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Original Article

NLRC5 silencing improves cardiac fibrosis by regulation of TGF-β1/Smad3 signaling pathway

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Abstract: Myocardial fibrosis is one kind of cells calcification diseases caused by continuity and repeatability myocardial anti-ischemia and anti-anoxia in progression of coronary arteriosclerosis, which can lead to chronic ischemic heart disease. Evidences have the proliferation of cardiac fibroblasts and excessive deposition of extracellular matrix (ECM) is the main pathological characteristics of cardiac fibrosis. Previous study has indicated that Nucleotide-binding oligomerization-like receptor family caspase recruitment domain-containing 5 (NLRC5) has been reported to be associated with the pathological processes of fibrosis. The purpose of this study was to analyze the pathology function NLRC5 in the development of myocardial fibrosis and investigate the potential molecular mechanism of NLRC5-induced signaling pathway in myocardial cells *in vitro*. We also studied the NLRC5 gene expression in cardiac cells with fibrosis and normal cardiac cells *in vitro*. We also explored the role of NLRC5 and its relationship between NLRC5 and TGF-β1/Smad3 signaling pathway in the progression of cardiac fibrosis *in vitro*. RT-qPCR, western blot, small interfering RNA (siRNA) transfections and immunohistochemistry were used to analyze the role of NLRC5 in the progression of cardiac fibrosis. *In vivo* results showed that NLRC5 was up-regulated in myocardial fibrosis mice. *In vitro* results demonstrated that transforming growth factor beta 1 (TGF-β1)-induced cardiac fibroblasts. In addition, *in vitro* results showed that NLRC5 knockdown markedly inhibited cell proliferation and migration of myocardial cells. NLRC5 knockdown also suppressed myofibroblast differentiation and expression of pro-fibrotic molecules in TGF-β1-treated cardiac fibroblasts. Furthermore, we found that knockdown of NLRC5 decreased TGF-β1-induced expression and phosphorylation of Smad3 in cardiac fibroblasts *in vitro*. Restoration of TGF-β1 can abolish the inhibitory effects of NLRC5 knockdown on expression and phosphorylation of Smad3 and proliferation and migration of myocardial cells. Taken together, these results indicate that NLRC5 silencing can ameliorate cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway, suggesting that NLRC5 might be a novel target for the treatment of cardiac fibrosis.

Keywords: Cardiac fibrosis, NLRC5, cardiac fibroblasts, TGF-β1/Smad3

Introduction

Cardiac fibrosis is pathological changes in cardiac fibroblasts that frequently caused by fibrous connective tissue inflammation, myocardial injury, myocardial ischemia or other damage of organ violation [1]. Cardiac fibrosis often occurs in the fibrous connective tissue caused by flexibility and calcification, which leads to heart the infringement in endocardial fibrosis and endocardial thickening in left ventricular [2, 3]. These damages seriously affect the normal function of the heart and greatly influence the efficiency of the heart, cardiac hypertrophy, congestive heart failure and even secondary to hardening of the arteries [4-6]. Currently, cardiac fibrosis is an important pathological feature of cardiac remodeling in heart diseases [7] and remains a major cause of morbidity and mortality in a variety of cardiovascular diseases, including myocardial infarction, cardiac hypertrophy, heart failure and severe arrhythmia [8]. Therefore, understanding the pathogenesis of cardiac fibrosis to avoid arrhythmia or heart failure is crucial for the treatment of cardiac fibrosis. Herein, we aimed to explore the role of NLRC5 and its molecular mechanisms in the progression of cardiac fibrosis.

NLRC5, as the largest member of nucleotide-binding domain and leucine-rich repeat (NLR) family, has been shown to play a pivotal role in the development of hepatic fibrosis [9, 10]. Xu et al have indicated that NLRC5 can regulate TGF-beta1-induced proliferation and activation of hepatic stellate cells during hepatic fibrosis [11]. In addition, NLRC5 has recently been identified as a critical regulator of immune responses through negatively regulating NF-kappaB that is associated with the development of hepatic fibrosis [12]. Furthermore, research also has indicated that NLRC5 plays essential role in cardiac fibroblasts proliferation and differentiation by regulation of different signaling pathways [13-15]. These reports suggest that NLRC5 plays important role in the progression of fibrosis. However, it remains unknown whether NLRC5 is involved in the pathogenesis of cardiac fibrosis.

Although significant therapeutic progresses of cardiac fibrosis have been investigated over the past decades, the molecular mechanisms underlying the development of cardiac fibrosis remain not well understand [16-18]. Cardiac fibroblast is one of the most prevalent cell types in the heart and plays a key role in regulating normal myocardial function. [19, 20]. Previous study has showed that cardiac fibroblast is associated with ischemia/reperfusion via regulation IGF-1 through both PI3K/Akt and MEK-ERK pathways [21]. Further, the proliferation of cardiac fibroblasts and excessive deposition of extracellular matrix (ECM) are the main pathological characteristics of cardiac fibrosis [22]. Moreover, it was known that transforming growth factor beta (TGF-β) plays a pivotal role in mediating cardiac fibroblast function and cardiac fibrosis [23]. Interestingly, cardiac fibroblasts are differentiated to cardiac fibroblasts (CMF) by TGF-β1, and these differentiated cells are actively involved in cardiac fibrosis [20].

In this study, we investigated the expression and potential roles of NLRC5 in cardiac fibrosis. We confirmed that NLRC5 is involved in the pathogenesis of cardiac fibrosis. We have explored the role of NLRC5 and its mechanisms in regulation of cardiac fibrosis. We aimed to explain the signal pathway by which knockdown of NLRC5 contributes to prevention of cardiac fibrosis through inhibition of TGF-β1/Smad3 pathway in cardiac fibroblasts.

Materials and methods

Ethics statement

Animals study was implemented legitimately according to the Guide for the Care and Use of Laboratory Animals of Anesthesiology of the First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine. All surgical operations and euthanasia were made to minimize suffering.

Cells culture

Cardiac fibroblasts were harvested from C57BL/6J mice and cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma, USA). Cells were cultured in a 5% CO_2 incubator with a humidified atmosphere at 37°C. Cells were treated with TGF-β1 (2 mg/ml, Sigma, USA) for 12 h at 37°C for further analysis.

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

Total RNA was extracted from cardiac fibroblasts by using Trizol reagent (Takara Biotechnology, Dalian, China). One microgram of total RNA was reverse-transcribed to complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The qRT-PCR reaction was performed with the SYBR green detection system (Bio SYBR Green Master Mix, Takara, Japan). All the forward and reverse primers were synthesized by Invitrogen. The ratio of the relative expression of target genes to β-actin was calculated by using the delta-delta method from threshold cycle numbers.

Small interfering RNA (siRNA) transfections

Cardiac fibroblasts were cultured to 80% confluence and transfected with siRNA that targeted NLRC5 (Si-NLRC5) or Si-vector using Lipofectamine™ RNAi MAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. siRNA targeting rat NLRC5 and scrambled siRNA were from GenePharma (Shanghai, China).

Cells migration assay

Cardiac fibroblasts were transfected with Si-vector or Si-NLRC5 to analyze the effects of NLRC5 on migration and invasion. For migration assay, cardiac fibroblasts were transfected

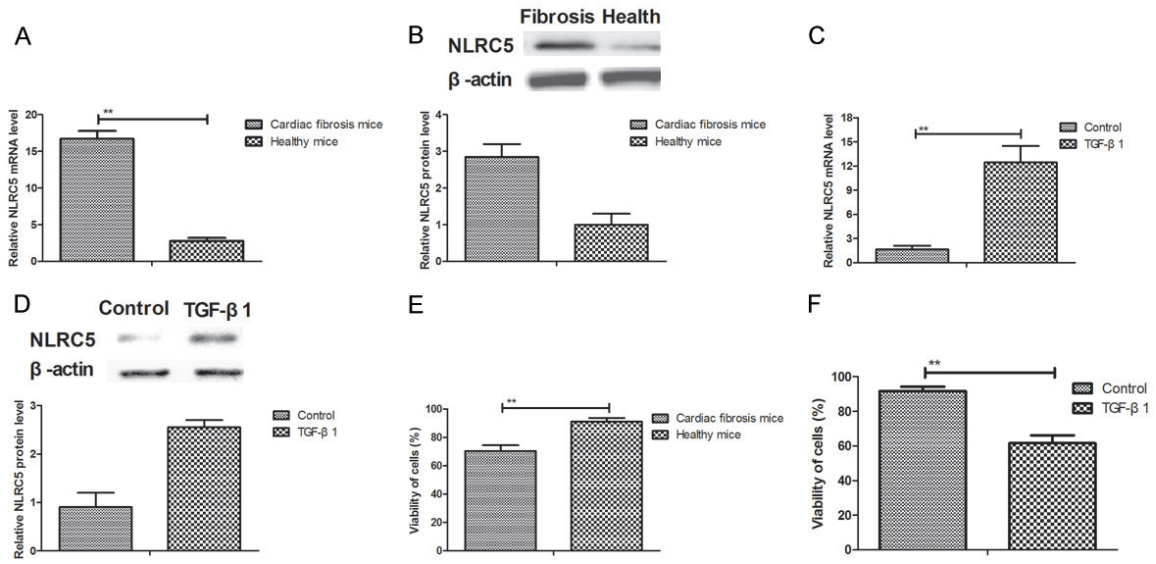

Figure 1. Analysis of NLRC5 expression in mice with cardiac fibrosis and TGF-β1-induced cardiac fibroblasts. (A, B) NLRC5 mRNA (A) and protein (B) expression levels in mice with cardiac fibrosis. (C, D) NLRC5 mRNA (C) and protein (D) expression levels were analyzed in TGF-β1-induced cardiac fibroblasts. (E) Viability of cardiac fibroblasts in mice with cardiac fibrosis. (F) Viability of cardiac fibroblasts in TGF-β1-induced cardiac fibroblasts. The results were expressed as mean and SD of three independent experiments. $^{**}P<0.01$ vs control group.

with Si-NLRC5 or Si-vector and incubated for 96 hours by using a control insert (BD Biosciences) instead of a Matrigel Migration Chamber. All procedures were performed according to the manufacturer's instructions. Migration and invasion of migration Cardiac fibroblasts were counted in at least three randomly stain-field microscope every membrane.

Cell proliferation assay

The MTT assay was used to measure cell proliferation. Briefly, CFs were seeded at a density of 1×10^4 cells/well into 24-well plates and transfected with Si-NLRC5 or Si-vector for 24 hours. The cells were treated with TGF-β1 (10 ng/ml) for another 24 hours. Subsequently, 20 μl of MTT (5 mg/ml) was added to each well and incubation continued at 37°C for 4 h, followed by removal of the culture medium and addition of 100 ml of dimethyl sulfoxide (Sigma, St. Louis, MO, USA). The absorbance at 450 nm was measured using an ELISA microplate reader (Invitrogen, Carlsbad, CA, USA).

Western blot

Cardiac fibroblasts were lysed in the RIPA buffer containing a phosphatase inhibitor and the protease inhibitor cocktail. Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, USA). Equalamounts of proteins (40 μg/lane) were loaded and separated by SDS-PAGE assay. The primary antibodies were used to incubate the primary antibodies for 120 minutes at 37°C. Then second antibodies were added to member after PBS for 60 minutes at 37°C. The results were visualized by using chemi-luminescence detection system.

Animal experiments

Six-eight female C57BL/6J (SPF) mice were purchased from Orient Bio Korea. All mice were free to access food and water, and housed with a 12 hours light-dark artificial cycle. TGF-β1 (10 mg/kg body weight) was used to induce cardiac fibrosis according to precious study [24].

Histological assay

Cardiac slices isolated from mice with cardiac fibrosis were prepared and fixed in 4% paraformaldehyde. Cardiac slices were conducted by an avidin-biotin-peroxidase technique. Paraffin-embedded tumor tissue sections were prepared and epitope retrieval was performed for further analysis. The paraffin sections were subjected with hydrogen peroxide (3%) for 10~15 minutes, which subsequently were blocked by a regular blocking solution for 10~15

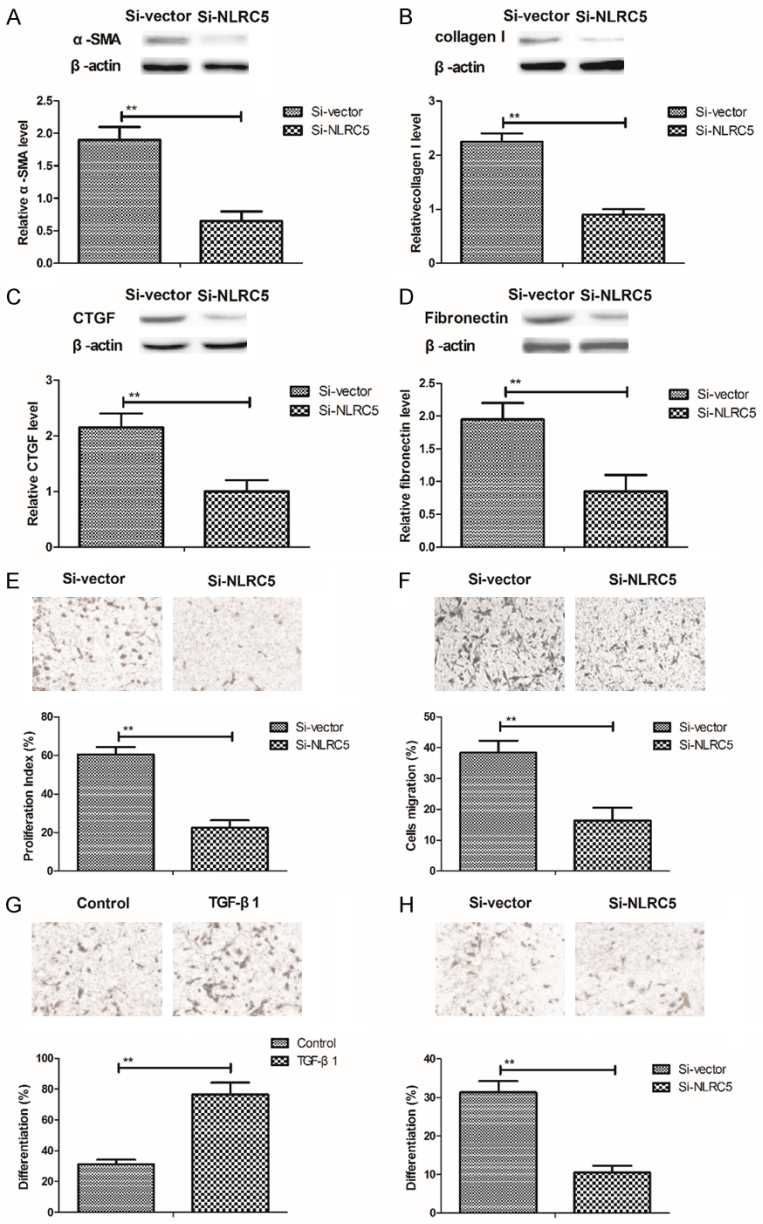

Figure 2. Effects of NLRC5 on proliferation, migration and differentiation induced by TGF-β1 in cardiac fibroblasts *in vitro*. (A, B) NLRC5 mRNA (A) and protein (B) expression levels in cardiac fibroblasts after treated by Si-NLRC5. (C, D) Proliferation (C) and migration (D) were analyzed in TGF-β1-induced cardiac fibroblasts. (E, F) Proliferation (E) and migration (F) of cardiac fibroblasts were analyzed after knockdown of NLRC5. Magnification, 40x. (G, H) Differentiation of cardiac fibroblasts was analyzed after treated by TGF-β1 (G) and knockdown of NLRC5 (H). Magnification, 40x. The results were expressed as mean and SD of three independent experiments. ***P*<0.01 vs control group.

minutes 37°C. Finally, the sections were incubated with goat anti-mouse anti-NLRC5, anti-Smad3, anti-TGF-β and anti-α-SMA, respectively, at 4°C for 12 hours after blocking. Sections were stained with the rabbit anti-goat secondary antibody after washed with PBS three times, respectively. Morphological images of cardiac fibrosis in experimental mice were shown Supplementary Figure 1.

Statistical analysis

Results are expressed as mean and standard deviation (SD). Comparisons between two groups and among multiple groups were conducted by Student t-test and one-way ANOVA, respectively. *P<0.05 and *P<0.01 is considered significant.

Results

NLRC5 expression is upregulated in TGF-β1-induced cardiac fibroblasts and mice with cardiac fibrosis

We first analyzed the expression of NLRC5 in TGF-β1-induced cardiac fibroblasts and mice with cardiac fibroblasts. As shown in **Figure 1A**, **1B**, results showed that NLRC5 mRNA and protein expression levels were up-regulated in mice with cardiac fibrosis *in vivo*. Previous study showed that TGF-β1 can induce cardiac fibroblasts by induction of angiotensin II [25]. Therefore, we detected NLRC5 expression levels in cardiac fibroblasts after induced by TGF-β1 *in vitro*. Our results showed that NLRC5 expression levels were significantly increased in TGF-β1-induced cardiac fibroblasts (**Figure 1C**, **1D**). Further, we observed that viability of cardiac fibroblasts was down-regulated in mice with cardiac fibrosis (**Figure 1E**). Moreover, TGF-β1 treatment also decreased the viability of cardiac fibroblasts (**Figure 1F**). Taken together, these results suggest that NLRC5 expression is up-regulated in cardiac fibroblasts in cardiac fibrosis and TGF-β1-induced cardiac fibroblasts.

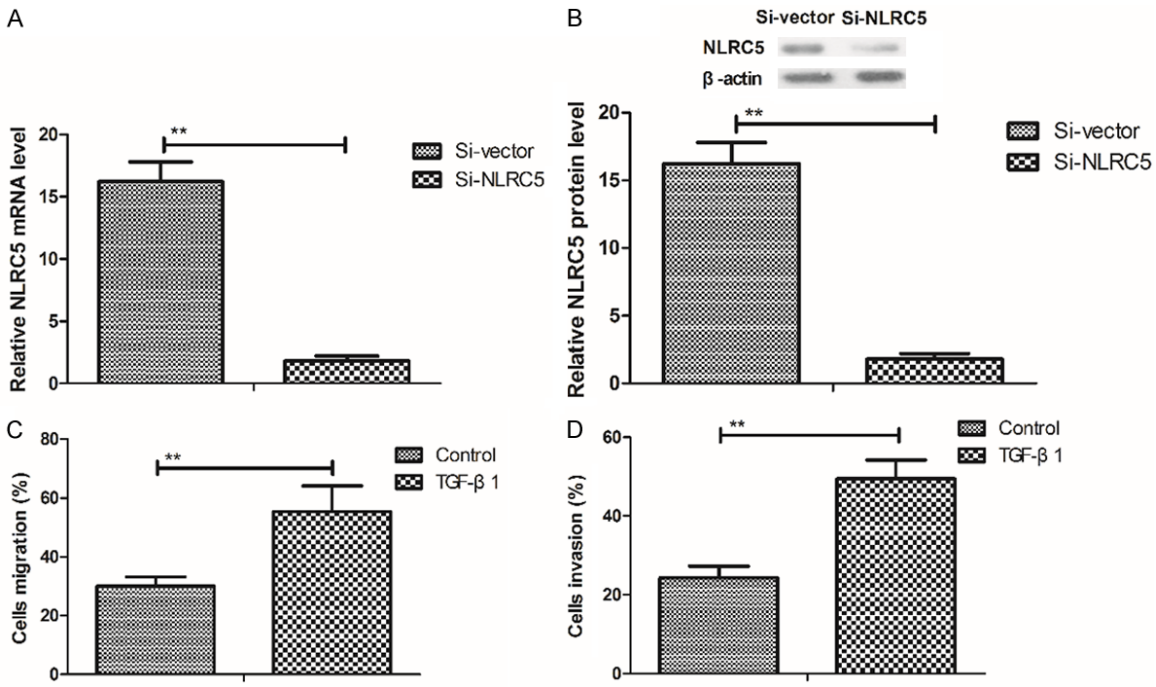

Figure 3. Effects of NLRC5 knockdown on α-SMA and pro-fibrotic molecules induced by TGF-β1 in cardiac fibroblasts *in vitro.* A. Protein level of α-SMA in NLRC5-knockdown cardiac fibroblasts. B. Protein level of collagen I in NLRC5-knockdown cardiac fibroblasts. C. Protein level of CTGF was analyzed in NLRC5-knockdown cardiac fibroblasts. D. Protein level of fibronectin was analyzed in NLRC5-knockdown cardiac fibroblasts. The results were expressed as mean and SD of three independent experiments. $**P<0.01$ vs control group.

Silencing NLRC5 inhibits cell proliferation, migration and differentiation induced by TGF-β1 in cardiac fibroblasts in vitro

To characterize the biological effect of NLRC5 on cardiac fibrosis, we analyzed proliferation, migration and differentiation in cardiac fibroblasts after treatment with TGF-β1 or knockdown of NLRC5 in cardiac fibroblasts by using siRNA. As shown in **Figure 2A**, **2B**, we found that knockdown of NLRC5 markedly decreased expression of NLRC5 determined by RT-PCR and Western blotting. In addition, we observed that TGF-β1 induced proliferation and migration of cardiac fibroblasts (**Figure 2C**, **2D**). However, knockdown of NLRC5 inhibited proliferation and migration of cardiac fibroblasts induced by TGF-β1 (**Figure 2E**, **2F**). Furthermore, we also found that TGF-β1 induced differentiation, while knockdown of NLRC5 inhibited xx (**Figure 2G**, **2H**). Taken together, these results suggest that NLRC5 knockdown can suppress proliferation, migration and differentiation in-duced by TGF-β1 in cardiac fibroblasts.

NLRC5 knockdown suppresses the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in cardiac fibroblasts in vitro

In order to evaluation the effects of NLRC5 on cardiac fibrosis, we next analyzed the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in cardiac fibroblasts. The results in **Figure 3A** showed that protein levels of α-SMA were down-regulated in NLRC5-knockdown cardiac fibroblasts, which is a hallmark of myofibroblast differentiation. In addition, we found that collagen I, CTGF and fibronectin expression levels were also down-regulated in cardiac fibroblasts after knockdown of NLRC5 (**Figure 3B**, **3D**). Taken together, these results suggest that NLRC5 knockdown can suppress the expression of α-SMA and pro-fibrotic molecules induced by TGF-β1 in cardiac fibroblasts.

Knockdown of NLRC5 attenuates myocardial fibrosis trough TGF-β/Smad3 signaling pathway in cardiac fibroblasts in vivo

In order to analyze the molecular mechanism of NLRC5-mediated myocardial fibrosis, we inves-

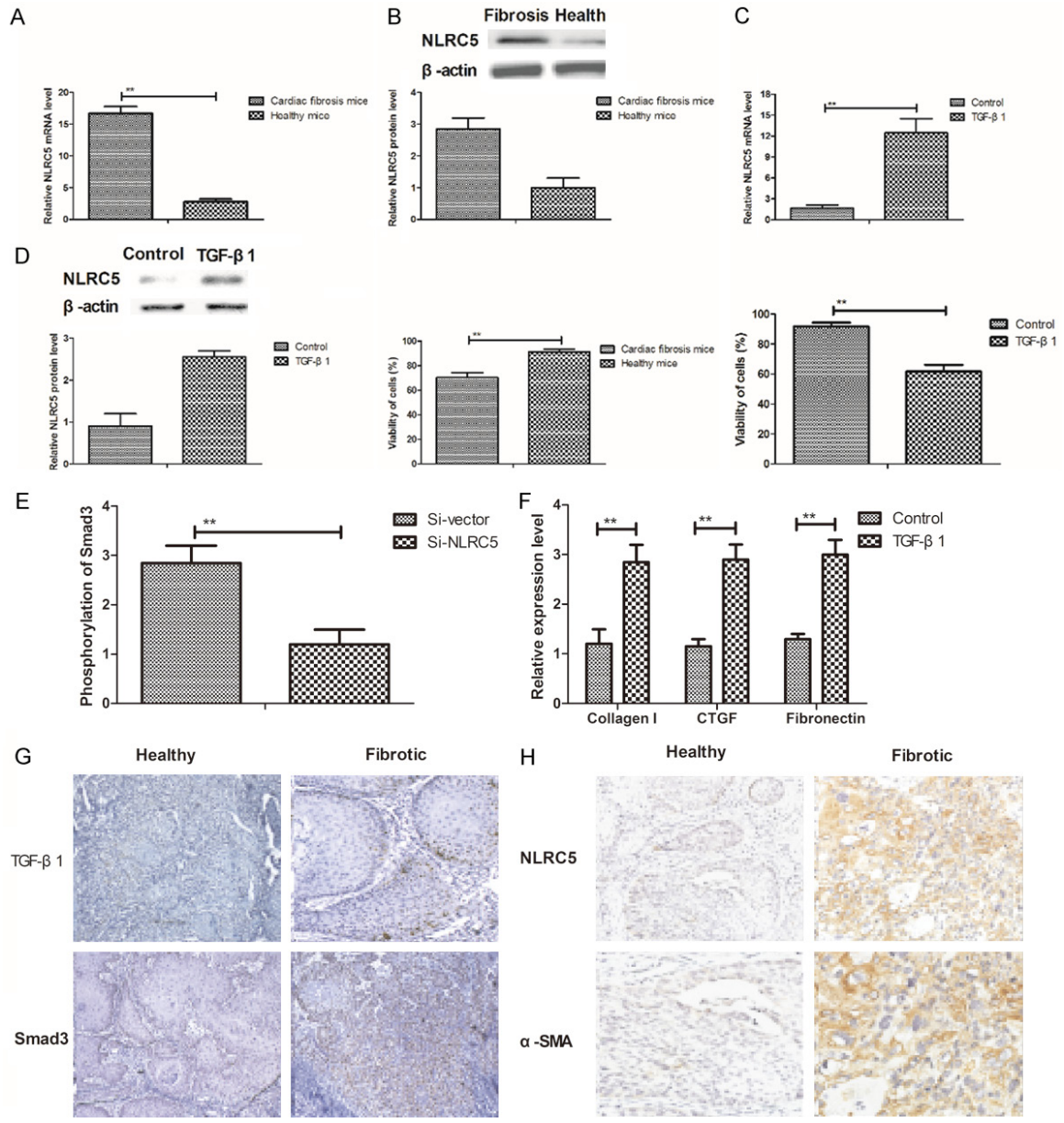

Figure 4. Silencing NLRC5 improves myocardial fibrosis trough TGF-β/Smad3 signaling pathway in cardiac fibroblasts *in vivo*. (A, B) Smad3 mRNA (A) and protein (B) levels were detected in cardiac fibroblasts induced by TGF-β1. Smad3 mRNA (C) and protein (D) levels were detected in cardiac fibroblasts after Knockdown of NLRC5. (E) Phosphorylation of Smad3 in cardiac fibroblasts after Knockdown of NLRC5. (F) Restoration of TGF-β1 abolished the effects of NLRC5 silencing on expression levels of pro-fibrotic molecules in cardiac fibroblasts. (G) Histological analysis of TGF-β1 and Smad3 expression levels in cardiac fibroblasts. Magnification, 40x. (H). NLRC5 and α-SMA expression levels in cardiac fibroblasts in mice with cardiac fibrosis. Magnification, 40x. The results were expressed as mean and SD of three independent experiments. $^{**}P<0.01$ vs control group.

tigated TGF-β/Smad3 signaling pathway in cardiac fibroblasts. As shown in **Figure 4A**, **4B**, our results demonstrated that mRNA and protein levels of Smad3 were up-regulated in cardiac fibroblasts induced by TGF-β1. Knockdown of NLRC5 inhibited mRNA and protein levels of Smad3 in cardiac fibroblasts induced by TGF-β1 (**Figure 4C**, **4D**). In addition, the similar results of phosphorylation of Smad3 were observed in cardiac fibroblasts (**Figure 4E**). We also found that restoration of TGF-β1 abolished the effects of NLRC5 silencing on expression levels of pro-fibrotic molecules in cardiac fibroblasts (**Figure 4F**). Histological analysis also

showed that TGF-β1 and Smad3 expression levels were significantly up-regulated in cardiac fibroblasts (**Figure 4G**). Further, we also found that NLRC5 and α-SMA expression levels were markedly increased in cardiac fibroblasts (**Figure 4H**). Taken together, these results suggest that Knockdown of NLRC5 attenuates myocardial fibrosis trough TGF-β/Smad3 signaling pathway in cardiac fibroblasts.

Discussion

Cardiac fibrosis is a pathological changes occurring in cardiac fibroblasts that frequently leads to myocardial injury, myocardial ischemia and other damage of organ violation [26]. Evidences have indicated that NLRC5 expression level is associated with the progression of cardiac fibrosis and exhibits an increasing regulation in patients with cellular fibrosis [11]. In addition, it has been reported that the activation of TGF-β1/Smad3 signaling play an important role in the development and progression of cellular fibrosis [27]. Furthermore, study indicates that NLRC5 may play a crucial role in regulating the reversal of hepatic fibrosis through NF-kappaB signaling pathway [12]. In this study, we investigated the role of NLRC5 and molecular mechanism of NLRC5-mediated signal pathway in cardiac fibroblasts in the progression of cardiac fibrosis. In the present study, we demonstrated that NLRC5 was up-regulated in TGF-β1-induced cardiac fibrosis. We found that knockdown of NLRC5 not only can inhibit cell proliferation and migration, but also suppress myofibroblast differentiation and expression of pro-fibrotic molecules in TGF-β1-incubated cardiac fibroblasts. In addition, results also indicate that knockdown of NLRC5 attenuates TGF-β1-induced Smad3 phosphorylation in CFs. Our outcomes have indicated that NLRC5 acts as a key regulator of pathological cardiac fibrosis, and NLRC5 silencing ameliorates cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway. These results suggested that NLRC5 might be a novel target for attenuating cardiac fibrosis.

Currently, nucleotide-binding domain and leucine-rich repeat (NLR) protein families act crucial roles in innate immune responses as pattern-recognition receptors. NLRC5 is one of the important member of the NLR protein family, contains three structural domains including the N-terminal atypical caspase activation and recruitment domain (CARD), the centrally located NACHT (named after NAIP, CIITA, HET-E, and TP-1 proteins) and 27 leucine-rich repeats (LRRs) at the C-terminal. A growing body of evidence indicates that NLRC5 plays important roles in regulating the immune responses [28-30]. Fanton *et al* have suggested that TLR- and NLR-independent signaling pathways involves in the processes of cardiac fibrosis and may prove useful to test future therapeutic strategies to cardiac fibrosis [31]. Further, Yilmaz et al also reported that NLR is a promising and inexpensive inflammation marker that correlates with histological grade and fibrosis stage in NASH patients [32]. Moreover, Staehli et al have reported that NLRC5 is abundantly expressed and required for the regulation of MHC I expression in lymphocytes [33]. These reports suggested that NLRC5 is a potential target for the treatment of cardiac fibrosis. Our results also showed that NLRC5 silencing inhibited TGF-β1-induced proliferation, migration and differentiation cardiac fibroblasts.

In recent year, study showed that 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 [34]. However, it remains unknown whether NLRC5 is involved in the pathogenesis of cardiac fibrosis. Herein, we aimed to explore the role of NLRC5 and its mechanisms in regulating cardiac fibrosis. NLRC5 is recently proven to be a critical modulator in liver fibrogenesis. In addition, NLRC5 was significantly up-regulated in human liver fibrotic tissues [34]. Consistent with the results of prior study, our results observed that NLRC5 was upregulated in TGF-β1-induced cardiac fibroblasts and mice suffered cardiac fibrosis, suggesting that NLRC5 may play crucial role in the initiation and progression of cardiac fibrosis. Furthermore, proliferation of cardiac fibroblasts is the main pathological characteristics of cardiac fibrosis [35]. It was reported that myofibroblasts originate fromresident fibroblasts, and invade and repair injured tissues by secreting and organizing the ECM [36]. Our results found that knockdown of NLRC5 can inhibit cell proliferation and migration. These results suggest that siRNA-NLRC5 exerts anti-fibrotic effect through inhibiting cardiac fibroblasts proliferation and migration.

Previously, differentiation and activation of fibroblasts into myofibroblasts which expressα-SMA are essential for cardiac fibrosis [37]. Excessive collagen deposition in the heart contributes to cardiac fibrosis [38]. CTGF, a crucial pro-fibroticfactor, also greatly contributes to myofibroblast differentiationand activation and it is a marker for activatedfibroblasts in cardiac fibrosis [39]. Previous studies have shown that TGF-β1 can stimulate collagen synthesis and inhibit the degradation of collagen [40, 41]. Our results have found that TGF-β1 treatment greatly induced the expression levels of α-SMA, collagen I and CTGF. However, silencing NLRC5 inhibited pro-fibrotic molecules in TGF-β1-treated cardiac fibroblasts.

In conclusion, we have found that NLRC5 is up-regulated in TGF-β1-treated cardiac fibroblasts and mice with cardiac fibrosis. All evidences in this study also have indicated that the TGF-β1/Smad signaling pathway plays crucial roles in the myocardial remodeling process. It has been shown that TGF-β1 can activate cardiac fibrosis predominantly through the TGF-β1/Smad signaling pathway. Although previous study has confirmed that TGF-β1-mediated induction of collagen type III and tenascin-C in isolated cardiac fibroblasts was dependent on Smad3, its pathological mechanism has not been investigated [42]. Also, another study reported Smad3 up-regulation can impair differentiation of cardiac fibroblasts by reducing migratory potential and capacity to contract collagen pads upon TGF-β1 stimulation [43]. Taken together, our results indicate that knockdown of NLRC5 can attenuate TGF-β1-induced Smad3 phosphorylation in cardiac fibroblasts, suggesting that NLRC5 may be a potential target for the treatment of cardiac fibrosis by inhibiting the TGF-β1/Smad3 signaling pathway in cardiac fibroblasts.

Disclosure of conflict of interest

None.

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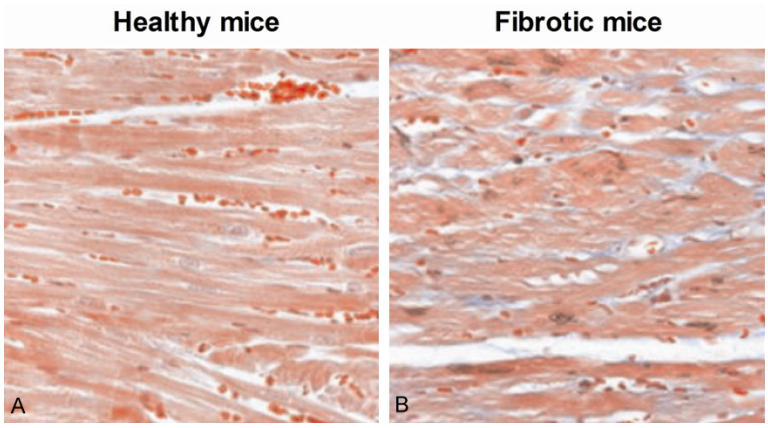

Supplementary Figure 1. Morphological evidence of cardiac fibrosis in experimental mice *in vivo.* (A) Cardiac fibrosis was accessed using histological sections stained by Masson's Trichrome. Presented are representative images (40x) depicting cardiac fibrosis from Healthy mice (A) and (B) fibrotic mice.