Original Paper

MiR-22 may Suppress Fibrogenesis by Targeting TGF β R I in Cardiac Fibroblasts

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Key Words

Myocardial infarction • Cardiac fibrosis • MicroRNA-22 • TGF β RI

Abstract

Background/Aims: Cardiac fibrosis after myocardial infarction (MI) has been identified as a key factor in the development of heart failure, but the mechanisms underlying cardiac fibrosis remained unknown. microRNAs (miRNAs) are novel mechanisms leading to fibrotic diseases, including cardiac fibrosis. Previous studies revealed that miR-22 might be a potential target. However, the roles and mechanisms of miR-22 in cardiac fibrosis remained ill defined. The present study thus addressed the impact of miR-22 in cardiac fibrosis. **Methods:** After seven days following coronary artery occlusion in mice, tissues used for histology were collected and processed for Masson's Trichrome staining. In addition, cardiac fibroblasts were transfected with mimics and inhibitors of miR-22 using Lipofectamin 2000, and luciferase activity was measured in cell lysates using a luciferase assay kit. Western blotting was used to detect the expression of collagen1, α -SMA and TGF β RI proteins levels, and real time-PCR was employed to measure the Col1 α 1, Col3 α 1, miR-22 and TGF β RI mRNA levels. **Results:** In this study, we found that miR-22 was dynamically downregulated following MI induced by permanent ligation of the left anterior descending coronary artery for 7 days, an effect paralleled by significant collagen deposition. Inhibition of miR-22 with AMO-22 resulted in increased expression of Col1 α 1, Col3 α 1 and fibrogenesis in cultured cardiac fibroblasts. Conversely, overexpression of miR-22 in cultured cardiac fibroblasts significantly abrogated angiotensin II-induced collagen formation and fibrogenesis. Furthermore, we found that TGF β RI is a direct target for miR-22, and downregulation of TGF β R may have mediated the antifibrotic effect of miR-22. **Conclusion:** Our data clearly demonstrate that miR-22 acts as a novel negative regulator of angiotensin II-induced cardiac fibrosis by suppressing the expression of TGF β RI in the heart and may represent a new potential therapeutic target for treating cardiac fibrosis.

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Introduction

Cardiac ischaemia leading to post-infarction heart failure particularly in patients with large myocardial infarction is associated with a high mortality. Excessive fibrosis leads to ventricular dilation, infarct expansion, and heart failure [1]. During cardiac remodeling, fibroblasts differentiate into myofibroblasts. These are fast-proliferating, α -smooth muscle cell actin (α -SMA)-positive cells with pronounced contractile and secretory properties [2]. Myofibroblasts can be induced by transforming growth factor (TGF)- β and lead to excessive matrix accumulation [3]. Therefore, elucidating the molecular mechanism of cardiac fibrosis is of great value for the clinical therapy of it. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level by either degradation or translational repression of a target mRNA [4]. Lots of studies have showed that miRNAs are involved in the process of kinds of diseases, especially in various fibrotic diseases, including cardiac fibrosis, idiopathic pulmonary fibrosis, liver fibrosis, and so on [5-7]. In the previous study, van Rooij et al. found that miR-22 was decreased in the border zone region of mice both 3 days and 14 days after MI [8]. And, a study from Guo et al. showed that miR-22 inhibited cell proliferation, migration, and invasion of osteosarcoma cells [9]. However, the role and mechanisms of miR-22 on cell proliferation of cardiac fibroblasts and myocardial fibrosis is largely unknown.

In this study, we found that miR-22 was down-regulated in the mice of experimental cardiac fibrosis induced by myocardial infarction. And, inhibition of miR-22 caused fibrogenesis in cultured cardiac fibroblasts, whereas over-expression of miR-22 attenuated Ang II-induced collagen content enhancement *in vitro*. Furthermore, our data showed that miR-22 is involved in the process of cardiac fibrosis by regulation of TGF β RI. These results indicate that miR-22 is a novel therapeutic target for myocardial fibrosis.

Materials and Methods

Surgical Procedures

All animal protocols were approved by the Institutional Animal Care. Adult C57BL/6 male mice were anesthetized with 2.4 % isoflurane and placed in a supine position on a heating pad (37°C). Animals were intubated with a 19G stump needle and ventilated with room air, using a MiniVent mouse ventilator (Hugo Sachs Elektronik; stroke volume, 250 μ l; respiratory rate, 210 breaths per minute). Via left thoracotomy between the fourth and fifth ribs, the left anterior coronary artery (LCA) was visualized under a microscope and ligated by using a 6-0 prolene suture. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Leica) by discoloration of the occluded distal myocardium. Sham operated animals underwent the same procedure without occlusion of the LCA.

Masson's trichrome staining

Tissues used for histology were incubated in Krebs-Henselheit solution, fixed in 4% paraformaldehyde, sectioned, and processed for Masson's Trichrome staining [10].

Cell culture and treatment

Cardiac fibroblasts (CFs) were isolated as previous described [3]. Briefly, hearts were excised from anesthetized neonatal 1 to 2-day-old Sprague-Dawley rats (Harlan Sprague-Dawley), minced, and digested with pancreatin 0.1 %. Cells were plated on primaria plates for 2 h, and the medium that contained the cardiomyocyte fraction of the digested tissue was removed. Cardiac fibroblasts attached and proliferated much more rapidly than cardiac myocytes; this produced virtually pure fibroblast cultures after the first passage, which was confirmed by repeated differential plating and microscopic evaluation. Cells were detached with 0.05 % trypsin for passaging, and culture studies were performed at passages 2 to 4. Cells were grown in high glucose (4.5 gm/l) DMEM containing 10 % heat-inactivated FBS and antibiotics (penicillin and streptomycin).

Transfection procedure

Rat cardiac fibroblasts were transfected with mimics and inhibitors of miR-22 (100 nM, Sangon, Shanghai, China) using Lipofectamin 2000 (Invitrogen, Carlsbad, CA).

Real time-PCR

After experimental treatment, total RNA samples were isolated from cardiac tissues and cultured cardiac fibroblasts using Trizol reagent (Invitrogen, CA, USA) according to manufacturer's protocol. RNA was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) to obtain first-strand cDNA. Levels of Col1α1, Col3α1, miR-22 and TGFβRI mRNA were determined using SYBR Green I incorporation methods on ABI 7500 fast Real Time PCR system (Applied Biosystems), with U6 as an internal control of miRNA or GAPDH as an internal control of mRNA.

Western blotting

Protein lysates were prepared as described [11]. We used primary antibodies against TGFβRI, collagen1, α-SMA, GAPDH (Santa Cruz, CA), as well as appropriate secondary antibodies.

Luciferase assays

HEK293 cells were transfected with 0.1 mg p-MIR-report plasmid (Ambion) containing the 3'-UTR of rat TGFβRI RNA and 50 nM of miR-22 using Lipofectamine 2000 (Invitrogen). Forty-eight hours later luciferase activity was measured in cell lysates using a luciferase assay kit (Promega).

Statistical assays

Average data are presented as mean ± SEM. Statistical analysis was carried out using the Prism software (GraphPad). For statistical comparison of two groups, we used unpaired, two-tailed Student's t-test; for the comparison of three or more groups, we used ANOVA followed by Fisher's post-test. Differences were considered significant when $p < 0.05$.

Results

miR-22 is decreased in mice after myocardial infarction (MI)

In an effort to identify the role of miR-22 in post-MI remodeling, we first constructed MI by occlusion of the left coronary artery (LCA). After seven days, Masson staining showed that there are markedly collagen deposition in MI mice compared sham group (Fig. 1A). And, real-time PCR assay found that the mRNA levels of Col1α1, Col3α1 were significantly increased in MI mice (Fig. 1B & 1C). Furthermore, Western blot showed that the expression of collagen1 and α-SMA were significantly up-regulated in the MI mice than that in sham mice (Fig. 1D). At the same time, real-time PCR found that miR-22 was decreased in the border zone of the infarction region compared with that of the sham-operated mouse hearts (Fig. 1E), indicating that miR-22 may participates in the process of post-MI remodeling.

Inhibition of miR-22 increased the production of collagen in cardiac fibroblasts

To investigate whether miR-22 plays important roles in the process of cardiac fibrosis, we administrated AMO-22, the antisense oligonucleotide of miR-22, into cultured cardiac fibroblasts and evaluated the fibrogenesis of these cells. As illustrated in Fig. 2A, AMO-22 inhibited the endogenous expression of miR-22. And, inhibition of miR-22 caused significantly increased of Col1α1 and Col3α1 at mRNA levels (Fig. 2B & 2C). Furthermore, using western blot assay, we found that the expression of collagen1 and α-SMA were elevated in the cultured cardiac fibroblasts after transfection of AMO-22 (Fig. 2D).

Overexpression of miR-22 alleviated Ang II-induced collagen content

To determine whether overexpression of miR-22 was capable of reducing collagen expression, we exposed fibroblasts to a miR-22 mimic. As shown in Fig. 3A, the level of miR-22 expression in cultured fibroblasts was increased by as much as 100-fold after transfection

Fig. 1. miR-22 is down-regulated in the border zone of the infarcted region after MI. (A) Masson Trichrome staining of mouse heart sections shows collagen deposition and interstitial fibrosis in the border zone of the infarcted region 7 days after MI. Both the mRNA levels of Col1α1 (B) and Col3α1 (C) were up-regulated in the MI mice compared with sham group. (D) Western blot assays showed the increased collagen1 and α-SMA expression in the heart of with MI. (E) Real-time PCR analysis confirms the decreased miR-22 in response to MI compared with sham operated animals. $n = 3$ ** $p < 0.01$ vs. sham group.

Fig. 2. *In vitro* silencing of miR-22 caused fibrogenesis in cultured cardiac fibroblasts. (A) Real-time PCR analysis the inhibition of miR-22 after transfection of AMO-22 in cultured cardiac fibroblasts. Inhibition of miR-22 induced up-regulation of Col1α1 (B) and Col3α1 (C) at mRNA level. (D) Silencing of miR-22 increased collagen1 and α-SMA expression at protein level in cultured cardiac fibroblasts. $n = 3$; * $p < 0.05$, ** $p < 0.01$ vs. NC group.

of miR-22. And, real-time PCR analysis indicated that forced expression of miR-22 diminished the up-regulated Col1α1 and Col3α1 caused by Ang II in cultured cardiac fibroblasts (Fig. 3B

Fig. 3. Forced expression of miR-22 attenuated Ang II-induced fibrogenesis in cultured cardiac fibroblasts. (A) Transfection of miR-22 enhanced the expression of miR-22 in cardiac fibroblasts. Overexpression of miR-22 abrogated increased Col1 α 1 (B) and Col3 α 1 (C) caused by Ang II. (D) Forced expression of miR-22 attenuated Ang II-induced up-regulation of collagen1 and α -SMA expression at protein level in cultured cardiac fibroblasts. (E) Show Ang II-induced on the expression of miR-22 in fibroblasts. $n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. NC or Control group. # $p < 0.05$ vs. Ang II.

& 3C). At the same time, miR-22 alleviated the Ang II-induced alteration of fibrotic-related proteins, including collagen1 and α -SMA (Fig. 3D). The Ang II-induced on the expression of miR-22 in fibroblasts (Fig. 3E).

TGF β RI mediated the anti-fibrotic effect of miR-22

To exploit the underlying mechanism of miR-22 in myocardial fibrosis, we performed miRNA gene target prediction using Target-Scan 6.0 database, and we indeed identified a binding site in the 3'-UTR of TGF β RI mRNA for miR-22, which is highly conserved among human, rat and mouse (Fig. 4A). At the same time, TGF β RI was also increased in the heart of mice after occlusion of LCA (Fig. 4B), these results suggest a targeting relationship between miR-22 and TGF β RI. We then experimentally verified the regulation of TGF β RI by miR-22 with luciferase activity assay in HEK-293 cells. And, luciferase assay showed that miR-22 significantly inhibited luciferase activity elicited by the pMIR-REPORTTM luciferase vector containing the target sequence (Fig. 4C). Furthermore, our following data confirmed that

Fig. 4. TGFβRI is a direct target for miR-22. (A) The predicted duplex of miR-22 and its targets sites in the 3'-untranslated region (3'-UTR) of TGFβRI. (B) Western blot analysis the alteration of TGFβRI in the mice after MI. (C) Dual luciferase assay was performed in HEK293 cells transfection of luciferase reporter construct alone or cotransfected with miR-22 or NC. Firefly luciferase activity was normalized to Renilla luciferase activity for each sample. (D) Western blot was performed to verify the effect of miR-22 on TGFβRI at protein level. (E) Real time PCR analysis the effect of miR-22 on TGFβRI at mRNA level. n = 3; *p < 0.05 vs. Sham or NC group.

miR-22 inhibited the expression of TGFβRI at protein level (Fig. 4D), whereas has no effect on its mRNA level (Fig. 4E). These results indicated that miR-22 regulate TGFβRI in a post-transcriptionally manner.

Discussion

The data presented here show that miR-22 participate in the pathogenesis of myocardial fibrosis. Our data found that miR-22 is decreased in the mice after MI, which along with increase of collagen deposition. And, inhibition of miR-22 caused fibrogenesis in cultured cardiac fibroblasts. Furthermore, forced expression of miR-22 abrogated the Ang II-induced elevated the production of collagen. Meanwhile, we found that TGFβRI is one of the targets for miR-22. These findings indicate that miR-22 is an important mediator for cardiac fibrosis and suggest that miR-22 may be a novel target in treating myocardial fibrosis.

More and more studies have provided strong evidence that miRNAs play important roles in the process of cardiac fibrosis [4]. van Rooij et al. found that miR-29 family, including miR-29a, miR-29b and miR-29c were downregulated in the region of the heart adjacent to the infarct. And, over-expression of miR-29 in fibroblasts reduces collagen expression by targeting a cadre of mRNA that encodes proteins involved in fibrosis [8, 12-14]. Zhao et al. illustrated that miR-101a exerts anti-fibrotic effects by targeting TGFβRI, suggesting that miR-101a plays a multi-faceted role in modulating TGFβ signaling pathway and cardiac fibrosis [15]. Yin et al. found that low ambient temperature could cause enlarged heart, ultrastructure damage of myocardium and weakened functions, and Resveratrol treatment could inhibit the increase of miR-328 and effectively suppress these changes at least partially via inhibiting cardiomyocyte apoptosis [16]. MiR-21 is another master regulator of cardiac fibrosis [17-19]. Thum et al. found that miR-21 induces cardiac fibrosis by targeting sprouty homologue 1 (Spry1), and silencing of miR-21 inhibits pressure-overload-induced interstitial fibrosis and attenuates cardiac dysfunction in mouse [20]. In addition, others researchers also

confirmed that miR-21 contributes to the pathological process of cardiac fibrosis [1, 7]. Rudy F. Duisters and colleagues found that miR-133 and miR-30 was decreased in pathological left ventricular hypertrophy and contributes to collagen synthesis. And, overexpression of these two miRNAs decreased CTGF, which was accompanied by decreased production of collagens [21]. However, the role and underlying molecular mechanism of miRNAs in cardiac fibrosis remains unclear.

Increasing evidence showed that miR-22 participated in kinds of disease, such as cancer, diabetes [22], and cardiovascular diseases [23]. Anmad et al. report that miR-22 is downregulated in peripheral blood mononuclear cells derived from chronic myeloid leukemia (CML) patients and in CML cell line K562 [24]. And, overexpression of miR-22 inhibits proliferation and alteration in cell cycle of K562 cells by regulating neuro-epithelial transforming gene 1 (NET1). A study from Kaur et al. showed that *in vivo* silencing of miR-22-3p by antagomiR administration lowered random as well as fasting glucose levels in diabetic mice. MiR-22-3p antagonism improved glucose tolerance and insulin sensitivity [22].

MiR-22 is an evolutionally conserved miRNA that is highly expressed in the heart. Extensive studies have demonstrated that miR-22 contributes to cardiac hypertrophy through modulates the expression and function of genes involved in hypertrophic response, sarcomere reorganization, and metabolic program shift during cardiac remodeling [23]. Huang et al. found that miR-22 regulates cardiac hypertrophy, and miR-22 deficient mice are unable to properly develop cardiac hypertrophy in response to isoproterenol administration [25]. In addition, another study from Gurha et al. showed that miR-22 is a regulator of Ca²⁺ homeostasis in the heart, and deletion of miR-22 in the heart promotes stress-induced cardiac dilation and contractile dysfunction [26]. At the same time, Gurha P and colleagues found that miR-22 promotes heart failure through post-transcriptionally inhibits peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), PPAR α and sirtuin 1 (SIRT1) expression [27]. In the previous study, we found that miR-22 is downregulated in the MI mice from the microarray data of van Rooij [8]. However, whether miR-22 involved in the process of cardiac fibrosis is still unknown. The present study found that miR-22 is decreased in the heart of mice after MI, and overexpression of miR-22 inhibits Ang II-induced fibrogenesis in cultured cardiac fibroblasts. These results highlight the potential anti-fibrotic function of miR-22 in the treatment for the cardiac remodeling after MI. However, further studies will be necessary to explain the functional and mechanisms of miR-22 *in vivo*.

Normal tissue repair requires the produced new connective tissue, which is performed by myofibroblasts, a specific cell type expresses the pro-contractile protein α -smooth muscle actin (α -SMA) [28]. However, inappropriately terminated tissue repair program and excessive deposition of scar tissue and extracellular matrix (ECM) result in chronic fibrotic diseases, which affect some organs such as heart, lung and liver, and finally lead to organ failure and resulting in death [29, 30]. As is well known, transforming growth factor β 1 (TGF- β 1) is a major contributor to fibrotic diseases, including cardiac fibrosis. Active TGF- β 1 binds to a complex of TGF β type I and TGF β type II receptors, resulting in the phosphorylation of the receptor-activated Smads (R-Smads), Smad2 and 3, by the TGF β receptor I kinase. Activated Smad2/3 binds Smad4 and become localized into the nucleus where they can activate transcription [28]. Previous studies have demonstrated that inhibiting TGF β RI with a TGF β RI inhibitor significantly attenuated left ventricular remodeling and improved systolic dysfunction in rat MI models [31]. In our study, we report that TGF β RI is a novel target of miR-22, and mediated the anti-fibrotic effect of miR-22 in cultured cardiac fibroblasts.

In summary, this study examined the alteration and the function of miR-22 on the progression of post-infarction left ventricular (LV) remodeling in mice. Our results showed that miR-22 expression was reduced in the heart of mice after MI. And forced expression of miR-22 in cultured cardiac fibroblasts attenuated Ang II-induced fibrogenesis by targeting TGF β RI. Additional studies will be needed to evaluate the potential role of miR-22 as a therapeutic strategy for cardiac fibrosis.

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Disclosure Statement

We declare no competing interests.

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