Tripartite Motif Protein 72 Regulates the Proliferation and Migration of Rat Cardiac Fibroblasts via the Transforming Growth Factor- β Signaling Pathway

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Key Words

Tripartite motif protein 72 · Cardiac fibroblasts · Proliferation · Migration

Abstract

Background: The proliferation and migration of cardiac fibroblasts are critical for the progress of cardiac fibrosis. Tripartite motif protein 72 (Trim72), also known as MG53, mediates the dynamic process of membrane fusion and exocytosis in striated muscle. However, the role of Trim72 in the proliferation and migration of cardiac fibroblasts is unknown. **Methods:** In the present study, we used small interference RNA (siRNA) to silence Trim72 and then investigated the effects of Trim72 on cardiac fibroblast proliferation and migration, which were activated during cardiac remodeling after myocardial infarction. Cardiac fibroblasts were isolated from 2- to 3-day-old neonatal Sprague-Dawley rats and transfected with siRNA. A cell-counting assay was used to determine the proliferation of cardiac fibroblasts. A Boyden chamber assay was performed to determine the migration of cardiac fibroblasts. **Results:** Our study has, for the first time, demonstrated that Trim72 regulates the cell proliferation and migration of rat cardiac fibroblasts. Furthermore, the data from the gene expression profile microarray analysis indicate that Trim72 depletion can cause downregulation of the transforming growth factor (TGF)- β signaling path-

way, suggesting that Trim72 regulates the proliferation and migration of cardiac fibroblasts probably via the TGF- β signaling pathway. **Conclusions:** We have demonstrated that Trim72 might play a pivotal role in the proliferation of neonatal rat cardiac fibroblasts, which could be a potential target for the treatment of cardiac fibrosis. However, the involvement of other signaling pathways and factors in the formation of cardiac fibrosis cannot be excluded.

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Introduction

The myocardium is mainly composed of the surrounding extracellular matrix (ECM), cardiomyocytes, and cardiac fibroblasts [1, 2]. Cardiac fibroblasts comprise two thirds of the nonmyocytes and have various functions in both physiological and pathological processes [3–5]. In response to chronic ischemia stimuli, cardiac fibroblasts proliferate, migrate, and excrete ECM to maintain myocardial function. However, if the repair phase is not terminated appropriately, excessive deposition of ECM will result in chronic fibrosis, ultimately leading to worse cardiac function and a poor prognosis [6–8]. During the development of cardiac remodeling after myocardial infarction, the proliferation and migration of cardiac fibroblasts play critical roles in heart failure.

Tripartite motif protein 72 (Trim72), also referred to as mitsugumin 53, is abundantly expressed in muscle [9–11]. Similarly to other Trim family members, Trim72 contains a Spry motif, i.e. a conserved RING, at the carboxyl terminus and a coiled coil motif at the NH2 terminus, which serve to distinguish Trims as specific isoforms. A previous study demonstrated that Trim72 functions as a critical component of the ‘cell membrane repair kit’ in these tissues [12–14]. In the experiment with Trim72 knockout mice, Trim72 depletion increased contractility tissue damage, which is reduced in older mice subjected to downhill running [12, 15, 16]. A recent study showed that recombinant Trim72 protein provides therapeutic benefits for skeletal muscle myo-necrosis following damage due to vigorous exercise [17]. Moreover, it has also been shown that Trim72 plays a cardioprotective role following ischemia-reperfusion injury [18]. However, the molecular mechanism of Trim72 has not yet been extensively studied in cardiac fibroblasts.

In order to investigate the role of Trim72 in the migration and proliferation of cardiac fibroblasts, we, in this study, knocked down Trim72 in rat cardiac fibroblasts to determine whether Trim72 depletion would affect cardiac fibroblast proliferation and migration. In addition, the underlying molecular mechanisms were explored.

Materials and Methods

Cardiac Fibroblast Isolation and Cell Culture

Cardiac fibroblasts were prepared from 2- to 3-day-old neonatal Sprague-Dawley rats as previously described [19, 20]. Briefly, an isolated heart was perfused using a Langendorff apparatus and treated with 0.02% collagenase for 20 min. The heart was minced in Dulbecco’s modified Eagle’s medium and cultured for 90 min in a 2% gelatin-coated culture dish. The floating cells were rinsed and the attached cells were used as cardiac fibroblasts. The cells were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin streptomycin Fungizone in a humidified incubator with 5% CO₂ at 37°C. Cardiac fibroblast cell passages 2–5 were used. These protocols were approved by the Animal Subject Committee of Chongqing Medical University.

Immunostaining Analysis

Cardiac fibroblasts were grown on glass coverslips, fixed with ice-cold 4% paraformaldehyde for 15 min, and blocked with phosphate-buffered saline (PBS) containing 10% fetal bovine serum for 30 min. Cells were incubated with anti-SMA in PBS at 4°C overnight, washed 3 times with PBS, incubated with Cy3-labeled secondary antibody (Beyotime, China) in PBS for 1 h at room temperature, and then analyzed using a confocal fluorescence microscope.

Cardiac Fibroblast Transfection

The sequences of the small interference RNA (siRNA) used to suppress Trim72 expression were as follows: Trim72-siRNA#1,

5′-GCAAGAUCUGGAGGCACATT-3′; Trim72-siRNA#2, 5′-AGGCACACGUGGAGGCCAATT-3′, and Trim72-siRNA#3, 5′-AACUGAGGCAG AUGGAGAATT-3′. The control siRNA sequence was: 5′-UUCUCCGAACGU GUAACGUTT-3′. One day before transfection, cardiac fibroblasts were placed in 500 µl growth medium without antibiotics such that they would be 60% confluent at the time of transfection. In order for each well to be transfected, siRNA duplex-Lipofectamine™ RNAiMAX complexes were prepared as per the following instructions: dilute 90 pmol siRNA duplex in 150 µl Opti-MEM® I Reduced Serum Medium without serum; mix Lipofectamine™ RNAiMAX gently before use, and then dilute 1 µl in 150 µl Opti-MEM® I Reduced Serum Medium; combine the diluted siRNA duplex with the diluted Lipofectamine™ RNAiMAX; mix gently and incubate for 20 min at room temperature; add the siRNA duplex-Lipofectamine™ RNAiMAX complexes to each well containing cells, and incubate the cells at 37°C in a CO₂ incubator for 24–48 h until assay for gene knockdown.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from cultured cardiac fibroblast cells using an EZNA Total RNA Kit (Omega Bio-Tek, USA), and cDNA was generated using a PrimeScript RT Reagent Kit with a gDNA Eraser (TaKaRa, Otsu, Japan). Quantitative real-time PCR was performed using SYBR Premix ExTaq II (TLiRNaseH Plus; TaKaRa) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, Calif., USA). Relative mRNA expression levels were calculated using the 2^{−ΔΔCt} method after normalization of the expression levels of Trim72 mRNA to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Western Blotting

Cardiac fibroblasts were lysed with RIPA buffer (Beyotime) and boiled for 5 min. The protein concentration of each lysate was measured using the BCA method (Beyotime). Equal quantities of protein from each cell lysate were separated on SDS-polyacrylamide electrophoresis gels and transferred to PVDF membranes (Millipore, Billerica, Mass., USA). The membranes were blocked with 5% skim milk, incubated with each primary antibody overnight at 4°C, washed with TBS-T buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), and incubated with secondary antibodies. The proteins were visualized using enhanced chemiluminescence (GE Healthcare Biosciences).

Microarray Assay

Cardiac fibroblasts transfected with Trim72-si or the control were collected and the total RNA was isolated using the TRIzol reagent and subjected to microarray analysis. The microarray experiments were performed at CapitalBio Corporation using Affymetrix Rat Transcriptome Assay 1.0. To verify the microarray findings, several differentially expressed genes were further analyzed by qRT-PCR and Western blot.

Migration Assay

Transwell Boyden chambers (24-well insert, 8.0-mm; BD Biosciences) were used to determine the migration of cardiac fibroblasts. After transfection, 1 × 10⁵ cells were seeded in the top chamber with the noncoated membranes. Cells were seeded in a serum-free medium and then migrated toward complete growth medium. The migrated cells were fixed with 70% methanol and stained with

Color version available online

Fig. 1. siRNA reduced the expression of Trim72 in rat cardiac fibroblasts. Activated fibroblasts were identified via immunofluorescence for SMA using a phase contrast microscope (**a**) and a fluorescence microscope (**b**). **c** The relative levels of Trim72 mRNA expression were determined by qRT-PCR, and GAPDH was used as the control. **d** The effects of siRNA on Trim72 protein expression were determined via immunoblotting. NC = Negative control. * $p < 0.01$ compared to the control.

0.5% crystal violet. Cells that migrated to the lower surface of the filters were counted in 5 random high-power fields. All experiments were carried out in triplicate.

Cell Proliferation Assay

A cell proliferation assay was performed via live cell counting using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) as described previously [21]. For the proliferation assay, the cells were grown and transfected with siRNA after 24 h.

Statistical Analysis

All data are presented as means $\pm$ SD. $p < 0.01$ was considered statistically significant. Numerical data were calculated using Graph-Pad Prism 5.0 and analyzed using SPSS 17.0 (SPSS Inc., Chicago, Ill., USA).

Results

Depletion of Trim72 in Rat Cardiac Fibroblasts

Cardiac fibroblasts were isolated from the hearts of 2- to 3-day-old Sprague-Dawley rats by proteolytic digestion as previously described. Isolated cardiac fibroblasts were identified as activated myofibroblasts via immunofluorescence. As shown in figure 1, almost all of the isolated cells showed positive immunoreactivity with antibodies to α -smooth muscle actin. To investigate the role of Trim72 in cardiac fibroblasts, we designed 3 siRNA targeting Trim72. After transfection with siRNA, the depletion effect of siRNA on Trim72 expression was exam-

ined using qRT-PCR and immunoblot analysis. The mRNA (fig. 1a) and protein expression of Trim72 was significantly inhibited compared to the cells transfected with control siRNA.

Depletion of Trim72 Suppressed the Proliferation and Migration of Rat Cardiac Fibroblasts

To investigate the impact of Trim72 depletion on cardiac fibroblast proliferation, CCK-8 assays were carried out. In CCK-8 assays, Trim72 depletion caused significant proliferation retardation in cardiac fibroblasts both at the early time point (24 h) and at the late time point (48 h), whereas control siRNA did not affect the proliferation of the cells (fig. 2a). The effect of Trim72 depletion on cardiac fibroblast migration was also determined via transwell migration assay. The transwell migration assay showed that Trim72 depletion also significantly inhibited the cellular transmigration ability compared to controls (fig. 2b, c). Taken together, these data demonstrated that Trim72 knockdown resulted in cell proliferation and migration of cardiac fibroblasts.

Microarray Analysis Identifies TGF- β

Pathway-Specific Signaling Downstream of Trim72

To gain further insight into the potential molecular mechanism underlying the Trim72 knockdown-induced suppression of the proliferation and migration of cardiac fibroblasts, we tested a gene expression profile microar-

Fig. 2. Silencing of Trim72 decreased the proliferation and migration of cardiac fibroblasts. **a** After transfection of the cardiac fibroblasts with siRNA for 24 h, a cell-counting assay was performed using the Cell Counting Kit-8. The normalized proliferation levels relative to the control siRNA are shown as means $\pm$ SD. * $p < 0.01$ compared to the control. **b** Cell migration was determined using a transwell chamber migration assay; 1×10^5 cells transfected with siRNA were added in the upper chamber. After 24 h, representative pictures showed migrated cardiac fibroblasts stained with crystal violet. **c** The migrated-cell numbers normalized relative to the control are shown as means $\pm$ SD. * $p < 0.01$ compared to the control. All of the data are from 3 independent experiments ($n = 3$). NC = Negative control.

ray assay to compare differential gene expression profiles between Trim72 depletion and negative control cardiac fibroblasts. Gene expression profiling revealed that 123 genes were upregulated (ratio >2.0) and 142 genes were downregulated (ratio, <0.5). Using the Kyoto Encyclopedia of Genes and Genomes, and Pathway Profiler analysis, the significant signaling pathways were categorized into different groups including the TGF- β signaling pathway and cell cycle regulation, among others. Important genes and pathways involved in this process are shown in figure 3a and b. QRT-PCR and Western blot demonstrated that Trim72 depletion caused the expression levels of TGF- β_1 and TGF β RI to decrease (fig. 3c, d). Overall, this data analysis showed that Trim72 regulates migration and proliferation via the TGF- β signaling pathway.

Discussion

Cardiovascular diseases are the leading cause of death worldwide [22]. In response to diverse stimuli, cardiac fibroblasts proliferate, migrate, and excrete ECM to maintain myocardial function. ECM metabolic imbalance is involved in various cardiovascular diseases such as myocardial infarction, heart failure, and atrial fibrillation, which may lead to cardiac remodeling [23–25]. Account-

ing for two thirds of the cells in human heart, cardiac fibroblasts are the most important cell type and have various functions in both ECM metabolism and cardiac fibroblast proliferation, which plays an important role in cardiac remodeling [26, 27]. Cardiac fibrosis is regulated by a multi-step and highly complex network of interacting genes and proteins.

It has been shown that in skeletal muscle cells breakage of the plasma membrane causes overexpression of Trim72 at the injury site, accompanied by a reduced extracellular dye penetration into the cell, suggesting the formation of a repair patch by Trim72 [28, 29]. The tripartite motif proteins are large and display diverse cell biological functions. Previous studies have shown that Trim protein function is related to several human diseases, including cancer and muscular dystrophy. To investigate the cell-molecular mechanisms of Trim proteins, studies have focused on the role of various protein motifs in mediating intracellular signaling events. Similar to other Trim family members, the molecular mechanism of Trim72 has not yet been extensively studied in cardiac fibroblasts [30, 31].

In the present study, we used siRNA to knock down Trim72 and then investigated the impact of Trim72 on cardiac fibroblast proliferation and migration. We found that Trim72 depletion significantly decreased the proliferation and migration of cardiac fibroblasts. Further-

Fig. 3. Microarray analysis and validation of Trim72 via the TGF- β signaling pathway. **a, b** Microarray-based gene expression profiling and important pathways affected by Trim72 depletion. **c, d** The expression levels of TGF- β_1 and TGF β RI were determined by qRT-PCR and immunoblotting in rat cardiac fibroblasts. * $p < 0.01$ compared to the control. NC = Negative control.

more, using gene expression profile microarray analysis, we have found that Trim72 depletion could result in downregulation of the TGF- β signaling pathway, suggesting that Trim72 regulates the proliferation and migration of cardiac fibroblasts through the TGF- β signaling pathway. Our study is consistent with previous studies that found that overexpression of myocardial TRIM72 could induce diabetic and other cardiomyopathies [29, 32–35]. However, other pathways such as angiotensin, endothelin, connective tissue growth factor (CCCN2), and platelet-derived growth factor may also be involved in the proliferation of cardiac fibroblasts. The Toll/IL-1 receptor domain-containing adaptor-inducing IFN- β (TRIF) may promote atrial fibrosis via microphages [36–38]. Rivaroxaban has been reported to inhibit AngII-stimulated cardiac fibrosis through nuclear factor- κ B and the mito-

gen-activated protein kinase pathway [39–41]. To the best of our knowledge, the present study has, for the first time, shown that Trim72 can regulate the migration of cardiac fibroblasts. Further clinical studies are needed to confirm this observation.

In summary, our study demonstrates that Trim72 might play a pivotal role in the proliferation of neonatal rat cardiac fibroblasts, which could be a potential target for the treatment of cardiac fibrosis. However, the involvement of other signaling pathways and factors in the formation of cardiac fibrosis cannot be excluded.

Conflict of Interest

There is no potential conflict of interests to disclose.

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