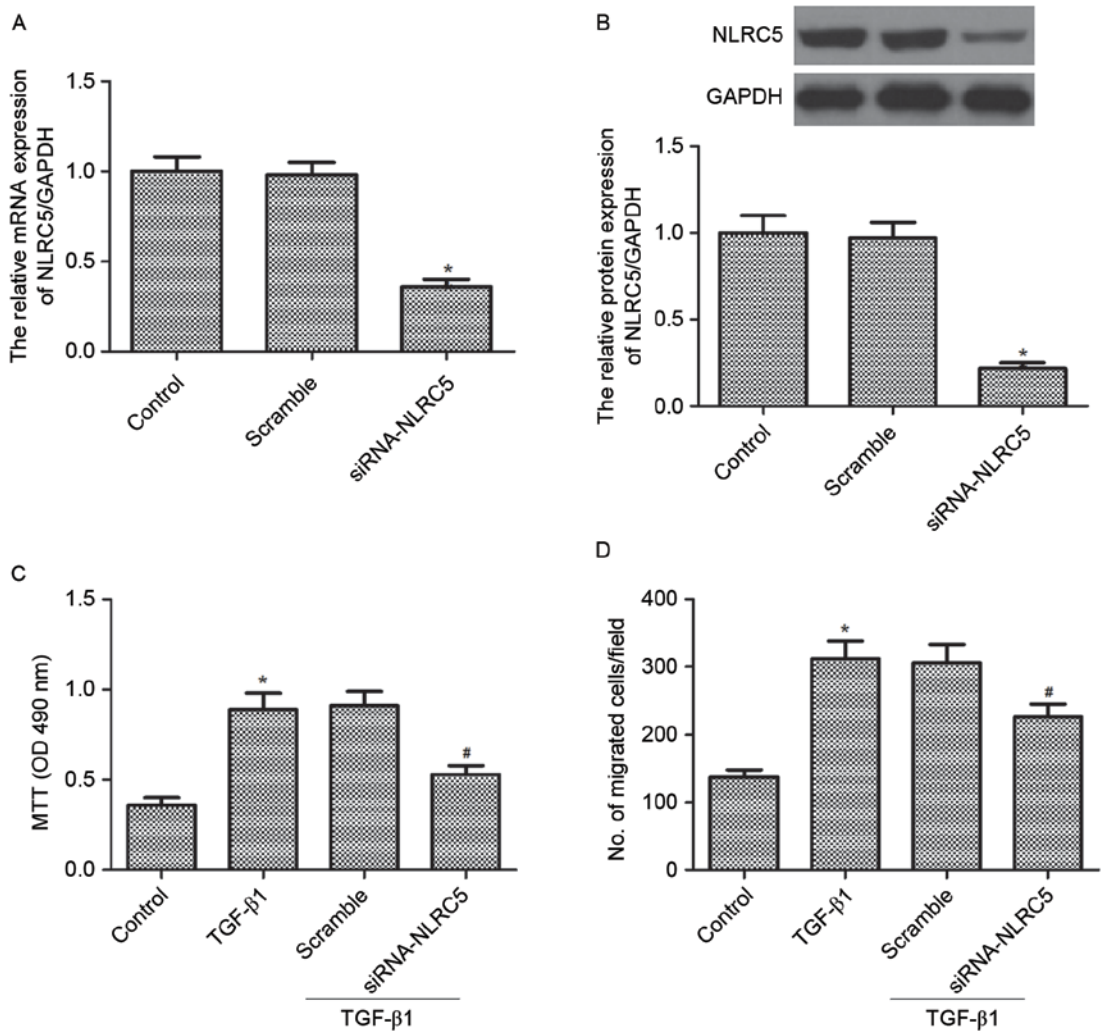

Figure 2. Silencing NLRC5 inhibits cell proliferation and migration induced by TGF-β1 in CFs. CFs were seeded at a density of $1x10^4$ cells/well into 24-well plates and transfected with siRNA-NLRC5 or scramble for 24 h. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA level of NLRC5. (B) Western blot analysis was performed to detect the protein level of NLRC5. CFs were transfected with siRNA-NLRC5 or scramble for 24 h, then stimulated with 10 ng/ml TGF-β1 for 24 h. (C) Cell proliferation was measured using an MTT assay. (D) Cell migration was measured using a Transwell chamber assay. The results are expressed as the mean ± standard deviation of three independent experiments. *P<0.05, vs. control group; #P<0.05, vs. scramble siRNA+TGF-β1 group. CFs, cardiac fibroblasts; siRNA, small interfering RNA; TGF-β1, transforming growth factor-β1; OD, optical density.

NLRC5 inhibited cell proliferation and migration, it also suppressed myofibroblast differentiation and the expression of pro-fibrotic molecules in the TGF-β1-treated CFs. Furthermore, the knockdown of NLRC5 attenuated the TGF-β1-induced phosphorylation of Smad3 in the CFs.

NLRC5 was previously shown to be a critical modulator in liver fibrogenesis, in which NLRC5 was significantly upregulated in human liver fibrotic tissues (11). Consistent with the results of this previous study, the present study observed that NLRC5 was upregulated in TGF-β1-induced CFs, indicating that NLRC5 might be in the development of cardiac fibrosis.

The proliferation of CFs is the primary pathological characteristic of cardiac fibrosis (14). It has been reported that myofibroblasts originate from resident fibroblasts, and invade and repair injured tissues by secreting and organizing the ECM (15). In the present study, it was found that the knockdown of NLRC5 inhibited cell proliferation and migration. These results suggested that siRNA-NLRC5 exerted an anti-fibrotic effect through inhibiting the proliferation and migration of CF.

The differentiation and activation of fibroblasts into myofibroblasts, which express α-SMA, are essential in cardiac fibrosis (16). Excessive collagen deposition in the heart contributes to cardiac fibrosis (17). CTGF, a crucial pro-fibrotic factor, also contributes to myofibroblast differentiation and activation, and is a marker for activated fibroblasts in cardiac fibrosis (18). Previous studies have shown that TGF-β1 can stimulate collagen synthesis and inhibit the degradation of collagen (19,20). In the present study, it was found that TGF-β1 treatment induced the expression levels of α-SMA, collagen I and CTGF. However, silencing NLRC5 inhibited the expression of pro-fibrotic molecules in the TGF-β1-treated CFs. These results suggested that siRNA-NLRC5 exerted an anti-fibrotic effect through inhibiting myofibroblast differentiation and the expression of ECM in CFs.

Previous evidence indicates that the TGF-β1/Smad signaling pathway is crucial in the myocardial remodeling process, particularly in cardiac fibrosis (21-24). As a primary downstream signal transducer of TGF-β1, Smad3 can be

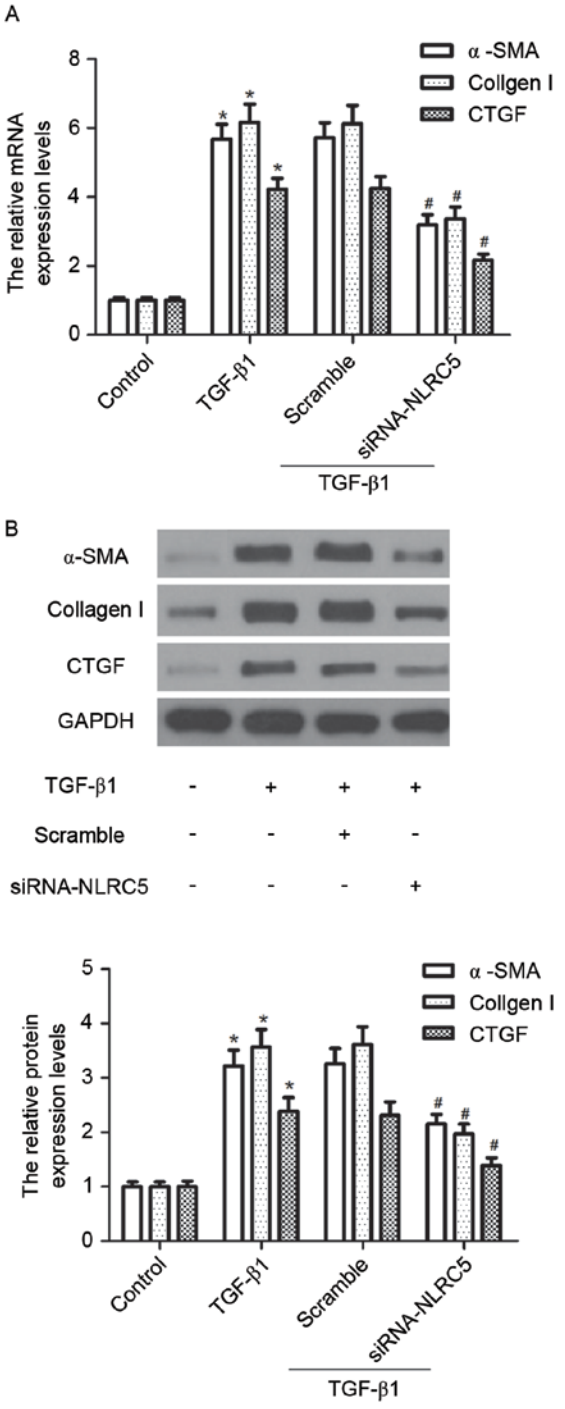

Figure 3. Silencing NLRC5 inhibits the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in CFs. CFs were seeded at a density of $1x10^4$ cells/well into 24-well plates and transfected with siRNA-NLRC5 or scramble for 24 h, then stimulated with 10 ng/ml TGF-β1 for 24 h. (A) mRNA expression levels of α-SMA, collagen I and CTGF were detected using reverse transcription-quantitative polymerase chain reaction analysis; (B) protein expression levels of α-SMA, collagen I and CTGF were measured using western blot analysis. Relative quantitative analyses of protein levels of α-SMA, collagen I and CTGF were normalized to GAPDH. The results are expressed as the mean ± standard deviation of three independent experiments. *P<0.05, vs. control group; #P<0.05, vs. scramble siRNA+TGF-β1 group. CFs, cardiac fibroblasts; siRNA, small interfering RNA; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor.

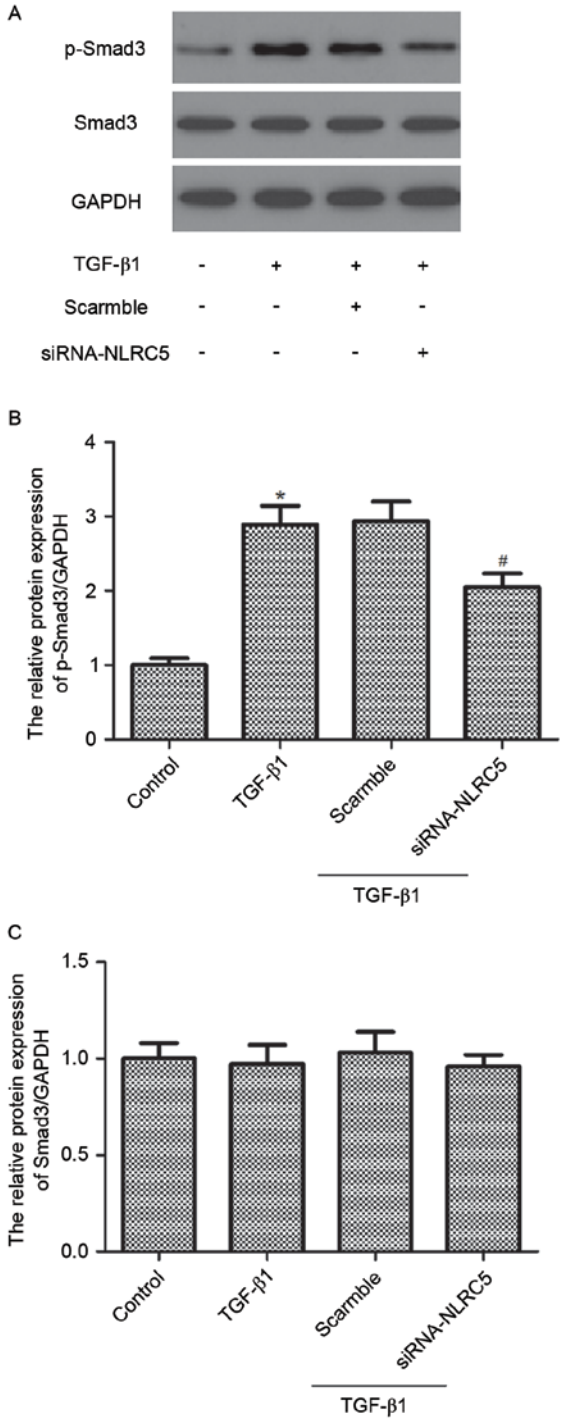

Figure 4. Silencing NLRC5 attenuates TGF-β1-induced phosphorylation of Smad3 in CFs. CFs were seeded at a density of $1x10^4$ cells/well into 24-well plates and transfected with siRNA-NLRC5 or scramble for 24 h, then stimulated with 10 ng/ml TGF-β1 for 1 h. (A) The protein expression levels of p-Smad3 and Smad3 were measured using western blot analysis. Relative quantitative analyses of protein levels of (B) p-Smad3 and (C) Smad3 were normalized to GAPDH. The results are expressed as the mean ± standard deviation of three independent experiments. *P<0.05, vs. control group; #P<0.05, vs. scramble siRNA+TGF-β1 group. CFs, cardiac fibroblasts; TGF-β1, transforming growth factor-β1; siRNA, small interfering RNA. Smad3, small mothers against decapentaplegic; p-Smad3, phosphorylated Smad3.

phosphorylated by the activated type I receptor of TGF-β1, followed by the formation of a complex with Smad4 and translocation into the nucleus, where it acts as a transcription factor and regulates the expression of target genes, including type I, type III collagen, α-SMA and CTGF (25,26). It has been shown in several experiments that TGF-β1 activates cardiac

fibrosis, predominantly through the TGF-β1/Smad signaling pathway. Bujak *et al* confirmed that the TGF-β1-mediated induction of procollagen type III and tenascin-C in isolated CFs is dependent on Smad3 (26). Another previous study reported that Smad3 null fibroblasts showed impaired myofibroblast transdifferentiation, reduced migratory potential and reduced capacity to contract collagen pads upon TGF-β1 stimulation (27). In the present study, it was found that the knockdown of NLRC5 attenuated the TGF-β1-induced phosphorylation of Smad3 in CFs. These results suggested that NLRC5 silencing ameliorated cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway in the rat CFs.

The results of the present study indicated that NLRC5 acted as a key regulator of pathological cardiac fibrosis, and that NLRC5 silencing ameliorated cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway. These results suggested that NLRC5 may be a novel target for attenuating cardiac fibrosis.

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